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TRAIL-FOLLOWING RESPONSES OF THE ARGENTINE ANT, *Iridomyrmex humilis* (MAYR), TO A SYNTHETIC TRAIL PHEROMONE COMPONENT AND ANALOGS

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Abstract—Behavioral evidence indicates that (*Z*)-9-hexadecenal (*Z*9-16:ALD) is a trail pheromone component of *Iridomyrmex humilis,* and that the true trail pheromone may be multicomponent. Trail-following responses of *I. humilis* workers to several concentrations of synthetic *Z*9-16:ALD, a constituent of the Pavan's gland, were found to be comparable to responses to gaster extract trails containing ca. 100 times less *Z*9-16:ALD. Of the five aldehyde analogs tested, only (*Z*)-7-hexadecenal (*Z*7-16:ALD) elicited significant trail-following. However, following responses to several *Z*9-16:ALD–*Z*7-16:ALD combinations were lower than responses to *Z*9-16:ALD alone. Trails on filter paper of biologically relevant concentrations of *Z*9-16: ALD lose activity within 2 hr in the laboratory. The release rate of *Z*9-16:ALD measured from filter paper trails was 0.25 ± 0.10 pg/cm-sec. This was used to estimate the trail-following threshold for this compound of Argentine ant workers.

Key Words—Argentine ant, *Iridomyrmex humilis,* Hymenoptera, Formicidae, trail-following, structure-activity, (*Z*)-9-hexadecenal, threshold, trail emission rate, synthetic trail longevity, bioassay.

INTRODUCTION

The failure of attempts to biologically control many honeydew secreting pests, particulary in citrus, can be traced to the presence of Argentine ants, *Iridomyrmex humilis* (Mayr), which interfere with the activites of natural predators and parasites (DeBach et al., 1951a, b). At present, since the removal of chlordane from the market, economical control of this ant cannot be

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obtained with conventional insecticides currently sold in this country, but detailed quantitative analysis of its behavior, particularly pertaining to the retrieval of food, may lead to the development of an alternative control scheme such as an effective bait program. Laboratory attempts to enhance bait pick-up in other species by incorporation of synthetic trail pheromones have been successful. However, field tests indicate that further research is needed for pheromones to be useful in ant control (Robinson and Cherrett, 1973, 1975; Cross et al., 1979).

Recently, (*Z*)-9-hexadecenal (*Z*9-16:ALD) was isolated and identified from ventral gland extracts of *Iridomyrmex humilis* ants, and implicated as a component of the trail pheromone complex by its aggregative and attractive qualities (Cavill et al., 1979, 1980). However, because of insufficient information concerning the assay procedure and because trail-following activity was not reported, conclusions about the behavioral importance of this compound could not be made.

This report is a quantitative analysis of the trail-following activity elicited by *Z*9-16:ALD and several analogs. In addition, measurement of the *Z*9-16:ALD release rate from trails on filter paper, and the duration of biological activity are presented.

METHODS AND MATERIALS

Chemicals. Tetradecanal (14:ALD), (*Z*)-9-tetradecenal (*Z*9-14:ALD), hexadecanal (16:ALD), (*Z*)-11-hexadecenal (*Z*11-16:ALD), (*Z*)-7-hexadecenal (*Z*7-16:ALD), and *Z*9-16:ALD were obtained from the Controlled Release Division of Albany International Corporation. Purity of these compounds was determined by gas–liquid chromatography, using 10% XF-1150 (50% cyanoethyl, methyl silicone) on Chromosorb W, AW DMCS, 100/120 mesh (2.5 m × 2 mm). The 14:ALD was greater than 99.9%, the *Z*9-14:ALD, *Z*7-16:ALD, and *Z*11-16:ALD greater than 98%, the *Z*9-16:ALD greater than 97%, and the 16:ALD greater than 95% free of other volatile impurities.

Biosassay. Laboratory rearing of the Argentine ant, *I. humilis*, has been described previously (Van Vorhis Key et al., 1981). Colonies were provided with new food sources in enclosed dishes immediately prior to bioassay periods. Once recruitment had been initiated through the tubing leading from the nest to the food source; individual unfed ants which had been recruited and were traveling towards the food source but had not yet arrived were redirected and introduced onto the experimental trail directly from the natural trail inside the tubing. Thus disturbance to the ants was minimal.

The techniques for application of trails, making gaster extracts, and bioassay were described previously (Van Vorhis Key et al., 1981). Briefly,

circular trails (50.7 cm circumference, 2 mm wide), were applied to filter paper disks (Whatman No. 1, 24 cm diam), and housed under a glass plate held 3 mm above the disk by a spacer ring. For 2 min after introduction of a single recruited worker ant onto the trail, observations were made of the duration and continuity of trail-following and, when appropriate, the rate of locomotion. Trail-following continuity [following duration (sec)/approaches] was calculated by dividing the time each ant spent within 15 mm of the center of the trail by the number of times it entered this area (approaches).

Dose–Response to Z9–16:ALD. Trail-following by recruited worker ants was measured on Z9–16:ALD trails of 0.01, 0.1, 1.0, 10. 100, and 1000 ng/50.7 cm trails. For comparison, solvent trails and gaster extract trails of an optimum concentration, 0.1 ant equiv/50.7 cm (Van Vorhis Key et al., 1981), were included. Each trail was used twice, and treatments were presented in a randomized complete block design. Trail-following continuity was calculated for 20 ants per treatment and was then compared among treatments using Duncan's new multiple range test on transformed data ($\log_{10}$).

In addition, for uninterrupted trail-following, the time required to travel a 90° arc of the circular trail was recorded for three 90° sections as soon as possible after introduction of the ant to the trail. From this, an average speed of locomotion was calculated for each concentration. Comparisons of rates of locomotion were made using Duncan's new multiple range test.

Longevity of Z9–16:ALD Trails. Trails of Z9–16:ALD, applied at 10 ng/50.7 cm or 1000 ng/50.7 cm to filter paper disks were aged in a fume hood (ca. 23.5° C; air velocity 5 mm above the paper surface = 0.15–0.3 m/sec), for 1, 2, 4, or 6 hr. New trails of each concentration and new and 1-hr-old solvent controls were also included in a randomized complete block design ($N = 15$). Ants were assayed as previously described; a recruited, unfed ant was introduced directly onto each trail as it reached its experimental age. The ants' trail-following responses were recorded and calculations of trail-following continuity made. Analysis was made using Duncan's new multiple range test on transformed data ($\log_{10}$).

Air Extraction of Synthetic Trails. Linear sections (2.54 cm) of new Z9–16:ALD trails, applied to filter paper disks at 1000 ng/50.7 cm were extracted under a nitrogen stream flowing at 30 ml/min, and volatiles were collected in a glass wool plug positioned downwind from the trail section (Baker et al., 1981). Recovery efficiency of this system for aldehydes, acetates, and alcohols was 90–100%. After 2 hr of collection, the glass wool was eluted with ca. 1.5 ml CS_2, and the eluant condensed by steam bath distillation after addition of 30 ng of the internal standard, *E*11–16:Ac. Gas–liquid chromatography was then performed using the XF-1150 column described above. Release rates of Z9–16:ALD were calculated by a peak height × retention time comparison with the internal standard for 5 trail segments extracted individually.

Analog Activity. Trail-following responses to 14:ALD, 16:ALD, Z9-14:ALD, Z7-16:ALD, Z9-16:ALD, and Z11-16:ALD were compared using trails of 10 ng/50.7 cm. Treatments were presented in a randomized complete block design, and each trail was used twice. Ants were introduced as previously described, and the trail-following continuity of 20 ants was measured. Velocity was calculated for ants responding to Z7-16:ALD and Z9-16:ALD ($N = 10$).

Z7-16:ALD + Z9-16:ALD Mixtures. Because of the significant activity of Z7-16:ALD, mixtures of Z9-16:ALD and Z7-16:ALD were tested to see if trail-following response could be increased. A series of ratios—10:0, 9:1, 5:5, 1:9, and 0:10 Z9-16:ALD–Z7-16:ALD—was applied totaling 10 ng of material/50.7 cm trail. The scoring distance from the trail center was reduced from 15 mm to 5 mm to increase the discriminatory power of the assay. Treatments were presented in a randomized, complete block design of 25 replicates per treatment. Trail-following continuity was compared using Duncan's new multiple range test on transformed data ($\log_{10}$).

RESULTS

Dose-Response to Z9-16:ALD. All Z9-16:ALD trail concentrations greater than 0.01 ng/50.7 cm, as well as 0.1 ant equiv gaster extract trails, elicited significantly greater trail-following continuity than solvent controls ($P < 0.05$; Figure 1). The 10, 100, and 1000 ng/trail treatments elicited responses not significantly different from each other nor from gaster extract, but greater than all lower concentrations ($P < 0.05$). The highest average responses (to 100 ng and 1000 ng trails) were 90 sec/approach, ca. 75% of the maximum possible 120 sec/approach, whereas suboptimal Z9-16:ALD concentrations (0.01–1.0 ng) elicited only 17–29% of the maximum. Response to solvent trails was minimal (4%). Displacement of trail-following away from 1000 ng trails of Z9-16:ALD was also observed, similar to that described for high concentrations of gaster extract (Van Vorhis Key et al., 1981). Ants trail-followed parallel to, but several mm away from, the center of these most concentrated trails.

Speed of locomotion, measured as time required to travel a 90° arc of trail, was concentration-dependent (Figure 2). Ants orienting along gaster extract trails and 100- and 1000-ng Z9-16:ALD trails traveled significantly faster (3.04, 2.93, and 2.61 cm/sec, respectively) than ants orienting along trails of 0.01 ng (1.89 cm/sec) ($P < 0.05$). The number of ants demonstrating uninterrupted trail-following for three 90° sections was also concentration-dependent, ranging from 3 (0.01 ng/trail) to 16 (1000 ng/trail).

Synthetic Trail Longevity. Trail-following to 10-ng Z9-16:ALD trails aged under laboratory conditions disappeared within 1 hr (Figure 3).

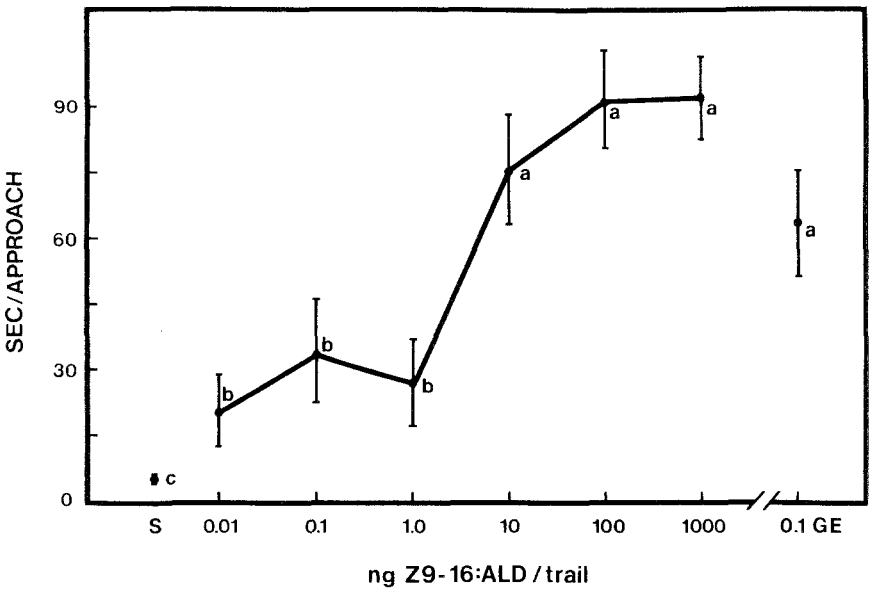

FIG. 1. Trail-following responses of ants to synthetic Z9-16:ALD trails ranging in concentration from 0.01 ng/50.7 cm to 1000 ng/50.7 cm, measured as the trail-following continuity (see text). S = solvent control; GE = gaster extract trail (0.1 ant equiv/50.7 cm); $N = 20$. Brackets around means denote standard errors. Means having no letters in common are significantly different (Duncan's new multiple range test, $P < 0.05$).

Following to 1000-ng trails aged 0–2 hr was significantly greater than to solvent controls. No significant decline occurred for 1000-ng trails during the 1st hour. Responses to 1000-ng trails aged 4 hr or more were not significantly different from responses to solvent controls.

Release Rate of Z9-16:ALD Trails. The mean emission rate from filter paper of 1000 ng Z9-16:ALD trails over the first 2 hr after application was 0.25 pg/cm/sec (± 0.10 SD; $N = 5$). The nitrogen flow rate of 30 ml/min in our apparatus produced a linear velocity of ca. 1 cm/sec, approximately 30 times slower than over the surface of trails being aged in the fume hood for behavioral assays. The low velocity in the apparatus was meant to approximate that which might be found at trail surfaces in the field or in our near static bioassay environment, not the trail aging conditions in our fume hood.

Analogs of Z9-16:ALD. Among the analogs of Z9-16:ALD tested, only Z7-16:ALD elicited significant trail-following activity. Trail-following continuity in response to Z7-16:ALD was similar to that to Z9-16:ALD ($P < 0.05$; Figure 4). Also, the mean speed of locomotion of ants following trails of Z9-16:ALD (1.84 ± 0.76 SD cm/sec) was not significantly different from that of ants following Z7-16:ALD trails (1.37 ± 0.57 SD cm/sec).

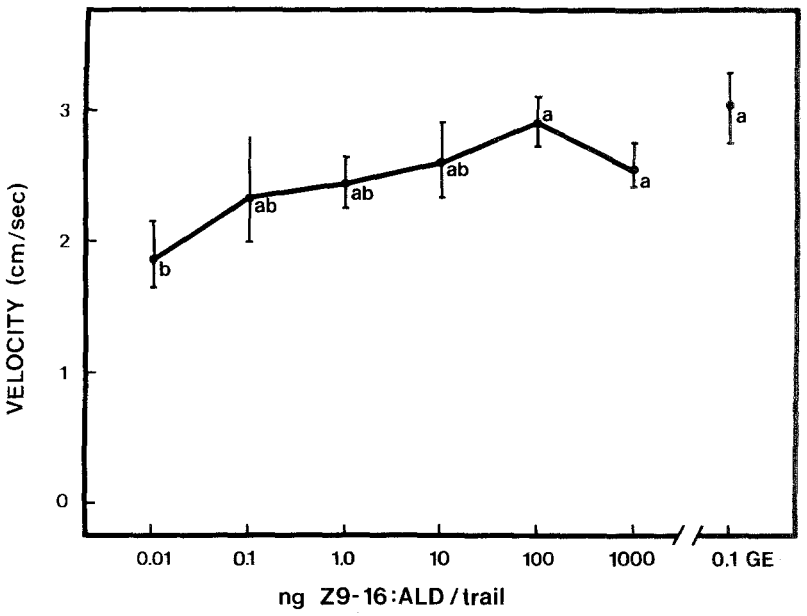

FIG. 2. Velocity of ants following trails of Z9–16:ALD ranging in concentrations from 0.01 ng/50.7 cm to 1000 ng/50.7 cm. GE = gaster extract trail (0.1 ant equiv/50.7 cm). Brackets around means denote standard errors. Means having no letters in common are significantly different (Duncan's new multiple range test, $P < 0.05$; $N = 3, 6, 7, 13, 15, 16$, and 14 for 0.01, .0.1, 1.0, 10, 100, and 1000 ng, and 0.1 gaster equiv trails, respectively.

Z9–16:ALD + Z7–16:ALD Mixtures. Trail-following continuity of ants following 2-component mixtures of Z9–16:ALD and Z7–16:ALD is illustrated in Figure 5. Addition of more than 10% of Z7–16:ALD to Z9–16:ALD significantly reduced trail-following compared to treatments containing 90% or more of Z9–16:ALD. Trails made from a 50:50 mixture elicited some trail-following response, but significantly less than all other treatments ($P < 0.05$). In contrast to the initial experiment where Z9–16:ALD and Z7–16:ALD trails were equally active, in this experiment Z9–16:ALD elicited more continuous trail-following than Z7–16:ALD. This apparent discrepancy in bioassay results probably can be attributed to the more rigorous criteria used to assess trail-following; to be scored as trail-following, ants had to remain within 5 mm of the center of the trail instead of within 15 mm as previously required.

DISCUSSION

For recruited workers of *I. humilis*, 10-, 100-, and 1000-ng trails of Z9–16:ALD elicited strong trail-following that was quantitatively similar to

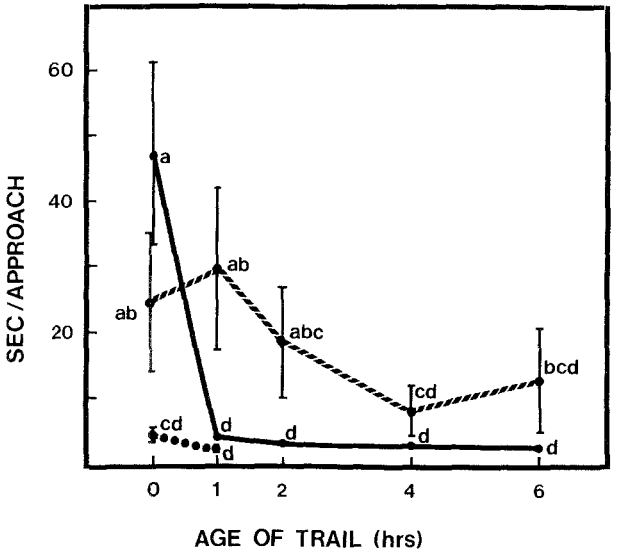

FIG. 3. Activity loss of aging trails of 10 ng Z9-16:ALD/50.7 cm, solid line, and 1000 ng Z9-16:ALD/50.7 cm, dashed line, measured as trail-following continuity (sec/approach). Solvent controls are represented by the dotted line. Activity was assessed for new trails and for trails which had been aged for 1, 2, 4, or 6 hr after application. Brackets around means denote standard errors. Means having no letters in common are significantly different according to Duncan's new multiple range test ($P < 0.05$; $N = 15$).

that elicited by 0.1 ant equiv whole gaster extract trails. More than 50% of the ants trail-followed during the entire test period without deviating from the trail. For all Z9-16:ALD concentrations of 0.1 ng/50.7 cm or more, the linear speed of trail-following ants was not significantly different from that of ants following an optimum concentration of gaster extract.

Cavill et al. (1979) reported a lower response (measured as attraction/aggregation in a multichoice olfactometer) to synthetic Z9-16:ALD than to Z9-16:ALD extracted from ants, and suggested that the concentrations used and/or the activity of minor components in the gaster extract may account for this difference.

Cavill et al. (1980) reported that 370 ventral glands of *I. humilis* workers contained ca. 300 ng of Z9-16:ALD. From this we calculate that the concentration of Z9-16:ALD in optimum (0.1 ant equiv) gaster extract trails is 1.6 pg/cm (0.081 ng/50.7 cm). However, to elicit equivalent trail-following response, trails of synthetic Z9-16:ALD must be $\geqslant$200 pg/cm (10 ng Z9-16:ALD/50.7 cm)—over 100 times more concentrated. This strongly indicates that minor components play a role in the trail pheromone system of *Iridomyrmex humilis*, although high concentrations of Z9-16:ALD alone elicit intense trail-following by recruited workers.

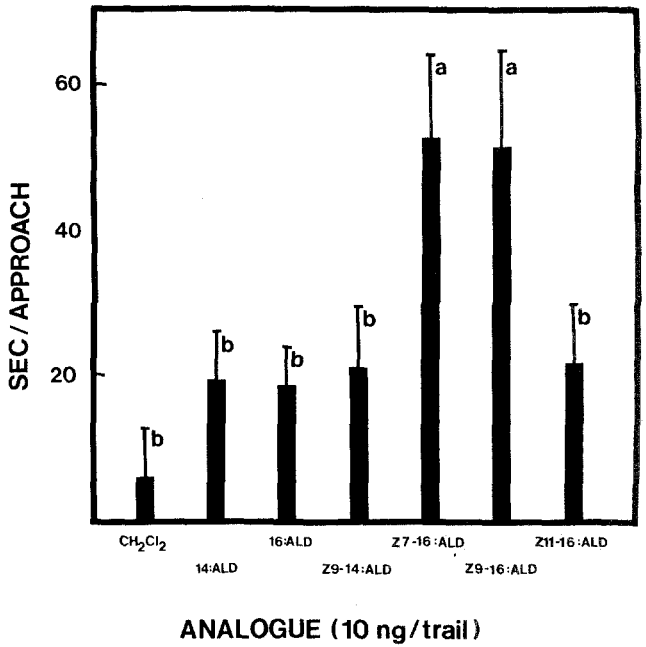

FIG. 4. Trail following responses to 10-ng trails of synthetic analogs of Z9–16:ALD and solvent controls (CH_2Cl_2). Activity is expressed as trail-following continuity (sec/approach). Brackets above means denote standard errors. Means having no letters in common are significantly different (Duncan's new multiple range test, $P < 0.05$; $N = 20$).

Structure–activity studies of trail pheromones in ants have been minimal, as chemical identifications of ant trail pheromones are few (Parry and Morgan, 1979). The structure–activity relationships of several analogs of the trail pheromone of *Atta texana* were elucidated by Sonnet and Moser (1972, 1973), and Caputo et al. (1979). Enantiomeric specificity has been found for alarm pheromone reception by *Atta texana* and *Atta cephalotes* (Riley et al., 1974) and by *Pogonomyrmex barbatus* (Benthuysen and Blum, 1974), and only compounds of similar size and geometry to 4-methyl-3-heptanone, the natural alarm pheromone, were found to be active in eliciting alarm in *Pogonomyrmex badius* (Blum et al., 1971).

Our analysis of the trail-following elicited by several aldehydes related to Z9–16:ALD revealed that Z7–16:ALD is highly active while Z11–16:ALD is not. This indicates discrimination between selected double-bond positions by the ants. Furthermore, the lack of response to Z9–14:ALD indicates sensitivity to changes either in chain length or distance from the double bond to the end of the chain (Gaston et al., 1972; Priesner et al., 1975; Roelofs and

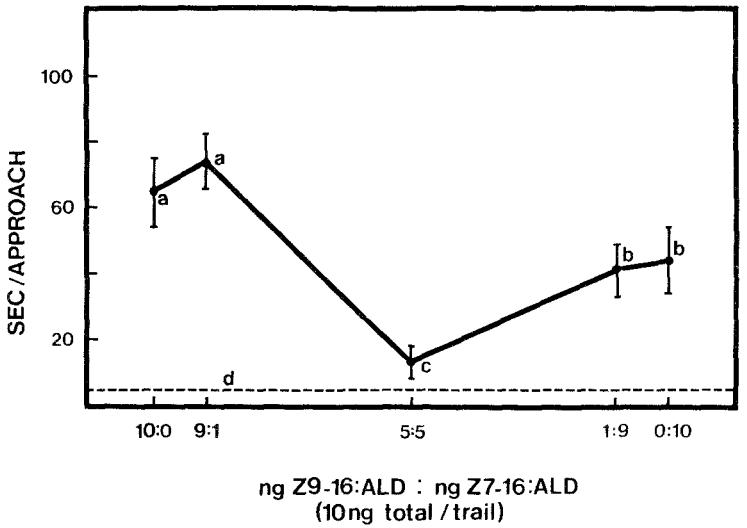

FIG. 5. Trail-following continuity of ants following 10 ng/50.7 cm trails of mixtures of Z9–16:ALD and Z7–16:ALD. Response to solvent controls is indicated by the dashed line. Brackets around means denote standard errors. Means having no letters in common are significantly different according to Duncan's new multiple range test ($P < 0.05$, $N = 25$).

Comeau, 1971). It would be interesting to test (*E*)-9-hexadecenal to determine whether *I. humilis* can discriminate between geometric isomers, but we were unable to obtain any.

Because Z7–16:ALD has not been found in *I. humilis* extracts, yet elicits significant trail-following, it may be mimicking the sensory input of Z9–16:ALD. Alternatively, since chemical and behavioral characterization of the trail pheromone of *Iridomyrmex humilis* is far from complete, Z7–16:ALD may be present in the deposited trail in an as yet undetermined ratio to Z9–16:ALD, although the ratios we tested did not significantly increase, and usually decreased, trail-following. The lower response to the 5-ng Z9–16:ALD–5-ng Z7–16:ALD mixture cannot presently be explained.

Our data concerning the longevity of synthetic Z9–16:ALD trails demonstrate that activity loss is rapid. In contrast, we found in a previous study (Van Vorhis Key et al., 1981), that 0.1 ant equiv crude gaster extract trails elicited significant trail-following for up to 8 hr. Cuticular waxes and other less volatile components of these crude extracts may be responsible for extending the normal lifetime of a trail, and other as yet unrecognized volatile active components may also help increase longevity.

The Z9–16:ALD from 1000-ng trails is emitted at 0.25 pg/cm/sec in the low (ca. 1 cm/sec) N_2 velocity of our collection device, a rate probably similar

to that in the "static-air" bioassay environment. This low rate is somewhat surprising in that only ca. 18% of the aldehyde will have been emitted during the first 4 hr, yet trail-following activity drops to near zero during that time. The higher wind velocity to which aging trails were subjected in the laboratory hood between tests might have accelerated evaporation of material compared to that in the collection device. Additionally, some aldehyde may have oxidized during the aging period, resulting in decomposition products such as acids, which might act antagonistically to reduce trail-following response to the remaining Z9–16:ALD.

Under the conditions of our assay procedure, the lowest concentration of pheromone causing at least 50% of the ants to follow a trail without interruption during the 2-min assay was emitted by 10-ng Z9–16:ALD trails and was designated as the threshold (Howard et al., 1976). We were able to collect and quantify emissions from 1000-, not 10-ng, trails in our apparatus. However, if we use this higher rate, an ant trail-following at its mean velocity of 2.61 cm/sec would encounter in 1 sec of following approximately 0.25 pg/cm/sec/2.61 cm = 9.6×10^{-14} g/sec (2.3×10^{8} molecules/sec). This then is a conservative estimate of the Z9–16:ALD trail-following threshold for *I. humilis*. The actual threshold is undoubtedly lower, since the above-threshold 10-ng trails should emit at lower rates than the 1000-ng trails we measured. Moreover, lateral and vertical diffusion of the molecules makes it likely that an ant's small receptor surface would sample only a small fraction of the emitted trail. Although ants may encounter large concentrations by antennal contact with the applied 10-ng trail, we have shown that trail volatiles alone elicit following and that contact is unnecessary (Van Vorhis Key et al., 1981). Similarly, ants trail follow several mm out from the edges of a 1000-ng Z9–16:ALD trail.

Barlin et al. (1976) reported that *Solenopsis richteri* ants which had no access to food for 3 days could follow 10 fg/cm of trail pheromone, while the trail-following threshold of fed ants was ca. 80 fg/cm. Tumlinson et al. (1972) reported that *Atta texana* workers can detect 80 fg/cm of their trail pheromone. Other such calculations have been made by Riley et al. (1974) for *Atta cephalotes,* Ritter et al. (1977) for *Monomorium pharaonis* (L.), and Cross et al. (1979) for *Atta sexdens rubropilosa* Forel. Ours, however, is the first study to estimate the threshold from volatilized, rather than deposited, trail pheromone concentrations.

Further chemical characterization of the trail pheromone of *I. humilis* and investigations of trail-following responses to other possible trail components (Cavill et al., 1980) are needed. Z9–16:ALD certainly must be considered a component of this species' trail-following system due to the intense following it evokes, and an assessment of its value to Argentine ant control programs is necessary.

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FIELD METHOD FOR SAMPLING CHEMICALS RELEASED BY ACTIVE INSECTS

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Abstract—An inexpensive, silent, maintenance-free suction/adsorption system is described for efficiently collecting submicrogram quantities of odors over a wide range of volatilities under field conditions. The samples can later be submitted to gas chromatographic–mass spectrometric analyses. An example is given with odors sampled from solitary bees engaged in digging and defensive behaviors at their nesting sites. Gas chromatograms of odor samples collected from known concentrations of synthetic chemicals are provided for standardization.

Key Words—Aeration of insects, Tenax, vacuum system (portable), collection of volatile compounds.

INTRODUCTION

The characterization of the biological functions of semiochemicals requires some qualitative and quantitative knowledge of the secreted chemicals within their natural contexts of release and reception. This need implies unbiased chemical sampling in the field over potentially broad ranges of volatility, polarity, and concentration. Both direct cryogenic condensation and adsorption have been successfully employed for the collection and concentration of airborne insect pheromones and allomones (Browne et al., 1974, 1979; Byrne et al., 1975; Peacock et al., 1975; Borden et al., 1979). Adsorption methods with polymers such as charcoal, Porapak Q, and Tenax GC may possess advantages of reduced water uptake, more efficient sampling of compounds with relatively high volatility, and less technical complication than cryogenic condensation. Previous studies indicate that the polymer adsorbant 2,6-

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diphenyl-*p*-phenylene oxide (Tenax-GC, Enka N.B., Holland) is especially suitable in these regards (Zlatkis et al., 1973a–c; Janak et al., 1974; Novotny et al., 1974; Bertsch et al., 1974; Neher and Jones, 1977; Lundgren et al., 1978; Juttner and Wurster, 1979) despite its overall lower adsorption capacity (Butler and Burke, 1976).

All systems utilize a low-flow vacuum system that can be precisely regulated. This has previously necessitated an electrical power source and a vacuum pump, both of which greatly restrict field applications. Portable, battery-operated vacuum disphragm pumps can be both expensive and subject to malfunctions. The system described below is designed to overcome these limitations and so provide the field biologist greater access to the study of chemical ecology.

METHODS AND MATERIALS

Construction. The device consists of a vacuum source, gas-flow regulator, gas-flow meter, and the adsorbant for sampling and retaining the volatile chemicals (Figure 1). A suction valve, or "suction ejector" (e.g., AGA suction ejector #323 190 101, AGA AB, Lidingö, S-181 20, Stockholm, Sweden) placed in line between a flow meter and compressed gas tank provides the low-flow suction needed for sampling. A high-velocity gas jet across the valve's chamber orifice reduces the air pressure in the chamber to less than

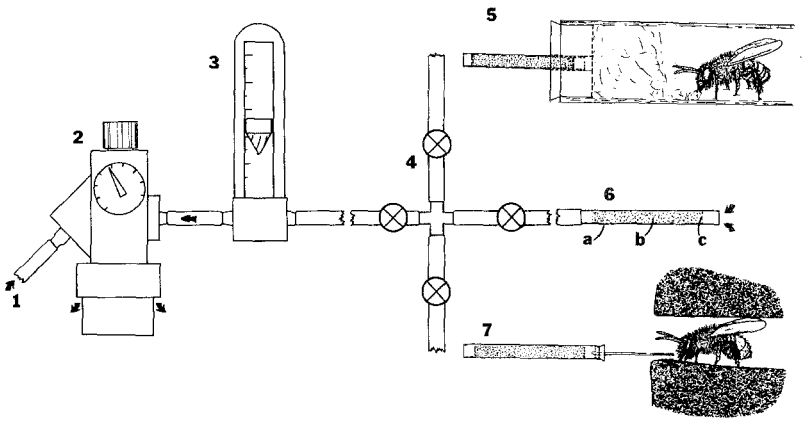

FIG. 1. Odor adsorption system. Air or nitrogen from a compressed gas tank (1) enters the suction valve (2) to generate a vacuum. Air flow from the sample tubes is monitored by an in-line gas-flow meter (3) and equalized using C clamps or valves (4). The glass sample tube (6a) is packed with Tenax (6b) and plugged with glass filter paper disks (6c). These were used to sample odors from bees engaged in digging (5) or defensive (7) behaviors.

ambient pressure to generate the vacuum. No moving parts are involved. Maximum gas pressure of 2400 mm Hg can yield a vacuum of 550 mm Hg. However, for normal sampling a gas pressure of 250–400 mm Hg is sufficient. At this rate, a 5-liter nitrogen tank will operate continuously for 8 hr. Such a tank weighs only 8 kg, so that it may be comfortably carried on a backpack frame for greater portability.

Sample flow rate is adjusted using the suction valve. Some such valves compensate for a decrease in vacuum (as when more sample tubes are added in parallel) by a simultaneous increase in gas flow rate, minimizing operator adjustments. Air sampling rates of 30 ml/min/sampling tube, which can be checked individually, are satisfactory for our particular design.

Odors are collected on the polymeric adsorbant Tenax-GC (Enka N.B., Holland), which is packed with vibration into glass tubes (4 mm inner diameter × 5 cm long, for our GC injection system). The adsorbant is held in place using single-thickness disks of glass fiber filter paper to plug the ends of each tube. In addition to the usual manufacturer's suggested preparation of the adsorbant, we also desorbed each tube with 30 ml/min dry nitrogen flow and sustained 200° C heat prior to field sampling. Packed tubes are reusable with this procedure. Since tubes are stored and transported in vials with dry nitrogen to minimize contamination and oxidation, we recommend using this inexpensive gas in the vacuum suction system. It can also be used for temporary insect anesthesia. Our sampling system readily handles 12 or more of these tubes in parallel at one time. One tube is used to sample ambient environmental volatiles as a control. Samples can be stored for extended periods under refrigeration (Zlatkis et al., 1973b; Lundgren et al., 1978).

Application. We used this system to document a glandular source for the mate attractants and defensive secretions of *Colletes* bees at their aggregation site (Cane and Tengö, 1981). Male bees of this genus locate virgin females after the latter have emerged from their natal subterranean cells but while they are still digging toward the ground surface. To simulate these conditions, we placed individual newly eclosed virgin male and female bees into glass tubes plugged with shredded glass wool into which these insects readily dug (Figure 1). Ambient sampling temperatures were maintained by shading the equipment. Air was drawn around them and through the packed sample tubes. Less volatile odors were rinsed from the cylinder's glass surfaces with pentane and concentrated by microdistillation. For defensive odors, the adsorption tubes were terminally fitted with 5-μl micropipets, which were used to probe and disturb the individual female bee at her nest entrance, while sampling from the airspace close (1–2 mm) to her mandibles (Figure 1). Later, the micropipet was placed with the sampling tube for simultaneous thermal desorption.

Our samples were analyzed using a capillary gas chromatograph (25-m FFAP/OV-17 column) fitted for splitless injection and coupled to a LKB 2091

mass spectrometer (Bergström 1973). The packed adsorption tube (and microcapillary) is slipped into the barrel of a stainless-steel syringe. Upon injection, the loaded syringe is heated to 185° C for 5 min in a thermal sleeve (Groth et al., 1977) while volatilized chemicals are swept onto the cooled GC column with helium carrier gas. Other thermal desorption systems using Tenax have been described (Zlatkis et al., 1973a,b; Bertsch et al., 1974; Ligon and Johnson, 1976; Juttner and Wurster, 1979). Alternatively, a packed glass syringe may be used for both sampling and subsequent injection (Lundgren et al., 1978), or the polymer may be extracted with an organic solvent for the adsorbed volatiles prior to GC–MS analysis (Borden et al., 1979).

The digging odors of *Colletes cunicularius* all contained linalool in the volatile fraction (Figure 2) and heneicosane, heneicosene, and tricosane in the pentane rinse of the digging tubes. These chemicals are found together only in the mandibular glands of these bees (Bergström and Tengö, 1978). These authors estimate a capacity of 10 μg/ gland. As few as three bees (25 min each) provided a sufficient chemical sample for our equipment. Linalool also predominated in materials collected from agonistic *C. cunicularius* females that were actively guarding their nest entrances.

Standardization. The absence of a sampling bias with regard to molecular weight was demonstrated using air samples from known quantities

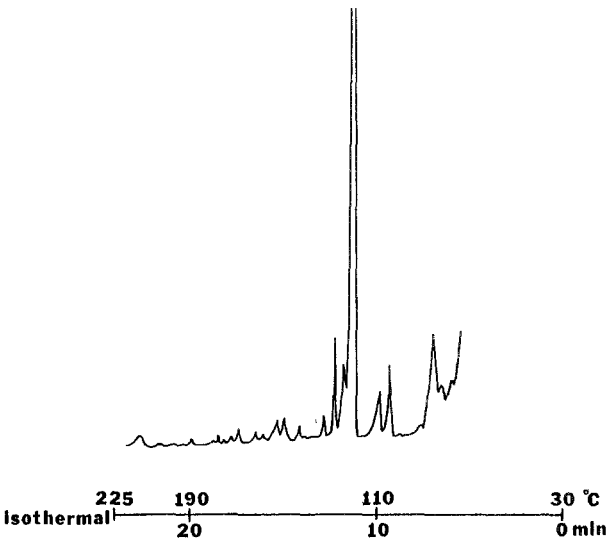

FIG. 2. Gas capillary chromatogram of odors released during digging by three sequentially sampled virgin female *Colletes cunicularius* bees, desorbed from Tenax. Total sample time of 85 min. Precolumn 185° C, 5 min, splitless. The dominant peak is linalool, of mandibular gland origin.

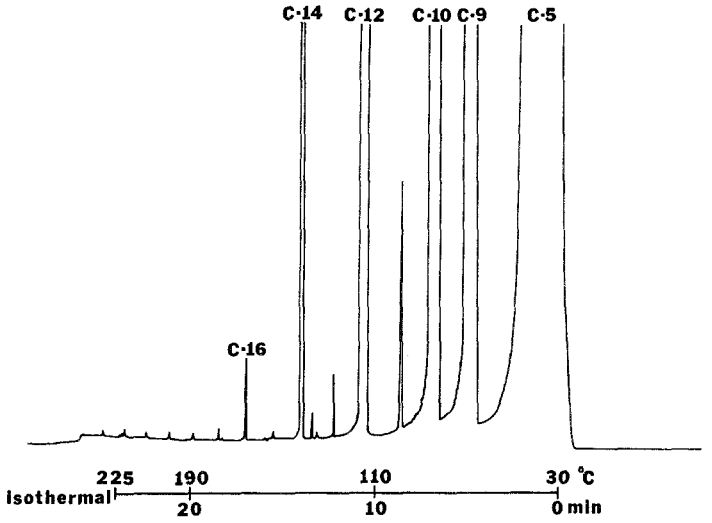

FIG. 3. Gas capillary chromatogram for a 30-min Tenax sample of a hydrocarbon series. The solution was added to the glass wool of a digging tube at 5-min intervals. The air flow rate was 30 ml/min. The composition of the total sampled standard was 80 μl *n*-pentane, 4 μl each *n*-nonane and *n*-decane, and 2 μl each of every even-numbered hydrocarbon from *n*-dodecane to *n*-docosane, inclusive. Precolumn injection was for 5 min (185° C), with the split being open until passage of the solvent peak (*n*-pentane), and then closed.

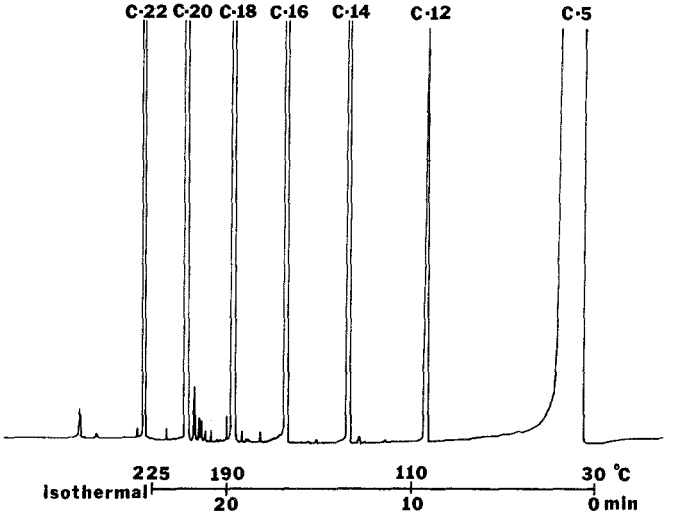

FIG. 4. Gas capillary chromatogram of the pentane wash from the digging tube and glass wool in the above standardization (Figure 3). Solute concentrated through microdistillation, followed by direct GC injection.

of a mixture of hydrocarbons (pentane through docosane). This test mixture was added to the glass wool plug of a "digging tube" every 5 min for 30 min while sampling at ambient temperature. The resulting chromatograms from the Tenax sample and the pentane rinse of the digging tube and its glass wool show that the entire complement of hydrocarbons is represented (Figures 3 and 4). Those hydrocarbons such as dodecane and tetradecane whose melting points are near ambient temperature (18° C) were present in both samples. The chromatogram peaks are quantitatively proportional in size to the volumetric proportions of each hydrocarbon in the test mixture.

DISCUSSION

The flexibility, sensitivity, and convenience of this odor-sampling system recommend it for a diversity of chemical ecological field problems. This is especially true for problems where the functional and/or behavioral significances of exocrine secretions remain undescribed, or where a glandular source of a semiochemical is sought, through comparisons of head-space samples with various glandular extracts. This system can facilitate the interception and identification of chemical messages in natural contexts of their release and reception.

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EFFECT OF EWE URINE AND VAGINAL SECRETIONS ON RAM INVESTIGATIVE BEHAVIOR

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Abstract—Urine from estrous ewes increased the investigative premating activity of the ram. Estrous vaginal tampons resulted in a decrease. The flehmen response of the ram was closely associated with urination by the ewe.

Key Words—Pheromones, urine, vaginal secretions, sheep, flehmen.

INTRODUCTION

In various mammalian species, premating behavior is thought to have olfactory components. Numerous studies have shown an increase in male investigative behavior and/or an effect on mating behavior, when males are presented with estrous female secretions or odors. Doty and Dunbar (1974) found that sexually experienced male dogs spent more time investigating urine and vaginal odors from estrous bitches than similar nonestrous secretions. Paleologou (1977) reported increased interest, and mounting attempts, when vaginal mucus from an estrous cow was rubbed on a dummy. Sambraus and Waring (1975) found that bulls used for natural service preferred nonestrous cows marked with estrous rather than nonestrous urine, whereas bulls used for semen collection at artificial insemination centers showed no such preference. Kiddy et al. (1978) trained dogs to detect by smell vaginal swabs from estrous cows. Subsequently dogs trained on vaginal swabs successfully selected estrous urine samples without being specifically trained for urine, suggesting that an odorous substance may be common to both urine and vaginal secretions.

In a few species, components of the secretions responsible for the attraction and arousal responses have been identified. Goodwin et al. (1979)

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found that methyl-*p*-hydroxybenzoate, a component in estrous bitch vaginal secretions, induced sexual arousal in male dogs, who attempted to mount nonestrous bitches smeared with this substance. Singer and Agosta (1976) found that dimethyl disulfide, a component in estrous hamster vaginal secretion, elicited approaching, digging, and sniffing by the male.

In sheep, ram courtship behavior has olfactory components, since it involves sniffing and nosing the vulva of estrous ewes. Lindsay (1965) found that anosmic rams approached all ewes at random, whereas normal rams preferred estrous ewes. Premating behavior in anosmic rams was considerably modified, but mounting was not affected. Fletcher (1968) also reported a reduction in anosmic rams' ability to find estrous ewes. Kelley (1937) found that smearing pregnant ewes with vaginal swabs from estrous ewes increased the investigative behavior of the ram.

The present study was designed to assess the importance of the olfactory information contained in estrous urine and vaginal secretions in sheep and to find a valid quantifiable assessment of ram interest.

METHODS AND MATERIALS

A total of three preference experiments were conducted. Experiment 1 compared ram reactions to estrous and ovariectomized or metestrous ewes (days 5–11 of the cycle) to establish the behavior pattern of the rams under the artificial test conditions. Experiment 2 investigated the effect of fresh (<3 hr old) estrous vaginal tampons on ram behavior. Experiment 3 investigated the effect of fresh (<3 hr old) estrous urine on ram behavior.

Ten mature Clun-cross ewes were used. Two were ovariectomized and the remaining eight, with normal estrous cycles (15–18 days), were used during the estrous and metestrous stage. Estrus was determined by use of a teaser vasectomized ram; metestrus was considered to be days 5–11 of the estrous cycle. The ovariectomized ewes were never marked by the teaser ram. Estrous vaginal mucus was sampled by inserting a small vaginal tampon (Lillets) for periods ranging from 1 to 20 hr. Urine samples were collected in small modified pediatric urine bags within a maximum time of 3 hr after diagnosis of estrous. Preference tests were conducted within 3 hr of sample collection. The ram population comprised five males, each with a minimum of three season's flock breeding experience, and two ram lambs without mating experience.

In a preference test, a pair of ewes was restrained, 2 m apart, facing away from the ram, who was confined behind a metal gate immediately at the rear of the ewes. All animals were habituated to the test situation for a minimum of 20 min each day for 2 weeks before the beginning of the test series. Each test lasted for 5 min. In all tests several behavioral criteria were used to quantify

ram interest. These included the number of approaches to each ewe, total time (in seconds) near each ewe (within a distance of 30 cm), flehmen responses, and a reaction score to each ewe consisting of the total of all sniffs, nudges, tongue flicks, chin rests, pawing or stamping the ground, vocalizations, and urinations. Ewe tail wags, urinations, and defecations were also recorded.

Experiment 1. Experiment 1 tested the reactions of the seven rams towards each of the eight ewes in estrous, paired with a control ewe. In half the tests the control animals were ovariectomized and in the remaining tests metestrous ewes were used.

Experiment 2. For experiment 2, estrous vaginal secretions from each of the eight ewes were presented to each of the seven rams. The tampon was rubbed around the genital area of the carrier ovariectomized ewe and attached with masking tape under its tail. The control ovariectomized ewe carried a tampon soaked in distilled water.

Experiment 3. For experiment 3, 2 ml of fresh estrous urine was applied to a small pad of absorbent paper under the tail of one ovariectomized ewe (ewe A) and a sample of 2 ml of fresh urine from ovariectomized ewe A was similarly placed under the tail of ovariectomized ewe B as a control. Experiment 2 preceded experiment 3, and both were always conducted on the same day. The genital area of the ewes was washed thoroughly with Hibitane solution between experiments 2 and 3.

A series of tests for experiment 1 was started before experiments 2 and 3, so that each ram had been tested at least three times with an estrous ewe, before going on to experiments 2 and 3. Experiment 1 was continued at regular intervals during the time experiments 2 and 3 were conducted. In all three experiments, the relative position of the ewes and their role as carrier or control was randomly determined for each test.

RESULTS

In experiment 1 there was no significant difference between ram reactions to ovariectomized and metestrous ewes, therefore ram reactions to these two control groups were pooled, and compared with ram reactions to estrous ewes. An analysis of the four variables (Table 1) in experiment 1 indicated that only investigation time and reaction score differed significantly between the estrous and control stimulus conditions, and these two criteria were therefore used to quantify ram interest for experiment 2 and 3.

Experiment 2 (Tables 2 and 3) showed significantly less ram interest in the ewe carrying the estrous tampon, according to both criteria. In experiment 3 (Table 2 and 3) the ewe carrying the estrous urine elicited a higher reaction score and investigation time. The data from experiment 3 were also analyzed

TABLE 1. RAM REACTIONS IN EXPERIMENT 1 (MEAN/TEST)[a]

	No. of tests		No. of approaches		Investigation time (sec)		Reaction score		Flehmen		Ewe urinations	
To control metestrous ewes	26	54	3.5	3.2	57.0	57.5	9.1	8.2	0.30	0.26	0.13	0.18
To control ovariectomized ewes	28		3.0 (NS)		60.0 (NS)		7.4 (NS)		0.22 (NS)		0.23 (NS)	
To estrous ewes	54		4.2 (NS)		170.0*		32.7*		0.37 (NS)		0.02*	

[a]Statistical analysis: t tests for ovariectomized vs. metestrous ewes. Wilcoxon's matched-pairs signed-ranks test for estrous vs. control ewes. *$P \leq 0.001$; NS = not significant.

TABLE 2. COMPARISON OF MEAN TIME RAM SPENT NEAR EWES[a]

Comparison groups	No. of tests	Mean investigation time (sec)		Difference between means	Significance level (*P*)
		Estrous ewe or carrier	Nonestrous ewe or carrier		
Experiment 1					
Estrous ewe/ control ewe	54	153	50	103	⩽0.001
Experiment 2					
Ovariectomized ewe with estrous tampon/ Ovariectomized ewe with control tampon	47	70	97	-27	⩽0.05
Experiment 3:					
Ovariectomized ewe with estrous urine/ ovariectomized ewe with control urine	58	116	75	41	⩽0.05
Ovariectomized ewe A/ ovariectomized ewe B	58	95	96	-1	NS

[a]Wilcoxon's matched-pairs signed-ranks tests.

TABLE 3. COMPARISON OF MEAN RAM REACTION SCORES TO EWES[a]

Comparison Groups	No. of tests	Mean reaction scores to:		Mean difference	Significance level (*P*)
		Estrous ewe or carrier	Nonestrous ewe or carrier		
Experiment 1					
Estrous ewe/control ewe	54	35	8	27	⩽0.001
Experiment 2					
Ovariectomized ewe with estrous tampon/ ovariectomized ewe with control tampon	47	14	23	-9	⩽0.05
Experiment 3					
Ovariectomized ewe with estrous urine/ ovariectomized ewe with control urine	58	26	15	11	⩽0.05
Ovariecomized ewe A/ ovariectomized ewe B	58	18	22	-4	NS

[a]Wilcoxon's matched-pairs signed-ranks test.

for ram bias towards one of the ovariectomized ewes. No significant differences were found.

When ewe reactions for all three experiments were analyzed, a very close association between the flehmen response and urination in nonestrous ewes emerged. In nonestrous ewes, urination rates ranged from 10 in 54 tests for ewes paired with estrous sheep, to 25 in 58 tests for carriers of estrous urine and were positively correlated with ram reaction score ($r = 0.216, p \leq 0.001$). In turn, ewe urination usually led to the flehmen response. All χ^2 tests for independence between ewe urination and flehmen response were significant at the 0.1% level.

The urination rate of estrous ewes was very low in spite of intensive ram courtship behavior. The flehmen response was observed independently of ewe urination and at a similar rate (Table 1) to that towards the control ewes.

DISCUSSION

The results of experiment 1 showed that under the artificial test conditions used, ram reaction scores and time spent near a ewe could be used to quantify ram responses elicited by the stimulus of an estrous ewe. The rams' responses to metestrous and ovariectomized ewes were similar, despite the hormonal differences between both groups. Thus it can be inferred that during metestrous the ovarian hormones did not change ewe attractiveness. Therefore, it was justifiable to use ovariectomized ewes as carrier animals in experiments 2 and 3.

The flehmen response was not a reliable indicator of an estrous stimulus. Rams showed this response to nonestrous ewes, where it was elicited by ewe urination. Estrous ewes had a much lower urination rate, yet the flehmen response was frequently observed. This suggests that other stimuli were present in the estrous ewe which elicited the flehmen response. Therefore the flehmen response ought to be seen as an indication of general olfactory investigation, not as a specific response to estrus.

The results of experiment 3 seem to implicate estrous urine as one potential source of a sex attractant pheromone. However, comparing ram interest in a ewe carrying an estrous urine sample with that in an estrous ewe shows that ram reactions were more definite and prolonged to the estrous ewe. This could have several different causes. Urine might only have a minor role to play as a chemical messenger, or the olfactory message of estrous urine could be contradicted by the frequent urinations of the carrier ewes. Behavioral clues, which we could not eliminate (e.g., tail wagging and firm stance), will also be important.

However, an alternative explanation is possible. The slight difference between ram interest in ovariectomized ewes carrying estrous and nonestrous

urine could be due to estrous urine lacking a signal indicating nonestrous, rather than estrous urine carrying an attractant signal. The high urination rates of nonestrous ewes in response to ram approaches could be a positive attempt on the part of the ewe to demonstrate her nonestrous physiological state.

If urine does not carry an attractant signal, the source of any such signal has to be sought elsewhere. Estrous mucus could be such a carrier. However, the results of experiment 2 did not support this hypothesis. In fact, the current data suggest a negative effect of the estrous tampons on ram behavior. This result does not agree with Kelley's findings (1937).

We can suggest several explanations for the present results. The observed negative response to vaginal tampons may be due to an alteration in the vaginal microflora following insertion of the tampon. The response could be due to a concentration effect. An odor which is attractive at physiological concentration may become a repellent at higher concentrations. If attractant odors of vaginal mucus develop in the posterior part of the vagina, our tampon collection technique might not have given adequate recovery. Finally, vaginal secretions per se may not be attractive.

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SITE OF SECRETION OF THE TRAIL MARKER OF THE EASTERN TENT CATERPILLAR

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Abstract—A new site of secretion of a chemical trail marker was found on the sternum at the tip of the last abdominal segment of the larva of the eastern tent caterpillar *Malacosoma americanum*. Larvae marked from this site by drawing their sterna along the substrate when they extended existing trails in search of food and again when they established recruitment trails to food-finds. Differences in the quantity or quality of the marker deposited by exploring and recruiting caterpillars may account for the greater activity of the recruitment trails.

Key Words—*Malacosoma americanum*, eastern tent caterpillar, Lepidoptera, Lasiocampidae, trail pheromone, pheromone secretory site, silk trail.

INTRODUCTION

A chemical basis for trail following in larval Lepidotera has been demonstrated for *Malacosoma americanum* (F.) (Fitzgerald and Gallagher, 1976), *Eriogaster lanestris* L. (Weyh and Maschwitz, 1978), *M. disstria* Hubner (Fitzgerald and Edgerly, 1979), and *Hemileuca oliviae* Cockerell (Capinera, 1980). All of these species produce silk trails, extracts of which yield chemical trail markers. Weyh and Maschwitz (1978) suggested that the marker is produced in the spinneret complex of *E. lanestris*. Capinera (1980) found that a methylene chloride extract of the anterior portion of the larva of *H. oliviae*, which contains the silk glands, elicited trail following. It has not been

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demonstrated, however, that the marker occurs as an intrinsic component of the silk strand of any species.

The trail system of *M. americanum* differs from that described for other species of trail-following Lepidoptera in that two distinct trail types are employed (Fitzgerald, 1976). Caterpillars lay down exploratory trails as they move from their tent in search of food. Caterpillars that find food and feed reinforce the trails that they follow back to the tent. Reinforced trails are more stimulating to the larvae than exploratory trails and are followed preferentially. They are analogous to the recruitment trails of eusocial insects since they serve, at least in part, to lead hungry tentmates to food-finds.

Although silk is a conspicuous component of the trail system of the eastern tent caterpillar, the caterpillars frequently do not spin silk when laying down recruitment trails (Fitzgerald, 1976). In addition, the last instar larva continues to mark trails even though it does not spin silk except to form its cocoon (Fitzgerald and Edgerly, 1979). These observations suggested that a previously unrecognized site of secretion of a trail marker might exist in this species and prompted the investigation reported here. We also sought to establish the role of silk as a trail component.

METHODS AND MATERIALS

Caterpillars were obtained from egg masses collected in Cortland County, New York and maintained at 2° C until needed. After hatching, larvae were maintained in Petri dishes and fed the leaves of black cherry (*Prunus serotina*). Whole colonies were also established in the laboratory on wooden stands that simulated the branch cradles in which larvae normally spin their tents (Figure 1).

The silk glands and the glands of Filippi, the ducts of which anastomose with the ducts of the silk glands (Snodgrass, 1924), were extracted in hexanes or ether. Extracts of the head, thorax and abdomen of 20 fourth instar caterpillars were also prepared. The abdomen was further divided by separating the posterior 5 mm from the rest of the segment. The body sections were ground separately in hexanes or ether. Artificial trails were prepared from the supernatant. Extracts were laid out in 20-mm-long lines on paper cards and the response of first or second instar caterpillars to the artificial trails observed.

Trails were prepared from silk pulled directly from the spinneret to determine if uncontaminated silk elicited a trail following response. A caterpillar was held by the back of its head with a vacuum forceps, and the strand of silk it secreted was wound onto a strip of chromatography paper, rotated slowly by an electric motor. Approximately 4 m of silk were wound onto 25-mm-long by 2-mm-wide strips. The response of first or second instar caterpillars to these strips was observed directly.

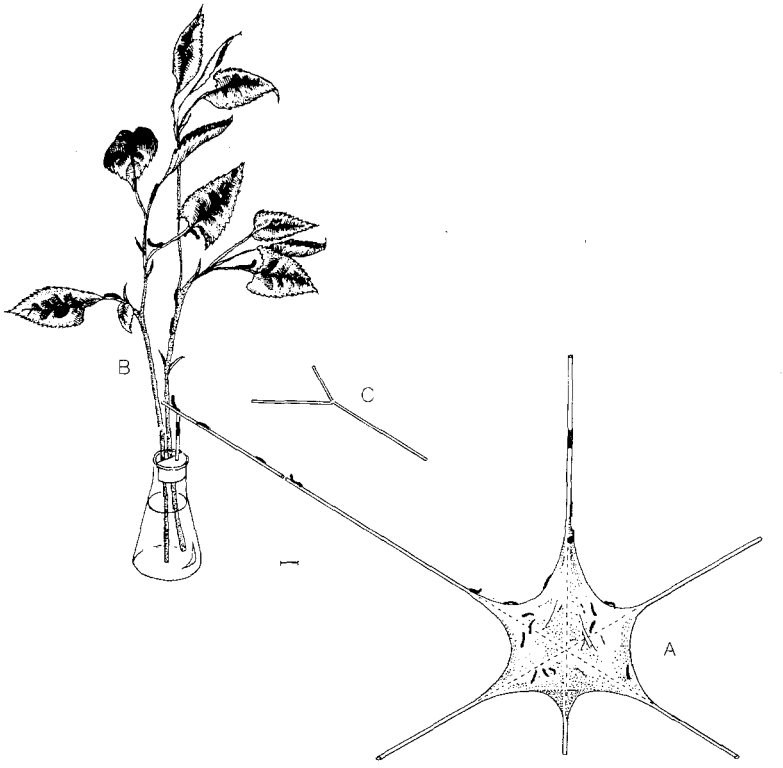

FIG. 1. Apparatus used to investigate the site of secretion of the trail pheromone of larval *Malacosoma americanum*. (A) rearing stand; (B) detachable feeding unit; (C) Y-maze. Scale bar = 1 cm.

The ability of fourth instar caterpillars to deposit silk was destroyed by excising their labia or cauterizing the tip of their spinnerets. Caterpillars so treated were allowed to fully recover before they were used. The trails of these caterpillars were compared to those of intact controls. Trails were obtained by allowing 20 caterpillars to climb a 30-cm-long by 4-mm-wide strip of chromatography paper suspended by one end. The strip was then cut into 20-mm-long sections. The relative strength of these trails was assessed by recording the time it took first or second instar caterpillars to move from one end of a strip to the other. The strips were arranged on a horizontal plane in the center of a sheet of chromatography paper and were used only once.

The effect of blocking or excising potential sites of pheromone secretion on recruitment trail marking was assessed by introducing treated caterpillars into colonies of otherwise intact larvae. Sites were blocked with paraffin applied at approximately 60° C with an electrically heated wire loop. The response of host caterpillars to trails deposited by the introduced larvae as

they returned from feeding sites was observed directly. Y-maze tests, described in detail later on, were also conducted to further establish the site of origin of the recruitment trail marker.

RESULTS AND DISCUSSION

Extracts of the glands, head, thorax, and anterior abdomen either failed to elicit trail following or elicited a weak response. Stronger response occurred to the extract of the tip of the abdomen. Serial dilutions of the extracts confirmed that the marker was concentrated at the posterior tip. Subsequent investigation showed that a trail marker accumulates on the surface of the abdomen in the region between the anal prolegs (Figure 2). Preliminary scanning electron microscopy and gross dissection revealed no apparent glands or glandular openings in this area. The marker does not appear to be stored in significant quantities.

The pheromone can be lifted from the caterpillar by wiping the creased edge of a folded sheet of filter paper across the sternal secretory site. First instar caterpillars followed trails prepared in this manner more readily than

FIG. 2. Sternum of the last abdominal segment of the larva of *Malacosoma americanum*. Excision of area isolated by dotted line precluded deposition of the trail marking pheromone. Drawn from scanning electron micrograph (42×).

they did trails prepared from tip extracts. The decrease in effectiveness of the extract was probably due to dilution and, possibly, also to a more rapid degradation of the marker following its dispersal. Artificial trails could not be established by wiping filter paper across other regions of the body. The trail marker of this insect is nonvolatile and persistent, although its effectiveness diminishes with time (Fitzgerald, 1976). The weak trail-following responses elicited by extracts of body regions other than the tip of the abdomen were likely due to the contamination of the caterpillars.

Exploratory Trail. Laboratory colonies of caterpillars were observed under low magnification as they moved to food. The larvae held the sternal secretory site above the substrate when they followed previously marked trails to food but lowered their sterna when they moved over unmarked branches (Figure 3). The marker thus appears to be a component of the exploratory trail that the larvae lay down when they extend their existing trail system into new areas.

Eastern tent caterpillars also deposit copious quantities of silk as they move over the branches of the host tree. Frequently used pathways eventually become marked with dense trails of silk. While it is clear from the observations reported above that caterpillars follow pheromone trails even when they lack silk, it has not been previously established if silk, per se, also elicits or augments a trail-following response. Strips of paper onto which uncontaminated silk was wound were only weakly stimulating to the caterpillars. In 15 tests, only 3 caterpillars walked the entire length of these strips during 3-min trails, although all walked part way. The same caterpillars walked the entire length of similar strips that had been wiped along the surface of the sternal secretory site of fourth instar caterpillars in 72 ± 14.0 sec ($\pm$SE) ($N = 15$). In contrast, silkless trails that were deposited on paper strips by caterpillars that had their labia excised or their spinnerets cauterized were significantly less effective in eliciting trail following than trails marked with

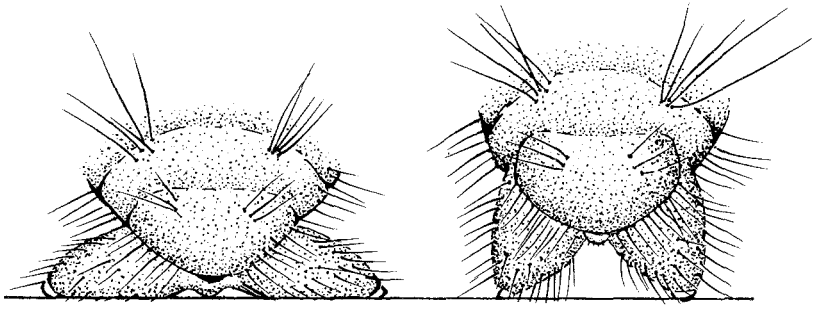

FIG 3. Position of the tip of the abdomen of larval *Malacosoma americanum* when marking (left) and when not marking (right).

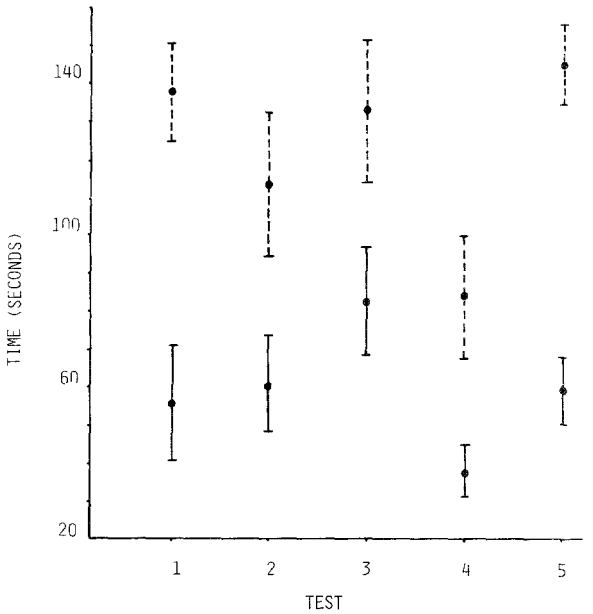

FIG. 4. Time taken ($X \pm$ SE, $N = 10$) by first or second instar *Malacosoma americanum* larvae to travel the length of trails deposited on paper strips by control larvae (—) or by treated larvae (---). Larval treatments: Tests 1–4, labialectomized; Test 5, spinneret cauterized. All within-test differences were statistically significant at $P < 0.05$.

silk that were deposited by intact caterpillars (Figure 4). The results of these studies suggest that while uncontaminated silk is in itself not effective in eliciting trail following, it enhances trail effectiveness when deposited along with the trail pheromone. It remains an unlikely possibility, however, that the silk pulled from the spinneret was ineffective because it was not laid out in a proper configuration. These results, furthermore, do not preclude the possibility that silk secreted by unrestrained caterpillars might contain an intrinsic factor that elicits trail following. The factor may be lacking in silk pulled from the subdued caterpillar.

Trails deposited on paper strips by labialectomized caterpillars that also had their sternal secretory sites covered with paraffin were largely ineffective in eliciting trail following. First instar caterpillars took 351.3 ± 32.0 sec ($N = 19$) to walk the length of 20-mm-long strips bearing such trails. This indicated that there were no other sites of pheromone secretion. Contamination of the paraffin seal and other parts of the caterpillar's body with small quantities of trail pheromone apparently accounted for the residual trail following that did occur.

Recruitment Trail. Labialectomized fourth instar caterpillars were marked with fluorescent powder and introduced into colonies of intact caterpillars. The introduced caterpillars were allowed to move to feeding sites. The response of their tentmates to the trails they deposited as they returned to the tent was observed. In each of four separate tests involving different groups of labialectomized caterpillars, the treated caterpillars clearly recruited other larvae to food-finds. These results supported previous observations that recruiting caterpillars often do not secrete silk (Fitzgerald, 1976).

Caterpillars in laboratory colonies were observed to press their sternal secretory sites against the substrate as they followed their exploratory trails back to the tent after feeding. In addition, caterpillars that were introduced into laboratory colonies after having their sternal secretory sites covered with paraffin appeared unable to recruit tentmates to their food-finds. These observations indicated that the recruitment trail marker originated from the sternal secretory site and suggested a follow-up experiment. The ventral surface of the tips of two groups of 30 caterpillars were excised with a razor blade as shown in Figure 2. Care was taken not to injure tissue immediately surrounding the anus. The operation had little apparent effect on the caterpillars, but as a precaution they were allowed to pass through one or more molts before their trail marking ability was assessed. Both groups were allowed to establish tents on separate stands as shown in Figure 1. Cherry leaves were provided at the ends of short, removable bridge sections (feeding units). A colony of approximately 200 intact caterpillars (controls) was established in a similar fashion.

To conduct the test, the caterpillars from the control colony were allowed to move onto a clean wooden Y maze (Figure 1) at the onset of one of their activity periods. After the caterpillars had established new exploratory trails on the maze, they were gently herded back to their tent by lightly touching them on the tip of the abdomen with a brush. The maze was then set aside and the feeding unit was abutted to the bridge of the control tent. Twenty caterpillars were allowed to move onto it. Twenty tip-excised caterpillars were allowed to move from their tent onto a similar feeding unit at the same time. While both groups of caterpillars were feeding, the feeding units were moved away from the tents and abutted to randomly selected arms of the maze. The two contingents of caterpillars moved from the feeding units onto the arms of the maze when they had finished feeding and were collected when they reached the end of the stem. Twenty larvae from the control colony were then allowed to move onto the stem of the maze, one at a time, and allowed to choose between the arms crossed by the groups of control and tip-excised caterpillars. Since the recruitment trails of fed, returning caterpillars are many times more effective in eliciting trail following than trails deposited by outbound, hungry caterpillars (Fitzgerald, 1976), each of the 20 larval choices was assumed to be

TABLE 1. NUMBER OF *Malacosoma americanum* LARVAE SELECTING ARM OF Y MAZE USED TO RETURN CONTROL (A) AND TIP-EXCISED (B) CATERPILLARS

	Trail selected	
Replicate	A	B
1	20	0
2	20	0
3	20	0
4	19	1
5	20	0
6	15	5

based upon the presence or absence of a recruitment trail rather than on the choice of preceding larvae. Six separate tests were conducted utilizing both colonies of tip-excised caterpillars. The results, presented in Table 1, show that the tip-excised caterpillars failed to establish recruitment trails.

Although the above observations indicated that a sternal secretion was used to mark both recruitment and exploratory trails, previous studies (Fitzgerald, 1976) showed that on a one-to-one basis recruiting caterpillars secrete stronger trails than explorers. The difference between the two trail types may be accounted for in several ways. It is possible that two distinct trail chemicals are employed, although chemical analyses currently underway suggest that this is not the case. In addition, we have found no evidence that chemical components of the food brought back to the tent in the guts of fed caterpillars are directly involved in recruitment trail marking. Although hungry caterpillars follow a crude ether extract of cherry leaves applied in a narrow trail to the surface of their tent, such trails are short-lived and noncompetitive with authentic recruitment trails.

Assuming only one pheromone is involved, the caterpillars might still differentiate the two trail types by secreting a smaller quantity of the marker when exploring than when recruiting. Alternatively, a partially degraded surface residue of the marker secreted during the last foraging trip might be used to mark exploratory trails. Recruitment trails may be marked with newly secreted, fully active pheromone. Histological studies of the sternal secretory site are currently under way.

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SYNTHESIS OF (±)-10-METHYL-1-DODECANOL ACETATE, THE CHIRAL COMPONENT OF THE SMALLER TEA TORTRIX MOTH (*Adoxophyes* sp.), WITH AN OPTION FOR ASYMMETRIC INDUCTION

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Abstract—A new route to (±)-10-methyl-1-dodecanol acetate, a minor component of the smaller tea tortrix moth (*Adoxophyes* sp.), is described. An optional sequence that permits the generation of the chiral center with enantiomeric excesses (ee's) as high as 74% (*R*) or 80% (*S*) employing the available (*S*)-(−)-prolinol as a chiral auxiliary may be included. High-performance liquid chromatography of diastereomeric intermediates allows preparation of products with greater ee's.

Key Words—Smaller tea tortrix moth, *Adoxophyes* sp., Lepidoptera, Tortricidae, asymmetric induction, 10-methyl-1-dodecanol acetate, ester, chirality.

INTRODUCTION

The structures of many insect sex pheromones contain one or more centers of asymmetry (Rossi, 1978). The current state of knowledge regarding relationships between molecular configuration and biological activity has been succinctly summarized (Silverstein, 1978). The continuing high interest in developing new methods of asymmetric synthesis and new techniques for measuring configurational composition of, for example, synthetics is therefore certainly apt. To date most of the asymmetric syntheses of insect sex pheromones have been initiated with commercially available chiral starting materials or have involved resolution of a key synthetic intermediate. Limited examples of syntheses employing techniques of asymmetric induction bespeak the difficulties inherent in achieving a high degree of stereoselection. The

current outpouring of research in asymmetric synthesis in organic chemistry (Valentine and Scott, 1978; Bartlett, 1980) will likely change this situation and, in fact, several elegant syntheses of insect pheromones by asymmetric induction have appeared very recently (Ade et al., 1980; Enders and Eichenauer, 1979; Farnum et al., 1979; Hofmann and Ladner, 1979; Larcheveque et al., 1979; Nishizawa et al., 1981; Sakito and Mukaiyama, 1979; Katsuki and Sharpless, 1980; Midland and Tramontano, 1980; Vigneron and Bloy, 1980).

We now describe a synthesis of 10-methyl-1-dodecanol acetate, a chiral component of the sex pheromone complex of the smaller tea tortrix moth (*Adoxophyes* sp.) (Tamaki et al., 1978; Suguro and Mori, 1979). The relative biological activities of the enantiomers has been described, and only slightly greater activity of the *R* isomer was reported (Tamaki et al., 1980). The synthetic route can be used to yield a racemic product or a product rich in one or the other of its enantiomeric forms. Involved in producing the latter type of product are alkylations of chiral amide anions via asymmetric induction, as recently described (Evans and Takacs, 1980). Also, we note that the consecutive reactions of stereochemically alkylating the amide at the carbon α to the carbonyl, hydrolyzing the amide, and then reducing the carboxyl group to a methyl group is a way to stereospecifically introduce methyl branching into a hydrocarbon moiety. Because many biologically active compounds isolated from insects do contain branched structures, their synthesis might be accomplished via the above-mentioned consecutive reactions.

METHODS AND MATERIALS

Gas–liquid chromatography (GLC) was performed with a Varian 2400® instrument (flame ionization detection, helium carrier) using columns and temperatures as indicated in Table 3. High-performance liquid chromatography (HPLC) was conducted with a system consisting of a Waters model M-6000® pump, a Rheodyne injector, and a Waters differential refractometer (model R-401®) employing columns and conditions as described in Table 3. Infrared (IR) data were obtained with a Perkin-Elmer 467 grating infrared spectrophotometer (CCl_4) and ^1H nuclear magnetic resonance (NMR) data were obtained with a Bruker WHX-90® spectrometer (10–15% solutions in acetone-d_6. Mass spectral (CIMS) data were obtained with a Finnegan model 3200® chemical ionization mass spectrometer (isobutane) that was equipped with a chromatographic inlet (Varian model 1400®) served by a 3% OV—101 column, 3.2 mm × 1.5 m. The (*S*)-(−)-prolinol was either purchased from Aldrich Chemical Co. or synthesized from L-proline by reduction with lithium aluminum hydride (LAH) in tetrahydrofuran (THF) (Gassman and Fentiman, 1967).

(±)-*2-Ethyl-10-undecenoic Acid, II.* Commercial 10-undecenoic acid (9.2 g, 50 mmol) was converted to a dianion by its addition to lithium diisopropylamide (LDA) made from diisopropylamine (16.8 ml, 120 mmol) and *n*-butyllithium (BuLi) (4.6 ml, 110 mmol) in THF (70 ml) and hexamethylphosphoric triamide (HMPT) (10 ml) under nitrogen in the usual manner (Pfeffer and Silbert, 1970). The solution was cooled to ca. −20° C and ethyl bromide (9.0 ml, 120 mmol) was injected. The resulting mixture was stirred at ambient temperature for 1.5 hr and then partitioned between 10% HCl and hexane. The product acid was obtained by (1) hexane extraction of the aqueous acid phase, (2) extraction of the organic phase with 1.25 N NaOH to permit removal of nonacidic material with the hexane, and (3) acidification of the alkaline extract and reclamation of the acid with hexane. Distillation afforded 9.5 g of acid (74% corrected for the presence of ca. 15% undecenoic acid as determined by GLC of a MeOH-BF_3-esterified sample): bp 125–127° C (0.01 mm); IR 1705 (C=O), 990, 910 ($CH{=}CH_2$), NMR δ 0.88 (t, 3H, CH_3CH_2), 2.0 (m, ~2H, $CH_2C{=}$), 4.8–5.6 (m, vinyl H); methyl ester: CIMS (*m/e*) 227 (P + 1).

(±)-*2-Ethyl-10-undecen-1-ol, III.* The acid II (7.42 g, 35 mmol) was added dropwise to a stirred solution of LAH (2.7 g, 70 mmol) in THF (50 ml) that was cooled in an ice bath. The resulting mixture was stirred under reflux for 16 hr, then worked up with 1 N NaOH, filtered, washed with water, and dried ($MgSO_4$) in the usual manner. Distillation gave 5.3 g (77%) of III: bp 100–103° C/0.1 mm; IR 3640 (OH), 1000 b, 910 ($CH{=}CH_2$); NMR δ 0.88 (bt, 3H, $C\underline{H}_3CH_2$), 2.1 (m, 2H, $C\underline{H}_2C{=}$), 3.15 (bs, 1 H, OH), 3.44 (m, 1 H, $C\underline{H}_2OH$), 4.9–5.9 (m, vinyl H); CIMS (*m/e*) 199 (P + 1), 181 (P + 1 − H_2O).

(±)-*10-Methyl-1-dodecene, IV.* The alcohol III (2.0 g, 10 mmol) was added to a stirred, cooled (0–10° C) suspension of triphenylphosphine dibromide (12 mmol) in methylene chloride (CH_2Cl_2) (20 ml). The resulting solution was allowed to stand at ambient temperature for 2 hr. It was evaporated to dryness, and the residue was triturated with hexane and suction filtered. The filtrate was concentrated, and the crude bromide was then passed through a column of silica gel (10 g) with pentane (100 ml). Concentration provided a bromide of sufficient purity for reduction to IV: NMR δ 0.88 (bt, 3H, CH_3CH_2), 2.0 (m, $CH_2C{=}$), 3.49 (d, J = 7, 2H, $CHC\underline{H}_2Br$), 4.8–5.8 (m, vinyl H); CIMS (*m/e*) 261,263 (P + 1), 181 (P + 1 − Br). The crude bromide 2.6 g, 9.9 mmol) was dissolved in THF (25 ml) under nitrogen and chilled in an ice bath. Lithium triethylborohydride in THF (19.8 ml, 19.8 mmol) was injected. The bath was removed, and the solution was allowed to stand overnight. The reaction mixture was worked up with $NaOH/H_2O_2$ in the usual manner (Brown and Krishnamurthy, 1973), and the alkene IV was obtained by careful concentration of the pentane phase. The olefin (1.9 g of crude product containing some residual solvent) was distilled bulb-to-bulb:

bath temp. 110° C (30 mm); IR 1000b, 910 ($CH{=}CH_2$); NMR δ 0.87 (bt, 3H, $C\underline{H}_3CH_2$), 2.0 (m, $C\underline{H}_2C{=}$), 4.8–5.6 (m, vinyl H); CIMS (*m/e*) 183 (P + 1).

(±)-*10-Methyl-1-dodecanol, V.* Alkene IV, obtained as described above (ca. 10 mmol), was added to a solution of disiamylborane prepared from diborane (10 ml of 1 M THF) and 2-methyl-2-butene (2.1 ml, 20 mmol) in the usual manner (Brown, 1975). After the usual work-up with NaOH H_2O_2, the crude alcohol was distilled bulb-to-bulb giving 1.5 g (75%): bath temp. 95° C (0.1 mm); IR 3640 (OH); NMR δ 0.87 (bt, 3H, $C\underline{H}_3CH_2$), 3.09 (bs, 1 H, OH), 3.51 (m, 2H, $C\underline{H}_2OH$); CIMS (*m/e*) 199 (P + 1), 183 (P + 1 − H_2O).

(±)-*10-Methyl-1-dodecanol Acetate, I.* Alcohol V (1.0 g, 5.0 mmol) was converted to its acetate with acetyl chloride (0.53 ml, 7.5 mmol) and triethylamine (1.05 ml, 7.5 mmol) in anhydrous ether (30 ml) in the usual manner. Distillation of the crude product bulb-to-bulb yielded acetate I (1.1 g, 83%): bath temp. 95° C (0.1 mm); IR 1740 (ester C=O); NMR δ 0.88 (bt, 3H, $C\underline{H}_3CH_2$), 1.99 (s, 3H, CH_3CO_2), 4.01 (5, J = 7, 2H, $C\underline{H}_2O_2C$); CIMS (*m/e*) 243 (P + 1), 183 (P + 1 − CH_3CO_2H).

(S)-(−)-*Prolinol, 10-Undecenamide, VIa and its O-Derivatives VIb–d.* The undecenoic acid (41.6 g, 226 mmol) was converted to its acid chloride using thionyl chloride (20 ml, 271 mmol) and dimethylformamide (2.2 ml, 27 mmol) in anhydrous ether (250 ml) in the usual manner (Bosshard et al., 1959). The mixture was stripped of solvent, and the crude acid halide was taken up in hexane, filtered through Na_2SO_4, and concentrated again. An ethereal solution of the crude product was then added dropwise to a chilled (10° C) solution of (*S*)-(−)-prolinol (10.1 g, 100 mmol) and triethylamine (42 ml, 300 mmol) in anhydrous ether (250 ml). The resulting mixture was stirred for 4 hr at ambient temperature, and the crude diacylated product was obtained by working the mixture up with aqueous acid and water washes (IR 1745, 1640). Saponification in a mixture of 170 ml of 6 N KOH, methanol (300 ml), and ethanol (100 ml) was complete in 4 hr at room temperature. The mixture was kept cool (<25° C) during initial mixing in order to avoid Claisen condensation, which yields di-9-decenylketone (IR 1720; CIMS, *m/e*, 307, P + 1). After concentration to ca. 1/2 volume, the mixture was diluted with water and extracted with ether. The crude product could be employed directly for *O*- and *C*-alkylation. The yield of crude VIa was 20.5 g (78%): Hickman distillation bath temp. 160° (0.02 mm); IR 3500b (OH), 1630 (amide C=O), 1000b, 910 ($CH{=}CH_2$); NMR δ 1.9 (m, $C\underline{H}_2C{=}$), 3.1 (s, 1H, OH), 3.50 (m, 2H, $C\underline{H}_2OH$), 4.8–5.8 (m, vinyl H).

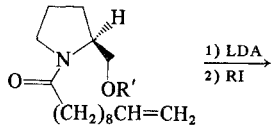
The ethers were prepared from the sodio derivative of VIa. Compound VIa was stirred for 1 hr in excess hexane-washed NaH and THF. The appropriate halide (methyl iodide for b, chloromethyl methyl ether for c, and methoxyethoxymethyl chloride (MEM-chloride) for d) was added in 10% excess, and the resulting mixture was stirred for 1 hr at room temperature. The crude ether was then obtained by dilution of the mixture with water and

extraction with hexane. The yields were essentially quantitative; satisfactory IR, NMR, and CIMS data were obtained for all compounds. For example, the MEM ether VId bulb-to-bulb distillation; bath temp. 185° C (0-015 mm); IR 1645 (amide C=O), 1000b, 910 ($CH{=}CH_2$); NMR δ 1.9 (m, $CH_2C{=}$); 3.29 (s, 3H, OCH_3), 3.5 (m, ~6H, OCH_2), 4.63 (m, ~2H, OCH_2O), 4.8-5.8 (m, vinyl H); CIMS (*m/e*) 356 (P + 1).

Asymmetric Alkylation of VIa and d. Although some experimental parameters are presented in Table 1, a detailed procedure for the alkylation of VIa leading to an 80% enantiomeric excess (ee) of the *S,S* diastereomer is given here. Amide VIa (15.8 ml of a 1.42 M THF solution, 22.5 mmol) was injected into a solution of LDA, which had been prepared from diisopropylamine (14.2 ml, 101 mmol) and *n*-BuLi (28.3 ml of 2.4 M, 68 mmol) in THF (90 ml) and HMPT (10 ml) at ice-bath temperature. The bath was removed, and the solution was allowed to stand for 0.5 hr. The mixture was then immersed in dry ice-ether (ca. −100° C), ethyl iodide (5.5 ml, 68 mmol) was injected, and the mixture was kept cold for 1 hr. The bath was removed and, after 0.25 hr, the mixture was diluted with water and extracted with hexane. The organic phase was washed with dilute HCl, then with water, and dried ($MgSO_4$). The crude product of an alkylation was generally converted in part to a MEM ether, as previously described, to allow analysis of diastereomer composition and could also be employed directly for hydrolysis to the acid II. A sample of ethylated VIa was distilled bulb-to-bulb: bath temp. 165° (0.02 mm); IR 3500b (OH), 1625 (amide C=O), 990, 910 ($CH{=}CH_2$); NMR δ 0.85 (bt, 3H, $C\underline{H}_3CH_2$); 1.9 (m, $CH_2C{=}$), 3.1 (bs, 1H, OH), 3.5 (m, 2H, $C\underline{H}_2OH$), 4.8-5.9 (vinyl H).

TABLE 1. ALKYLATION OF (*S*)-(−)-PROLINOL 10-UNDECENAMIDE AND ITS MEM ETHER IN THF

R′	R	Conditions	*S*:*R*
H	C_2H_5	*t*-BuLi, -50°C, 1 hr, 25°C, 1 hr, RI at -120°C	76:24
H	C_2H_5	*t*-BuLi, HMPT, -78°C, 1 hr, RI at -78°C, 1 hr	84:16
H	C_2H_5	LDA, HMPT, -78°C, 2 hr, RI at -78°C, 1 hr	88:12
H	C_2H_5	LDA, HMPT, 0-25°C, 0.5 hr, RI at -100°C, 1 hr	90:10
MEM	C_2H_5	LDA, HMPT, -78°C, 2 hr, RI at -78°C, 1 hr	13:87
MEM	CH_3	LDA, HMPT, -78°C, 2 hr, RI at -78°C, 1 hr	21:79



The ethylated MEM ether from VIa was purified by bulb-to bulb distillation: bath temp. 185° C (0.015 mm); IR 1645 (amide C=O), 1000b, 910 ($CH{=}CH_2$); NMR δ 0.85 (bt, 3H, $C\underline{H}_3CH_2$); 2.0 (m, ~2H, $C\underline{H}_2C{=}$), 3.29 (s, 3H, $OC\underline{H}_3$), 3.53 (m, ~6H, $OC\underline{H}_2$), 4.65 (m, 2H, $OC\underline{H}_2O$), 4.8–5.8 (m, vinyl H); CIMS (*m/e*) 384 (P + 1), 309 (P + 1 − $OCH_2CH_2OCH_3$).

(±)- *or* (−)-*2-Ethyl-10-undecenoic Acid, II.* Hydrolysis of VIa, the diastereomer mixture rich in (*S*)-(+)-II, was conducted in a two-phase system consisting of conc. HCl (ca. 0.5 ml/g amide) and hexane (10 ml/g amide) under reflux for 8 hr. After this period of time no amide remained, and a 50–60% yield of crude acid was obtained. Control experiments indicated no loss in ee for this operation, but a possible loss of 2% ee upon distillation of the acid. The rotation of pure (*S*)-(+)-II (corrected for the presence of 10% unalkylated acid as determined by GLC analysis of methyl esters) is calculated to be: $[\alpha]_D^{27}$ + 8.0 (c 9.2, $CHCl_3$).

The (*R*)-(−)-II was obtained by a two-step process in which the ethylated MEM ether, further purified by preparative HPLC, was first treated with anhydrous $ZnBr_2$ (4 g/g amide) in CH_2Cl_2 at room temperature. Reaction was sluggish and incomplete despite prior drying of the $ZnBr_2$ with acetic anhydride in benzene under reflux. Additions of $ZnBr_2$ were continued until <10% of the MEM ether remained. The product was worked up with water and CH_2Cl_2, and the crude prolinol amide then hydrolyzed to the acid as described above. Overall yields, however, were 30–40%, and the cleavage of the etherified amides generally could stand improvement.

The acids, (+)- and (−)-II were then carried through the same sequence of reactions as described for the racemic pheromone I. A sample of (*R*)-(−)-III had $[\alpha]_D^{27}$ − 2.6 (c 5.9, $CHCl_3$). The alcohol consisted of a 96:4 ratio of enantiomers as determined by GLC of amides based on (*S*)-α-methylbenzylamine. Hence, the calculated rotation of configurationally pure III would be 2.8°.

Samples of I were obtained which were highly enriched in either enantiomer: 92% ee of *R* and 80% ee of *S*. A sample of the *S* isomer was distilled to produce a material of 95% purity (GLC): $[\alpha]_D^{26}$ + 4.26 (16.2 $CHCl_3$). The value for configurationally pure I would therefore appear to be 5.60° provided the impurities are optically inactive (Suguro and Mori, 1979, report 4.85° neat). The discrepancy may be due to rotational power of the 5% non-I in our preparation and the insensitivity of our polarimeter, all of which serve to emphasize the value of absolute composition measurement.

RESULTS AND DISCUSSION

The racemic target compound I was synthesized by alkylating readily available 10-undecenoic acid with ethyl iodide using the method of Pfeffer and Silbert (1970) (Figure 1). Reduction to the carbinol III with lithium aluminum

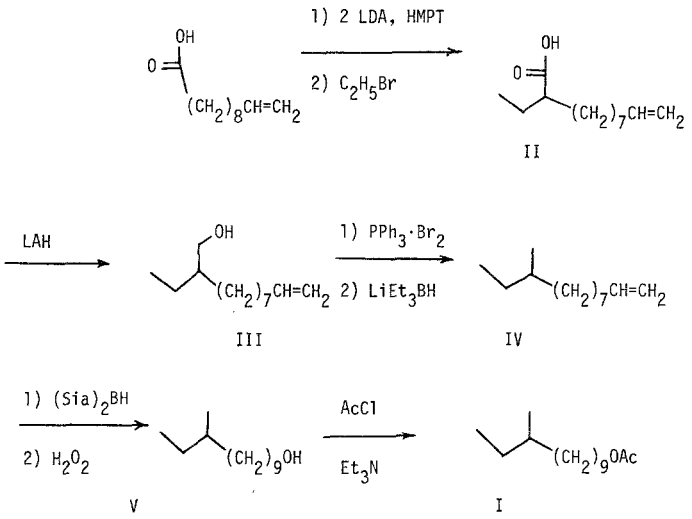


FIG. 1. Synthesis of (±)-10-methyl-1-dodecanol acetate.

hydride (LAH) was followed by conversion to the bromide by treatment of III with triphenylphosphine dibromide in methylene chloride. Alkene IV was then obtained by reduction of the bromide with lithium triethylborohydride. Hydroboration of IV with disiamylborane and oxidative work-up yielded alcohol V, which was then esterified with acetyl chloride and triethylamine. The several transformations that constitute the conversion of III to I could be conducted without rigorous purification of intermediates in ca. 70–75% overall yield. The yield of I from undecylenic acid was ca. 40–45% without any extensive effort at optimization.

Several successful syntheses of chiral α-alkylalkanoic acids, ketones, and aldehydes have been described (Meyers, 1978; Meyers et al., 1979; Larcheveque et al., 1979; Davenport et al., 1979; Evans and Takacs, 1980; Sonnet, 1980). These reactions proceed via metallated azaenolate or amide enolate intermediates that invite reaction preferentially with one face of the enolate double bond. Intriguingly, alkylation of amides of (*S*)-(−)-prolinol and (1)-(−)-ephedrine proceeded with opposite configurational biases when the free hydroxyl group of the amides is instead present as an ether. The ee's for such alkylations could be quite high for each configuration (82% ee with OCH_3): Sonnet and Heath, 1980; and up to 94% ee with OH: Evans and Takacs, 1980), thus a single chiral auxiliary would serve the purpose of constructing the desired molecule with a strong stereochemical bias in either configurational sense.

A modification of the route described (Figure 1) was devised whereby the amide of (*S*)-(−)-prolinol with 10-undecenoic acid was alkylated (Figure 2).

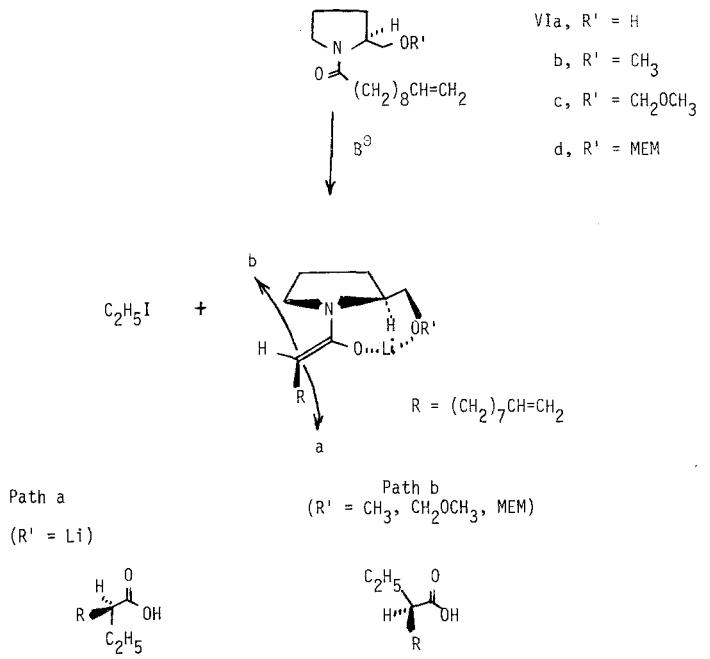


FIG. 2. The predominant configuration obtained upon reaction of the (*S*)-(−)-prolinol amide anion with ethyl iodide. Products shown are the acids obtained upon hydrolyzing the alkylated amide.

The amide was most conveniently synthesized by treating the amino alcohol with the acid halide using triethylamine as an acid scavenger. Since *N*- and *O*-acylation occurred competitively under these conditions, the reaction was conducted with a molar excess of 10-undecenoyl chloride. This resulted in the formation of the amide ester which, without any purification was saponified in aqueous alcoholic potassium hydroxide. If care was not taken in this reaction, di-9-decenyl ketone, resulting from a Claisen condensation, was isolated. Liberated 10-undecenoic acid was then recovered, and the crude amide could be alkylated directly. Ethers of this amide were prepared via the sodio derivative (sodium hydride—THF) and the appropriate alkyl halide (see Methods and Materials). These conditions do not jeopordize the asymmetric center.

Initial research with chiral amide anions (Evans and Takacs, 1980; Sonnet and Heath, 1980) indicated that the temperature for the alkylation step, the liganding materials, counterion, and bulk of the alkyl halide all played a role in determining diastereomeric composition. However, Evans and Takacs (1980) determined that essentially only a single anion was formed from (*S*)-(−)-prolinol propionamide and that the temperature for the deprotonation reaction was not critical. Several trial ethylations of (*S*)-(−)-

prolinol undecenoamide were conducted—a few are reported in Table 1. Hydrolytic cleavage of these alkylated amides proceeded readily in a two-phase system comprised of conc. HCl and hexane under reflux (4–6 hr). The asymmetric alkylation of 10-undecenoic acid (→ amide → alkylated amide → acid) could be conducted in overall 55% yield with an *S*-ee of 80%.

Methyl, methoxymethyl, and methoxyethoxymethyl (MEM) ethers of the prolinol amide were alkylated to produce *R*-enriched acid II. We examined the alkylation of the MEM ether more closely (Tables 1 and 2). It was noted that reaction of the intermediate anion with methyl, rather than ethyl iodide was significantly less selective. Stereoselectivity was greater for the ethylation reaction with lithium diisopropylamide (LDA) using a one-molar excess of diisopropylamine to LDA. At ratios of 1:1 and 3:1 (diisopropylamine to *n*-BuLi) the selectivity was appreciably diminished. Hydrolysis of the MEM ethers was effected with anhydrous zinc bromide in methylene chloride (Corey et al., 1976). Although cleavage was quite slow, the conditions did not compromise the new asymmetric center's stereochemistry. The crude product could then be hydrolyzed in the two-phase system just described. Attempts to hydrolyze the ether amide directly to the acid resulted largely in insoluble polymeric material. By contrast methyl ethers could be hydrolyzed directly but the reaction was slow (16–20 hr).

The asymmetric synthesis of acetate I could now be carried to completion. Acid II (*S*-ee 80%) gave I uneventfully. The alkylated MEM ether (*R*-ee 74%) was subjected to further purification by HPLC providing a sample of 90% ee that was subsequently converted to I. We feel that this route has two distinct advantages, namely: (1) broad composition options and (2) absolute measure of composition since the diastereomeric intermediates are readily analyzed.

Before proceeding to a discussion of these analyses, we should mention

TABLE 2. ETHYLATION OF MEM ETHER OF (*S*)-(−)-PROLINOL 10-UNDECENAMIDE[a]

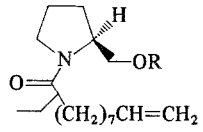
Diisopropylamine	*n*-BuLi	Free amine/chiral anion	*S*:*R*
1.5	1.5	0	26:74[b]
3.0	2.0	1.0	28:72
3.0	1.5	1.5	23:77[c]
4.0	2.0	2.0	13:87
5.0	2.0	3.0	19:81
10.0	2.0	8.0	26:74

[a]Millimoles of reactants per millimole of MEM ether have been tabulated. Each reaction conducted in HMPT/THF (1:5) and conducted (deprotonation, alkylation) at −78°C except as noted.
[b]Alkylation at −120°C.
[c]HMPT/THF (1:10).

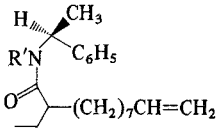
that the amides formed from (+)-II and α-methylbenzylamine are crystalline. Since this amine is readily available in both configurations, fractional crystallization of the resulting diastereomers could be an attractive procedure to obtain configurationally pure II. In fact, the 1:1 diastereomer mixture formed from (+)-II and (*S*)-α-methylbenzylamine was recrystallized three times from ethanol giving the *R,S* diastereomer in a 99:1 ratio, mp 95–97°. The *S,S* isomer was obtained by HPLC purification (mp 56–57°), although one notes that this particular compound could have been obtained by ethanol recrystallization of the amide from the (*R*)-amine. The recovery of the higher melting diastereomer was 40%. Unfortunately, amides resist hydrolysis, and a variety of methods either failed completely or doomed the asymmetric center to extensive racemization (Loew and Johnson, 1971; Olah and Olah, 1965; Vaughn and Rollins, 1975). For example, a reaction of a similar saturated amide that we synthesized (an α-methyl decanoamide) conducted in 25 N sulfuric acid/dioxane at 95° required 24 hr and caused a 16% loss in ee. Hydrolysis of the undecenamide was entirely unsuccessful presumably because of reaction involving the double bond of the acid residue. Additionally the *N*-methyl derivatives, unlike other tertiary amides (Brown and Kim, 1977) were totally resistant to $LiEt_3BH$ reduction. The use of nitrogen tetraoxide (White, 1955) or nitrosonium salts (Olah and Olah, 1965) could provide a satisfactory cleavage of such amides, providing the acid residue does not contain unsaturation. Nevertheless, the chiral auxiliary would be lost since the nitrosation process ultimately produces an alcohol from the original amine. In order to employ an approach based on crystalline amide resolution, therefore, an effective and general method for recovering the acid fragment must be devised or a β-amino alcohol chiral auxiliary capable of producing crystalline diastereomers must be found.

Analyses of the diastereomeric alkylated prolinolamides (Figure 2) were conducted with capillary columns coated with cholesteryl cinnamate (Heath et al., 1977) and Carbowax 20M® (Table 3). Diastereomeric MEM and methoxymethyl ethers could also be analyzed with the Carbowax-like Ultrabond-II® packing. That hydrolysis (aqueous HCl–hexane, reflux) had not compromised the new center was ascertained by reconversion of the acids to amides by treatment with thionyl chloride–dimethylformamide and then (*S*)-α-methylbenzylamine. These particular amides could be analyzed on all three columns. As has been previously noted (Pirkle and Hauske, 1977), *N*-methylation produced diastereomers exhibiting markedly reduced resolution. Interestingly, although methoxytrifluoromethylphenyl acetate (MTPA) esters of asymmetric alcohols can be employed routinely for chromatographic analysis and resolution (Bergot et al., 1978), the hydroxyl group apparently must be directly attached to the asymmetric center. Alcohol II could not be chromatographically resolved either as its MTPA ester or as a urethan based on α-methylbenzylamine.

TABLE 3. CHROMATOGRAPHIC DATA FOR DIASTEREOMERIC PAIRS OF AMIDES[a]



R	1[b]	2[c]	3[d]	HPLC[e]
H	U	2.31 (1.08)	2.56 (1.12)	U
CH_3	U	1.80 (1.06)	1.71 (1.07)	1.95 (1.08)
CH_2OCH_3	<1 (1.10)	3.98 (1.11)	1.67 (1.07)	2.11 (1.27)
MEM	<1 (1.15)	3.69 (1.09)	–[f]	1.51 (1.10)



R	1	2	3	HPLC
H	1.10	2.18 (1.11)	2.40 (1.06)	3.08 (1.45)
CH_3	U	U	U	<1 (1.12)

[a]Data expressed as "resolution (α)"; U = unresolved. In all cases, the *S*, *S* diastereomers were eluted first from the GLC columns and second from the HPLC column.
[b]Column 1: Ultrabond II, 3.2 mm × 1.5 m operated at 180–200°C.
[c]Column 2: Carbowax 20 M, 0.25 mm × 10 m WCOT operated at 200°C.
[d]Column 3: Cholesteryl cinnamate, 0.25 mm × 10 m WCOT operated at 190°C.
[e]LiChrosorb Si60 (5 μm), 6.4 mm × 25 cm column eluted with THF–ethyl acetate–hexane (*v/v/v*) 1:2:7 for the prolinol amides and 2:20:78 for the acyclic amides at 1.0 ml/min.
[f]Not obtained.

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HIGHLY POTENT GERMINATION INHIBITORS IN AQUEOUS ELUATE OF FRUITS OF BISHOP'S WEED (*Ammi majus* L.) AND AVOIDANCE OF AUTOINHIBITION

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Abstract—The aqueous eluate from fruits of *Ammi majus* (Bishop's weed, Umbelliferae) remarkably inhibited germination of adjacent seeds of *Anastatica hierochuntica*, lettuce, or tomato but had no effect on intact fruits of *Ammi*. Similar inhibition was found in dark or in light, except that seeds of *A. hierochuntica* were significantly more inhibited in the dark than in the light. Xanthotoxin was isolated, identified, and found to account for about a sixth of the inhibitory activity of the eluate. After fruits of *Ammi* were submerged in a large volume of water for 4 days, the fruits still exuded enough inhibitors to prevent germination of *A. hierochuntica*, lettuce, or tomato. Data support also the proposal that the phytotoxins are compartmentalized between the inner and the outer fruit envelopes. The inner layer excludes inhibitors from the embryo and autotoxicity is thus avoided, whereas the outer one ensures a gradual liberation of the phytotoxic compounds. This, as well as the high reactivity of the eluate, the high densities of *Ammi* fruits in nature, and their relatively limited annual germination, suggest chemical inhibition of neighboring plant species other than *Ammi*. Hence, in addition to their chemical protection against predators of either lower or higher organisms, furanocoumarins in fruits of *Ammi majus* may contribute to its success as a weed.

Key Words—*Ammi majus*, Bishop's weed, germination inhibitors, xanthotoxin, furanocoumarins, avoidance of autotoxicity, allelopathy.

INTRODUCTION

Fruits (mericarps) of *Ammi majus* (Umbelliferae, Figure 1a and b) contain at least 12 linear furanocoumarins (psoralens) of which xanthotoxin (Figure 2) is

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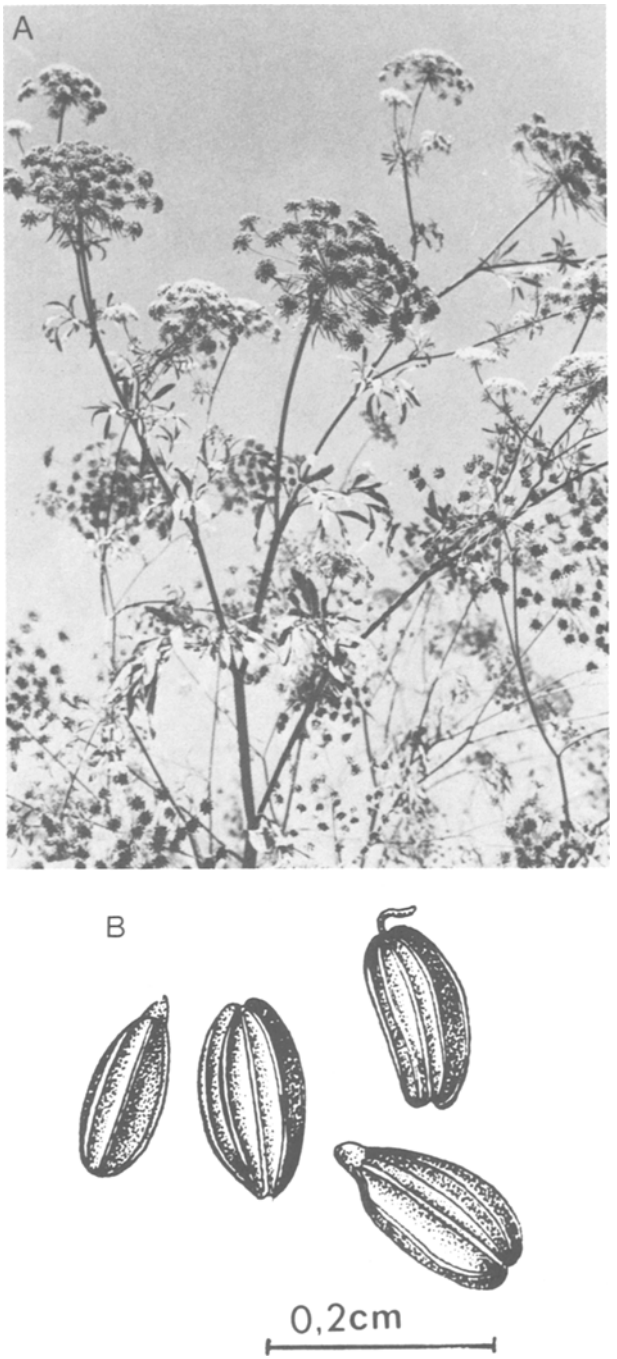
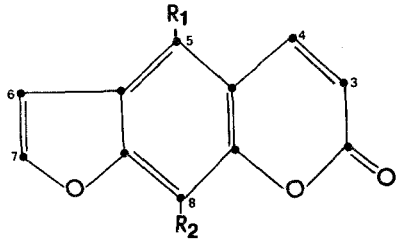


FIG. 1. *Ammi majus*: (a) flowering plants (Tel Aviv, April 1978) and (b) ripe fruits (mericarps).



R_1=R_2=H = Psoralen
R_1=H; R_2=OCH_3=Xanthotoxin

FIG. 2. Two linear furanocoumarins.

the most prevalent. Most of them are potent photosensitizers (Ivie, 1978). Xanthotoxin was found to inhibit germination and growth of lettuce (Rodighiero, 1954). Psoralen (Figure 2) arrests germination of radish seeds at 2×10^{-5} M (Fucushi, 1960) and lettuce achenes at 2×10^{-6} M (Shina-Roy and Chakraborty, 1976). In plants producing phytotoxins, the question of resistance or avoidance of autointoxication was approached by Camm et al. (1976), showing that cuttings of *Heracleum lantanum* were relatively resistant when exposed to xanthotoxin, the phytotoxin they produced. The phenomenon of avoidance or resistance to autotoxicity was discussed by Fowden and Lea (1979). Our purposes were to assess the inhibitory properties of the aqueous eluate of *Ammi majus* fruits and to show the method by which these fruits could avoid or resist autoinhibition.

METHODS AND MATERIALS

Various numbers (10–40) of fruits of *Ammi* (collection Ramat Hasharon, Israel, 1979) were placed in plastic Petri dishes, 5.0 cm in diameter, on two layers of filter paper with 2.5 ml distilled water and incubated in continuous darkness as well as under light (fluorescent and incandescent, 1000 lux). Twenty seeds of *Anastatica hierochuntica* (collected at Eilat, Israel, 1979) were set in the vicinity of *Ammi* fruits after these were so incubated for 24 hr, and the germination of *Anastatica* under the above conditions was followed. Fruits of *Ammi* were also similarly placed after being submerged in a large volume of distilled water (10 g in 4 liters) for 1, 2, 3 or 4 days, and the effect of the eluate of the leached fruits on the rate of germination on 20 seeds of *Anastatica* was observed. The normally rapid germination of *Anastatica* (starting 3 hr after wetting and attaining 90–100% after another 5 hr), provides a quick means for determining inhibition. Three replicas (Petri dishes) of each treatment were used, and all experiments were repeated at least twice. A suspension of ground *Ammi* fruits was prepared by stirring 10 g of ground

Ammi fruits, of particle size up to 0.5 mm in 100 ml distilled water for 30 min, centrifuged at 10^4 *g* for 15 min, and filtered. For the determination of xanthotoxin in fruits of *Ammi majus*, whole or ground fruits or dried eluate were extracted with methanol in a Soxhlet extractor for 4–5 hr. Such extracts were concentrated to 25 ml, and 3-μl aliquots were spotted (30 μl each) on TLC plates (0.2 mm SiO_2 Merck S6 254) along with comparable various concentrations of authentic xanthotoxin (from Sigma Chemicals, St. Louis, Missouri) dissolved in methanol. Plates were developed in benzene–ethyl acetate (9:1), and the separated spots were identified under UV, 364 nm. Spots corresponding in R_f to that of the standard xanthotoxin were scraped off and eluted in methanol, the extracts were filtered, and their absorption was read at 300 nm in a Varian spectrophotometer. For the isolation of larger amounts of xanthotoxin, 2-mm preparative TLC plates (Merck 60, F 254) were used. To locate xanthotoxin within the fruits, embryos were carefully isolated from them at early stages of their germination and xanthotoxin was separately determined in the embryos as well as in the fruit envelopes. To assess possible mechanical protection of the embryo by which it avoids autointoxication, fruits were scarified to measured levels by using fine sandpaper and, by weighing the fruits, the amount of scarification could be evaluated by percentage of weight reduction.

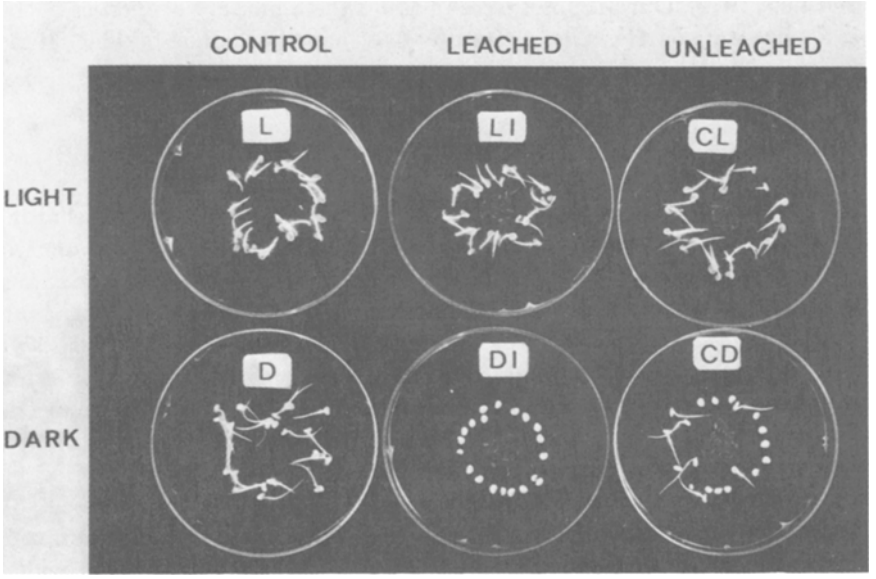


FIG. 3. Germination of seeds of *Anastatica hierochuntica* around 32 fruits of *Ammi* under light (L) and dark (D) with fruits leached 24 hr (1) and the control, unleached fruits (C).

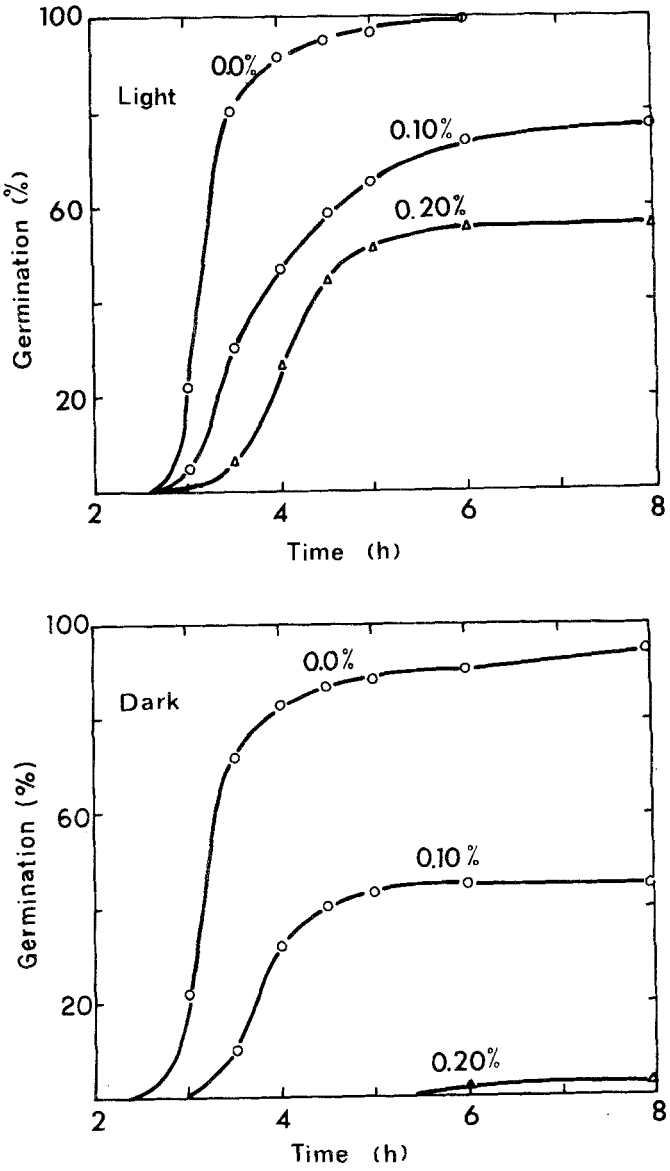


FIG. 4. The rate of germination of seeds of *Anastatica hierochuntica* L. exposed to aqueous suspensions of fruits of *Ammi majus* at concentrations of 0.0, 0.10, and 0.2%, in dark (Top) and under light (Bottom).

RESULTS

Thirty-two fruits of *Ammi*, equivalent to ~16 mg/Petri dish, or 6.4 mg/ml, after being incubated for 24 hr at 25° C, arrested germination of 20 seeds (~20 mg) of *Anastatica* (Figure 3) in the dark; this was true also when fruits of *Ammi*, prior to incubation, were submerged in water for 1, 2, 3, or 4 days. When exposed to aqueous slurries prepared from ground fruits of *Ammi*, germination of *Anastatica* seeds in the dark was impaired at concentrations as low as 0.02%, which is equivalent to 1 fruit of *Ammi* per Petri dish. Such inhibition was displayed also under light, although at a smaller rate (Figure 4). Germination of achenes of lettuce and seeds of tomato was also inhibited by the eluate or by aqueous slurries, but inhibition was similar under light or dark. Aqueous solutions of pure xanthotoxin also repressed germination of *Anastatica* (Figure 5). Here also inhibition was somewhat more severe in the dark, whereas in tomato or lettuce light did not reduce the inhibitory effect of xanthotoxin.

Germination of *Ammi* itself was not affected by increased numbers of *Ammi* fruits per Petri dish (Figure 6), nor by highly concentrated slurries of

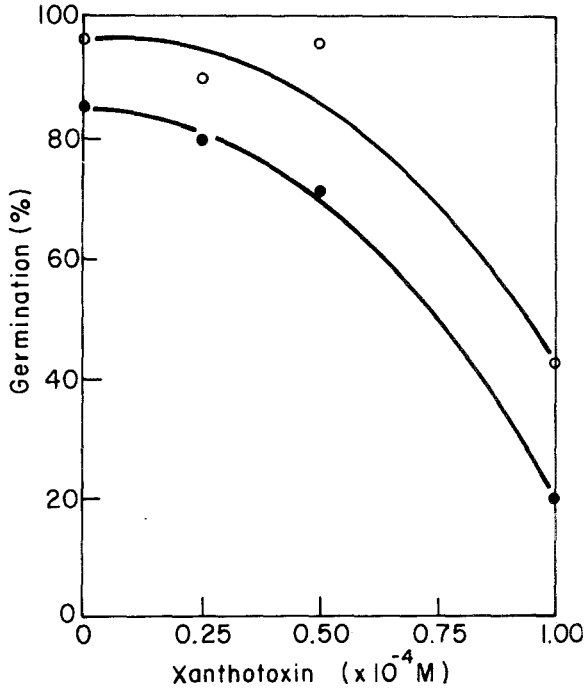


FIG. 5. The effect of xanthotoxin at various concentrations on germination of *Anastatics hierochuntica* in light (○) or dark (●) conditions.

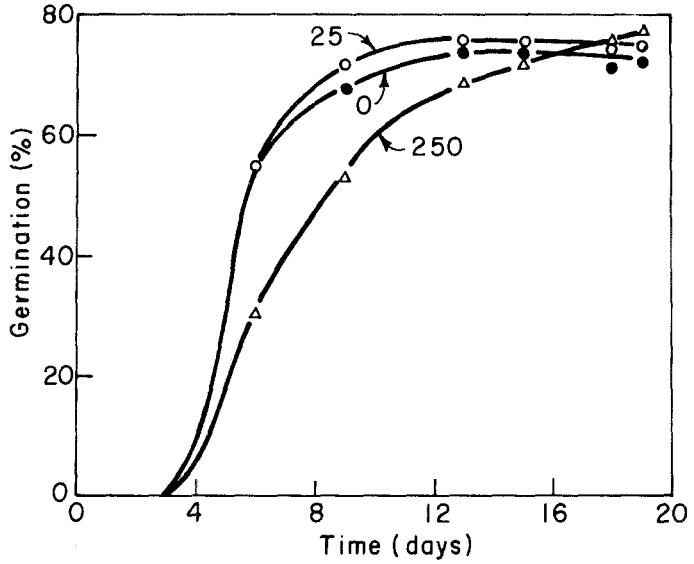


FIG. 6. The rate of germination of fruits on *Ammi majus* exposed to aqueous suspensions of ground fruits of *A. majus* at amounts of 25 or 250 mg/Petri dish, or equivalent to the ratio of 1:1 or 1:10 living fruits to ground ones.

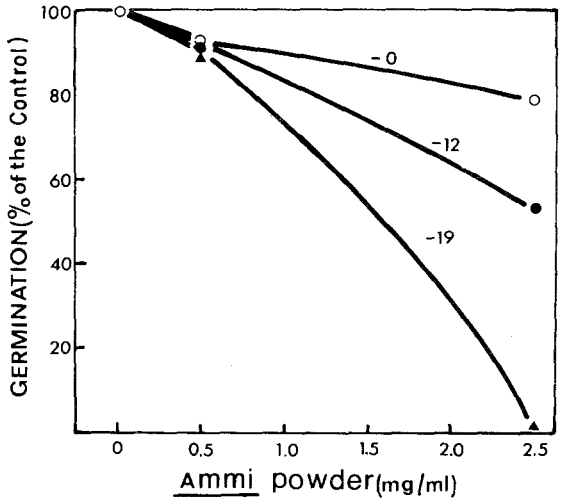


FIG. 7. Germination of fruits of *Ammi majus* scarified to various levels (−0, −12 and −19 loss of fruit dry weight in % of the initial fruit weight) and exposed to aqueous slurries of ground *Ammi* fruits at various concentrations (mg/ml).

TABLE 1. DISTRIBUTION OF XANTHOTOXIN IN FRUITS OF *Ammi majus* L.

	% of dry weight
Complete, nonscarified fruits	0.75
Fruits scarified to cause 29% reduction of dry weight	0.15
Fruit envelopes	1.05
Young seedlings	0.07
Aqueous eluate from fruits during 24 hr	0.014

ground *Ammi* fruits. Fruits of *Ammi* readily germinated at a ratio of 1:10 (i.e., when 50 fruits were exposed to aqueous extracts of 500 fruits), and the rate of germination was only slightly affected. However, germination was reduced when scarified *Ammi* fruits were exposed to aqueous extracts of *Ammi*, varying inversely with increased concentrations of the extracts and with increased levels of scarification (Figure 7). Inhibition occurred similarly when scarified *Ammi* fruits were exposed to the eluate as well as to xanthotoxin. Scarification to a level where fruits lost 29% of their initial weight also reduced fruit content of xanthotoxin from 0.75% to 0.15% based on fruits dry weight (Table 1). Fruit envelopes separated from the germinating embryos contained the highest amounts (1.05%) of xanthotoxin, while young seedlings contained only traces (0.07%). Fruits of *Ammi* submerged in distilled water for 24 hr liberated 0.014% xanthotoxin based on dry weight. This is only ~2% of the total amount stored in those fruits. In a Petri dish with 2.5 ml of distilled water this makes a concentration of 10^{-4} M xanthotoxin.

Identification of fractions recovered from TLC plates at R_f values equal to those obtained from standard xanthotoxin was verified by [^{13}C]NMR and showed about 80% xanthotoxin present. These fractions have also impaired germination of *Anastatica* at 20 ppm, i.e., 10^{-4} M xanthotoxin. However, other fractions recovered from the TLC plates (R_f 0.3–0.5) also showed inhibitory effects on such germination.

DISCUSSION

Inhibition of germination of *Anastatica* was about six times higher when seeds were exposed to aqueous solutions obtained from ground *Ammi* fruits than when subjected to pure xanthotoxin, e.g., to reduce germination to 50%, 18 μg/ml of pure xanthotoxin had to be applied, where the same effect was obtained with 3 μg/ml of the same compound in the leachate of ground *Ammi* fruits. Thus, we assume that substantial amounts of germination inhibitors other than xanthotoxin are stored within these fruits. Similarly they have a low rate of efflux, since fruits of *Ammi* (cf. Figure 3) after submergence in

water for four days not only did not lose their potency, but their inhibition potential even increased. The nature of these inhibitors has not yet been assessed, but they could be recovered from the TLC plates at R_f between 0.3 and 0.5. Photodeactivation of coumarins of furanocoumarins is widely reported (Evenari, 1949; Mayer and Shain, 1974; Baskin, et al., 1967). Nevertheless, our data and those of others suggest also light-independent activity (Shina-Roy and Chakraborty, 1976). The effect of light on the response of seeds to either the eluate of *Ammi* fruits or to xanthotoxin was found to be specific to the studied species. It is, therefore, inappropriate to attribute inhibition to interference with DNA replication as accepted for various lower organisms and higher ones (cf. Berenbaum, 1978). More work should be done to elucidate the mode of action of xanthotoxin and related compounds in higher plants.

Scarification of *Ammi* fruits resulted in a significant reduction of xanthotoxin (Table 1) content as well as an increase in the inhibitory effect on the scarified fruits of exogenously applied xanthotoxin as well as eluate of whole *Ammi* fruits. This suggests that autotoxicity is avoided by a physical barrier, situated between the outer fruit envelopes and the embryo. In *Ammi* this hard layer is known as woody "endocarp" (cf. Theobald, 1972), whereas the outer thinner layers keep the phytotoxins from rapid leakage. It is therefore possible that xanthotoxin as well as other germination inhibitors are stored within the secretory canals (vittae) typical to fruits of Umbelliferae. However, this is probably not the case in other umbelliferous fruits (e.g., *Foeniculum vulgare* Mill., *Cuminum copticum* Benth. & Hook., *Daucus carota* L., and *Coriandrum sativum* L.), in which Chaturvedi and Muralia (1975) showed autoinhibition of germination caused by their own eluate. In those species, the inhibitors function predominantly as germination controls which regulate densities within the population (intraspecific inhibition) and allow germination only after a sufficient amount of rainfall. Germination in fruits of *Ammi majus* is probably regulated by other means; the inhibitors, with their slow efflux, could account for decimation of species other than *Ammi* and thus reduce interspecific competition. This is probably a more advanced category of evolution which can be maintained through (1) storing inhibitors with high reactivity, (2) maintaining a slow efflux, and (3) sequestering the embryo from the phytotoxins.

Combining these findings with the arguments discussed above as well as with some field observations permits hypothesizing some ecological implications. Nearly all fruits of *Ammi* are dispersed in nature adjacent to the parent plants. Harvests of wild populations of *Ammi majus* indicated that yields may often attain densities between 5 and 15 fruits/cm^2. In the laboratory a density of 1.6 fruits/cm^2 caused a severe inhibition of *Anastatica*. Since *Ammi* fruits submerged in water liberate only 2% of the total stored xanthotoxin within 24 hr, inhibition in the vicinity of *Ammi* fruits may be maintained for long

periods under field conditions. Moreover, when *Ammi* fruits are planted in the soil, only a small fraction (3–10%) germinates; the rest stay quiescent. In semiarid regions they are an important contribution, enriching the seed bank and, as for many other species, serving as a reserve for renewing the population after years of shortage of rainfall. The time during which these fruits can retain the inhibitors was not assessed, but, in arid or semiarid regions where leaching is limited, they can also be regarded as small containers of highly active germination inhibitors. This may be why *Ammi majus* is an aggressive weed which, once established, can hardly be eradicated. Yet, some of these inhibitors such as xanthotoxin may also protect the seed (the dormant embryo and endosperm) against fungal infections (Chakraborty et al., 1957) or insects (Berenbaum, 1978) or against predation by higher organisms (Ivie, 1978). Inhibition of germination is certainly not eradication, but will postpone the process of seedling emergence and thus provides *Ammi* with an advantage at the crucial stage of plant life, i.e., seedling establishment. Possible interference between plants through chemical inhibition in natural habitats, i.e., allelopathy, is not yet well established by experimental analysis (see Harper, 1977). To better demonstrate such interference, inhibition should be tested also in the soil and on a wider array of plant species which could be expected to grow within stands of *Ammi majus*.

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DISTRIBUTION OF ADULT DEFENSE GLANDS IN CHRYSOMELIDS (COLEOPTERA: CHRYSOMELIDAE) AND ITS SIGNIFICANCE IN THE EVOLUTION OF DEFENSE MECHANISMS WITHIN THE FAMILY

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Abstract—Defense glands were examined in the adults of 65 species belonging to 10 different subfamilies. They were found in the pronota and elytra of members of the subfamilies Criocerinae, Chrysomelinae, Galerucinae, and Alticinae. It is suggested that these glands appeared monophyletically in the course of evolution and that the absence of glands in several species of the two most evolved subfamilies is a secondary event, explained by the presence of alternative efficient defensive behaviors: reflex bleeding in the Galerucinae and escape mechanism of jumping in the flea beetles. It is also suggested that a large distribution of the glands at the surface of the beetles is a primitive condition and that in the course of evolution only the glands most efficiently located along the edges of the pronotum and elytra were maintained. Such evolution has occurred several times. Alternative and complementary defensive mechanisms are also listed and discussed.

Key Words—Defense, glands, Coleoptera, Chrysomelidae.

INTRODUCTION

Defensive glands have, for a long time, been reported in the adults of a few species of chrysomelids, all belonging to the subfamily Chrysomelinae (Cuénot, 1896; Tower, 1906; Hollande, 1909; Patay, 1937).

When disturbed, the beetles emit a secretion from glands opening at the surface of elytra and pronotum; this behavior was considered to be responsible for their repellent quality against various vertebrate predators.

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Indeed, it has been demonstrated recently that the secretion of most species of the genera *Chrysolina*, *Chrysochloa*, and *Dlochrysa* contains cardenolides which are produced by the beetles themselves and not originally present in their host plant (Pasteels and Daloze, 1977; Daloze and Pasteels, 1979; Pasteels et al., 1979).

None of these works, however, accurately describe the structure of the glands and their distribution within the insects. In a previous paper this was attempted for the Colorado beetle (Deroe and Pasteels, 1977). We would like now to look more extensively at many species of the family in order to investigate how far this defensive mechanism is distributed within the chrysomelids, and how it could have evolved.

This paper will describe the distribution of defensive glands throughout the family, as well as their precise location within the insects. Only glands present in the adult stage will be considered. Details on the structure of the glands will be given in a following paper. The results will be considered together with other mechanical, behavioral, or chemical defensive mechanisms which could occur simultaneously or independently of the exocrine secretion.

METHODS AND MATERIALS

The beetles were collected in Belgium, except for three Canadian species (*Gonioctena americana*, *Altica ambiens*, *Entomoscelis americana*) and one species from South Africa (*Diamphidia nigroornata*). We have followed the classification adopted by Derenne (1963) for the Belgian fauna and the one recently established by Jolivet (1978) for the subfamilies of the world. For the nomenclature and subdivision of the Chrysomelinae, we have followed Jolivet and Petitpierre (1976).

The presence of defensive glands was recognized by the oozing of the secretion after disturbance. In a few species, the defensive glands could not be recognized in this manner. In such cases, the glands were considered defensive if they were homologous in either their structure or location to the glands for which a defensive function was proven. They were always very easily distinguishable from the much smaller dermal glands observed in the integument of all species. For most species the identity of the glands was confirmed by histology and transmission electron microscopy. These techniques will be described in another paper dealing with the comparative structure of the glands.

The mapping of the glands was established by observing whole elytra and pronota mounted in Canada balsam after moderate digestion in 5% KOH for 4 hr at 60° C. In the figures, each gland is represented by one dot.

RESULTS

Distribution of Defensive Glands in Adults

Species from ten subfamilies were examined. In six subfamilies, none of the examined species possessed defensive glands in the adult stage. In two subfamilies defensive glands were always observed, whereas in the two others defensive glands were lacking in some species but present in others.

Subfamilies in which Defensive Glands were Never Observed. Species examined: S.F. Donaciae: *Donacia appendiculata* Ahr., *D. cinerea* Herbst; S.F. Zeugophorinae: *Zeugophora subspinosa* F.; S.F. Clytrinae: *Clytra quadripunctata* L., *Gynandrophthalma cyanea* F., *Coptocephala unifasciata* Scop.; S.F. Cryptocephalinae: *Cryptocephalus biguttatus* Scop., *C. decemmaculatus* L., *C. labiatus* L., *C. moraei* L., *C. sericeus* L., *C. trimaculatus* Ros.; S.F. Hispinae: *Hispa testacea* L., *H. astra* L.; S.F. Cassidinae: *Cassida rubiginosa* Müll., *C. sanguinosa* Suffr.

Since only a small number of species could be examined, we cannot conclude that defensive glands are absent in all the species belonging to these six subfamilies. Phylogenetic considerations discussed later make, however, this tentative hypothesis attractive.

Subfamilies in which Defensive Glands were Always Observed.

1. S.F. Criocerinae. Four species were examined: *Lema cyanella* L., *L. melanopus* L., *Lilioceris lilii* Scop., and *Crioceris asparagi* L. Figure 1

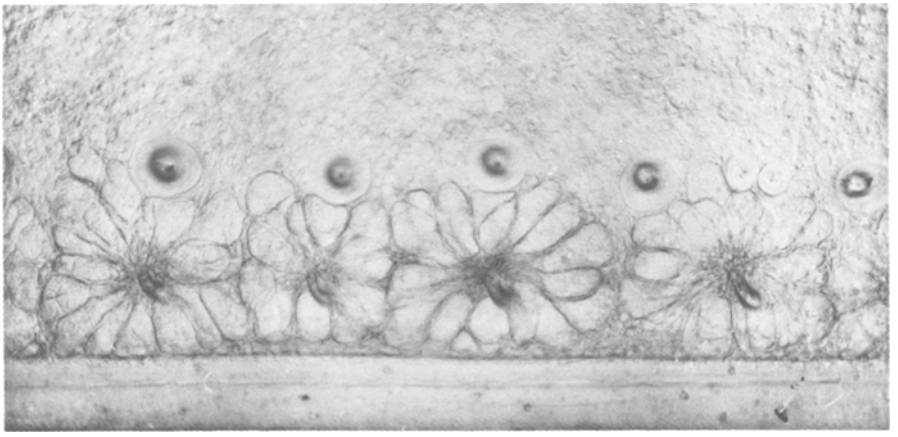

FIG. 1. Whole mount of a *Lilioceris lilii* elytron showing the lateral row of the defensive glands.

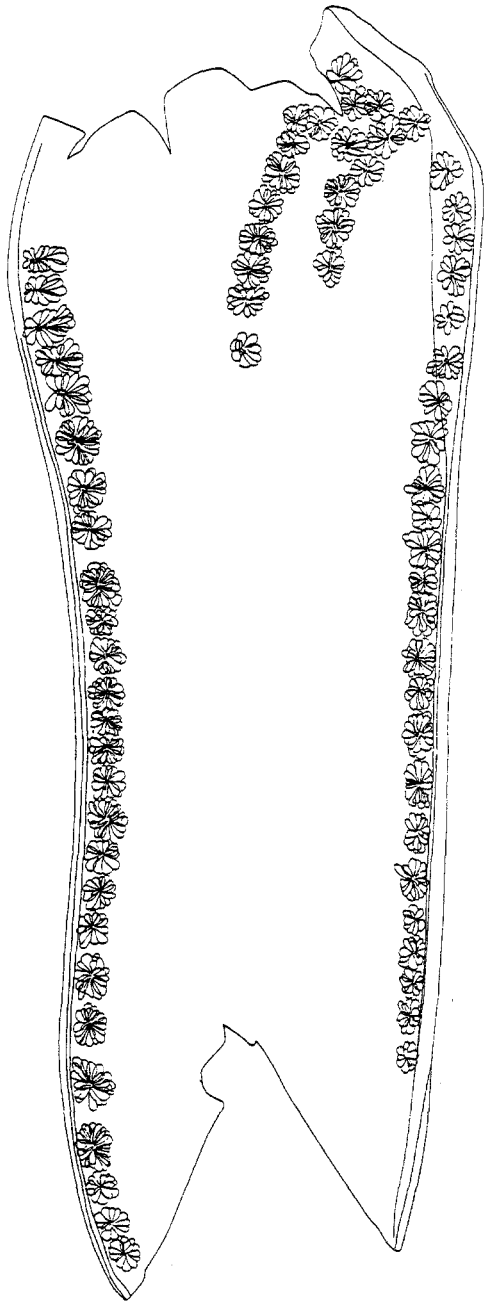

FIG. 2. Mapping of the defensive glands in an elytron of *Lilioceris lilii*. The elytron was broken at both ends during the mounting process.

illustrates how the glands can be recognized in a whole mount of the elytron of *Lilioceris lilii*, and Figure 2 gives the precise mapping of these glands. The distribution of the glands in the four species differs only by minor details. In *Lema melanopus*, the glands are largely distributed underneath both the pronotum and the elytra (Figure 3A). In the two other species, the distribution of the glands is more restricted. For example, only lateral glands were

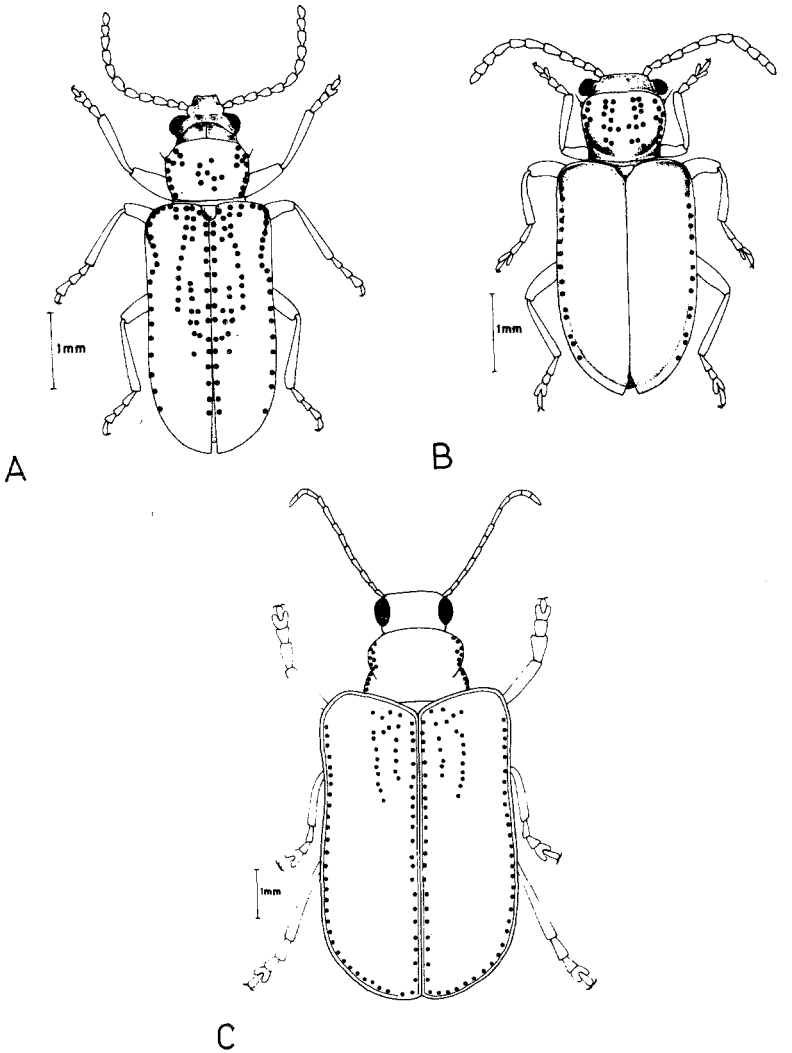

FIG. 3. Distribution of the defensive glands in selected Criocerinae: (A) *Lema melanopus*; (B) *Lema cyanella*; (C) *Lilioceris lilii.*

observed in the elytra of *Lema cyanella* and in the pronotum of both *Lilioceris lilii* (Figure 3B,C) and *Crioceris asparagi.*

2. S.F. Chrysomelinae. The following species were examined. (A) Chrysolinini: *Leptinotarsa decemlineata* Say, *Chrysolina coerulans* Scriba, *C. herbacea* Duftschm., *C. polita* L., *C. staphylea* L., *C. oricalcia* Müll., *C. varians* Schall., *C. hyperici* Förster, *C. brunsvicensis* Grav., *Dlochrysa fastuosa* Scop., *Chrysochloa cacaliae* Schrk. (B) Phaedonini: *Gastrophysa viridula* De Geer, *G. polygoni* L., *Plagiodera versicolora* Laich, *Chrysomela populi* L., *C. tremulae* F., *Hydrothassa marginella* L., *H. glabra* Herbst., *Prasocuris phellandrii* L., *Phaedon cochleariae* F., *P. veronicae* Bedel. (C) Phratorini: *Gonioctena (= Phytodecta) olivacea* Forst., *Gonioctena americana* Schaeffer, *Phratora* (= *Phyllodecta*) *vulgatissima* L., *P. laticollis* Suffr., *P. vitellinae* L. (D) Timarchini: *Timarcha goettingensis* L., *T. tenebricosa* F. (E) Entomoscelini: *Entomoscelis americana* Brown.

In the Chrysomelinae, the distribution of the glands in the insect may be more or less extensive. We were not able to recognize specific patterns which could be characteristic of a tribe. The distribution was even somewhat different between species belonging to the same genus or varied slightly from one specimen to another of the same species.

In all species, however, the glands are always present laterally in the pronotum and the elytra, where they open above the marginal groove which receives the secretion. This can be the sole location of the glands, as in the genera *Timarcha, Gastrophysa, Plagiodera, Hydrothassa, Prasocuris* (Figure 4A), *Chrysomela, Gonioctena americana*, some *Chrysolina* (*C. hyperici, C. varians, C. brunsvicensis*), and *Entomoscelis americana.*

In the pronotum, the lateral line of glands can be doubled by a few irregular rows of glands (*Chrysomela*). In the most extreme case (*Phaedon*), the glands are distributed on a surface reaching about one third of the pronotum on each side, (Figure 4B). Moreover, glands are often located near the anterior margin, mainly at the level of the corners of the pronotum, but they can form a nearly uninterrupted line as in *Leptinotarsa* (Deroe and Pasteels, 1977).

Furthermore, these glands are sometimes more widely distributed in the elytra too. In *Leptinotarsa decemlineata* (Deroe and Pasteels, 1977) and in *Chrysolina oricalcia* they are aligned in parallel longitudinal rows (Figure 4C). In *Chrysolina polita, C. coerulans*, and *C. herbacea*, one or two longitudinal rows of glands were observed along the inner margin of the elytra, and a few glands are usually close to the front margin (Figure 4D). A few incomplete longitudinal rows of glands are present also in the middle of the elytra of *Gonioctena olivacea* (Figure 5A), in this species the number of glands found in this location varies from specimen to specimen (Figure 5B).

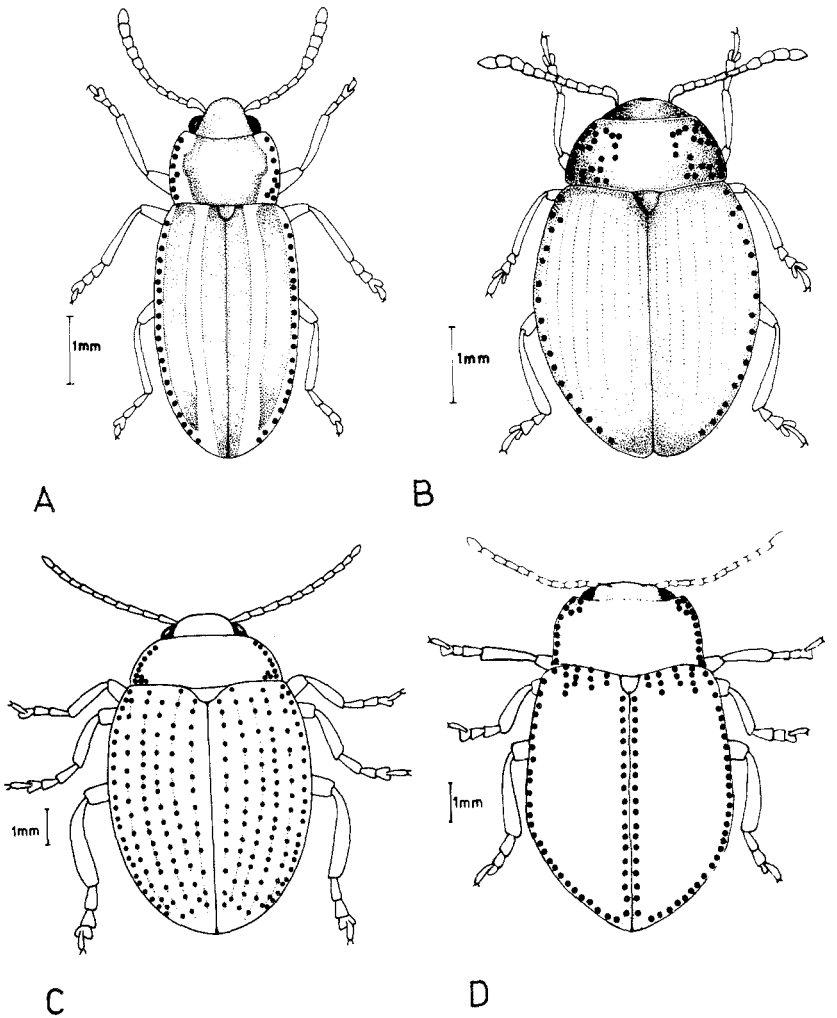

FIG. 4. Distribution of the defensive glands in Chrysomelinae: (A) *Prasocuris phellandrii;* (B) *Phaedon cochleariae;* (C) *Chrysolina oricalcia;* (D) *Chrysolina polita.*

Subfamilies in which Defensive Glands may be Present or Absent.

1. Galerucinae. The following species were examined. (A) Galerucini: *Galeruca taneceti* L., *Lochmaea suturalis* Thoms., *Galerucella calmariensis* L., *G. tenella* L. (B) Luperini: *Agelastica alni* L., *Sermylassa halensis* L., *Luperus circumfusus* Marsh, *L. longicornis* F.

In both tribes some species possess glands and others are devoid of them.

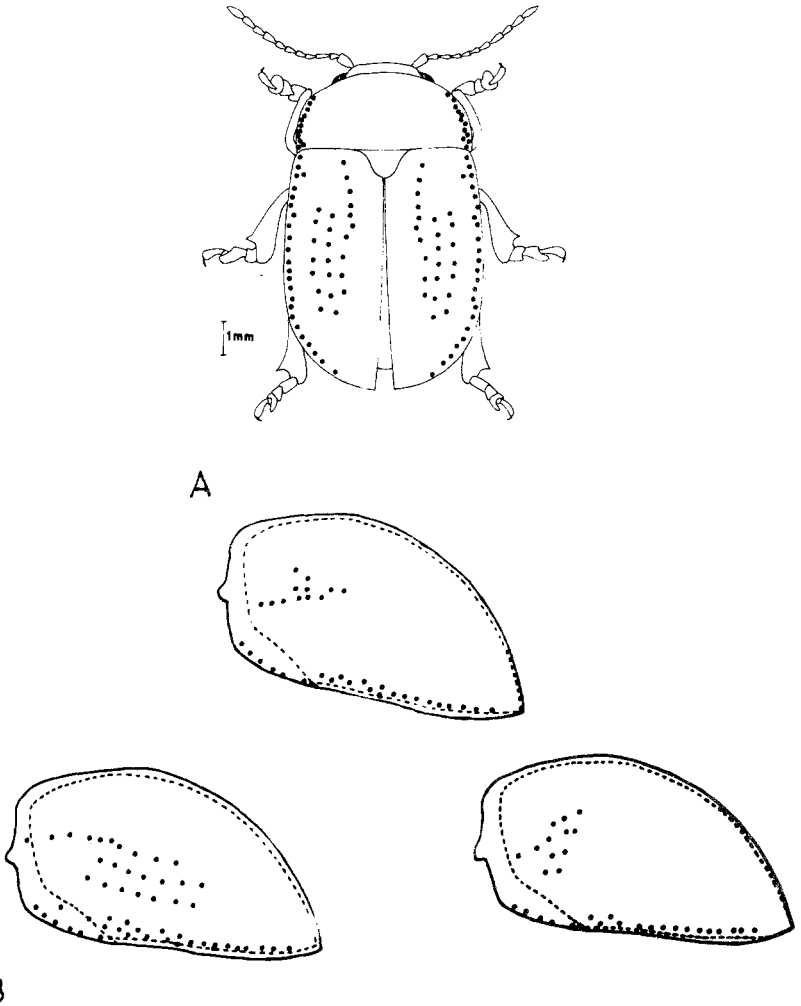

FIG. 5. (A) Distribution of the defensive glands in *Gonioctena olivacea*; (B) examples of variation in the distribution of the defensive glands within the elytra of *Gonioctena olivacea.*

Glands were found in *Lochmaea*, *Galerucella*, and *Sermylassa*, but not in other genera. In the elytra, the glands form a single lateral row in all four species. They are located anteriorly in the pronotum of *Galerucella* and *Lochmaea* (Figure 6A) but laterally in *Sermylassa* (Figure 6B).

2. Alticinae. Examined species: *Asiorestia transversa* Marsh., *Crepidodera* (= *Chalchoides*) *aurata* Marsh., *C. aurea* Geoffr., *Altica oleracea* L., *A.*

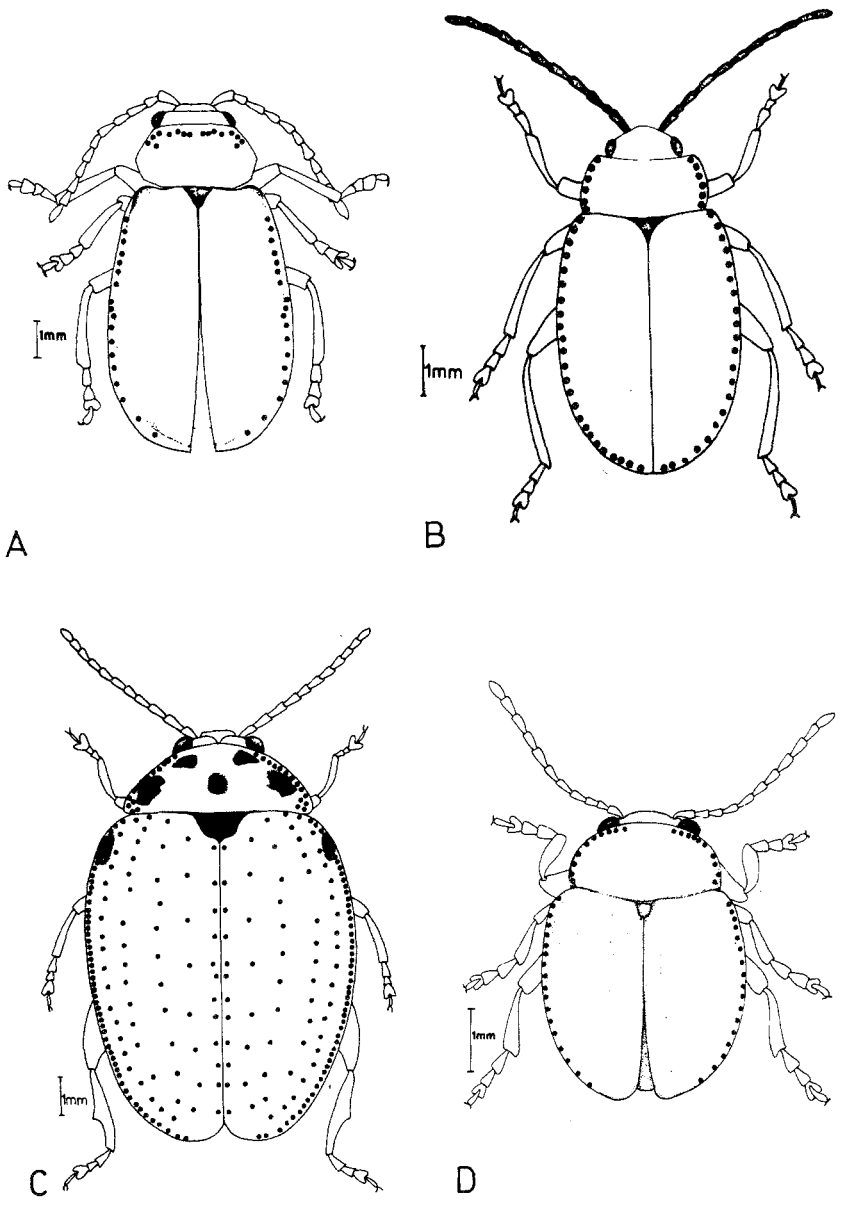

FIG. 6. Distribution of the defensive glands in selected Galerucinae (A) *Galerucella tenella* and (B) *Sermylassa halensis*, and Alticinae (C) *Diamphidia nigroornata* and (D) *Sphaeroderma testaceum*).

ambiens Le Conte, *Sphaeroderma testaceum* F., *Psylliodes affinis* Payk., *Diamphidia nigroornata* Stäl.

Glands were found in all the species except *Psylliodes affinis*. Again their distribution shows considerable variation between species. They are widely distributed in the elytra of *Diamphidia* (Figure 6C) but restricted to the lateral margin in the other species (Figure 6D). They are also located along the lateral margin of pronotum of most species but are concentrated in two patches at the posterior corner in *Altica oleracea* (Figure 7A).

Complementary and Alternative Defensive Devices

Nonglandular defensive mechanisms have not been systematically investigated. We will compile here information from the literature together with our own observations. Very little information could be found, however, with respect to tropical fauna. Again only the defensive mechanisms of adults will be considered.

Reflex Bleeding. Reflex bleeding and its efficiency against ants has been recently described for the Colorado beetle (Deroe and Pasteels, 1977). In the Chrysomelinae, buccal and sometimes tibiofemoral autohemorrhea are probably frequent in young adults when the membranes are still weak and have been reported several times [several species belonging to the genera *Chrysomela*, *Chrysolina*, *Timarcha*, and *Mesoplatys* (Cuénot, 1896; Hollande, 1909, 1911; Jolivet, 1946, 1948, personnal communication)]. Bleeding is rarely observed when the beetles are fully sclerotized and when the glandular secretion is abundant, but considerable variation exists between species. In *Timarcha*, reflex bleeding is prominent. It seems significant to us that in this species the defensive secretion can scarcely be observed and is often not emitted at all.

Reflex bleeding seems to be the main defensive mechanism in the Galerucinae, in which it is usually conspicuous. Buccal or tibiofemoral bleedings have been reported for the genera *Galeruca*, *Galerucella*, *Agelastica*, *Sermylassa*, *Luperus*, *Exosoma* (= *Malacosoma*) (Cuénot, 1896; Hollande, 1911, 1926), to which we may add *Lochmaea*.

The experiments made by Cuénot and Hollande demonstrated that several species, including Galerucinae and *Timarcha*, in which bleeding is an important defensive reflex, are chemically defended because the bleeding either repels, or is toxic to, vertebrate predators. To our knowledge, besides the Chrysomelinae and the Galerucinae, reflex bleeding has been reported only for two *Altica* (Alticinae) by Hollande (1911).

Crypsis, Aposematism, and Mimicry. In Europe, cryptic colorations seem to be the rule only in the Cassidinae, in which the flattened shape makes them even more concealed in the foliage (Figure 7B). In at least one American

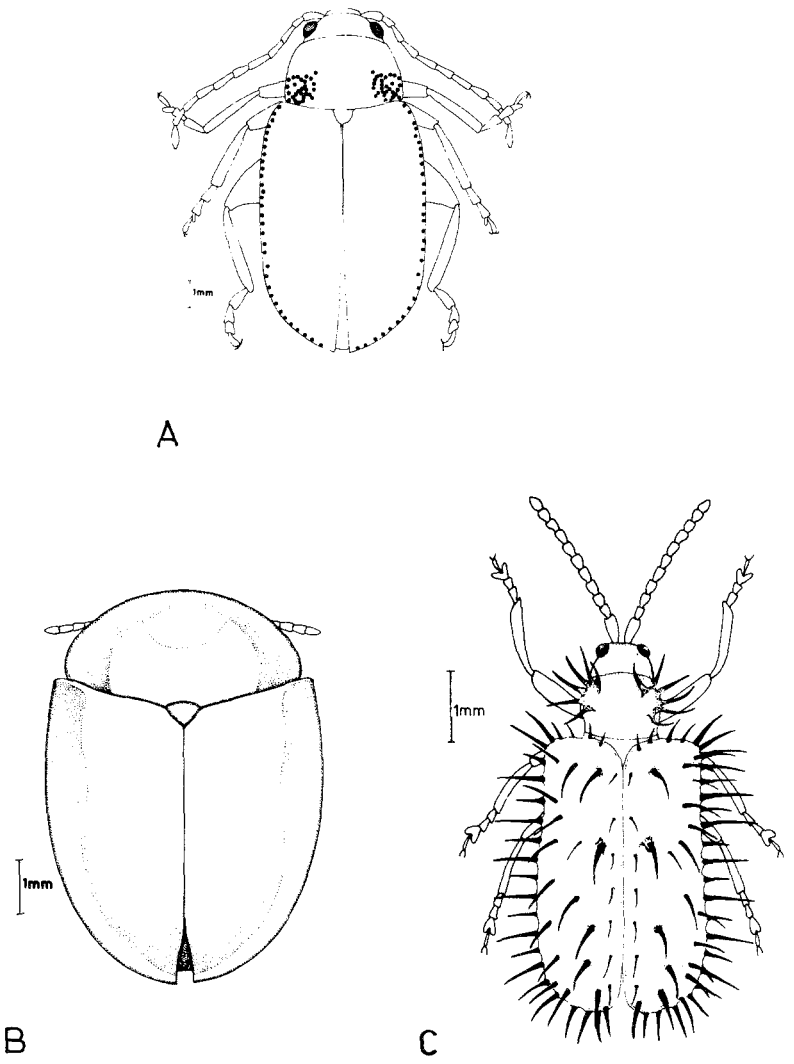

FIG. 7. (A) Distribution of the defensive glands in *Altica oleracea* (Alticinae); (B and C) alternative defensive devices in *Cassida* and *Hispa*.

species, special tarsal glands allow the beetles to have a firm grip on the substrate, which makes their capture by ants very difficult (Eisner, 1972).

As expected, aposematism is frequent in the four subfamilies in which defensive glands were observed: Criocerinae, Chrysomelinae, Galerucinae, and Alticinae. Bright coloration in these beetles, however, has not always been associated with chemical defense mechanisms. Lindroth (1971) suggested that

in some flea beetles (Alticinae) aposematism is linked to their ability to disappear suddenly by jumping, and Capinera (1976) inferred from his experiments with edible models given to blue jays and chickadees that the color pattern of *Crioceris duodecimpunctata* imparts by itself some protection against avian predators.

Müllerian and Batesian mimicry must be frequent among different chrysomelids and between them and other insects. The best documented cases of mimicry concerned some *Lebia* and *Lebistina* (Carabidae) and flea beetles (Jolivet, 1967; Balsbaugh, 1967; Lindroth, 1971) as well as the chrysomelid *Mesoplatys cincta* and the carabid *Cyaneodinodes ammon* in Upper Volta (Jolivet, personnal communication).

Mimicry could also occur between the completely black *Timarcha* sp. and young *Meloë proscarabeus* observed close to each other in early spring on herbaceous plants, or between the shiny blue *Agelastica alni* and *Chrysomela aenea*, sometimes found on the same *Alnus* tree. *Chrysolina coerulans* could also play a role in this last mimicry complex, even if it lives on *Mentha* sp. It possesses the same shiny blue color and is frequently encountered close to *A. alni* and *C. aenea.*

The several metallic green species observed on different herbs could form another mimetic complex. No experiments, however, have been done to demonstrate the effectiveness of these supposed cases of mimicry and to characterize them as Batesian, Müllerian, or even Peckhamian (aggressive) as suggested by Jolivet (personal communication) for the complex *Mesoplatys-Cyaneodinodes.*

Aposematic coloration also characterizes species which do not possess defensive glands. The most obvious examples are the bright colored Cryptocephalinae and Clytrinae. We do not know yet if they are Batesian mimics or if they possess a nonglandular chemical defense mechanism.

Some aposematic chrysomelids, apparently chemically protected, may adopt a concealment behavior when a predator is at close range. According to Capinera (1976), *Crioceris asparagi* and *C. duodecimpunctata* dodge evasively to the opposite side of the asparagus stem. Both are aposematic and at least the latter is unpalatable to birds (Jones 1932). *Crioceris*, as well as many other chrysomelids, whether or not chemically protected, frequently feign death when disturbed.

Quick Escape. Most chrysomelids are slow moving and cannot fly after the dispersion flight. But there are exceptions both among species in which defense glands are present and among those devoid of them. Flight is a frequent escape mechanism in some *Crioceris*, like *C. duodecimpunctata* (Capinera 1976). Species belonging to the Donaciae and *Luperus* (Galerucinae), all devoid of defensive glands, can escape quickly by flying too.

Quick escape by jumping is, of course, well known in the flea beetles

(Alticinae). This escape seems particularly efficient for very small specimens like *Psilliodes affinis* which are devoid of defense glands.

Mechanical Protection. Mechanical protection reaches dramatic proportions in the Hispinae, *Hispa*, in which no defense glands could be found. These beetles are protected by long sharp cuticular expansions of the elytra and pronotum (Figure 7C). The efficiency of such protection has not yet been tested.

Stridulation. Stridulation has only been observed in some Criocerinae. Capinera (1976) described it for two species of *Crioceris* and we have observed it with *Lilioceris lilii.*

Regurgitation and Defecation. Regurgitation and defecation are common reactions in many insects when they are seized. The same is true for many adult chrysomelids, and notably in some Chrysomelinae, in which regurgitation can occur at the same time as buccal bleeding, both liquids are mixed together.

DISCUSSION

The defensive strategies of the chrysomelids are quite diversified and can vary even in closely related species occupying very similar ecological niches (Capinera, 1976). Moreover the beetles' protection only rarely relies on a single device as opposed to a combination of several. Therefore, it is still difficult to understand fully how the various defense mechanisms have evolved within this family. Our survey of the adults' defense glands, however, adds some elements to this study.

First of all, it seems that the development of defense glands has been a monophyletic event. Indeed, if we consider the phylogenetic tree independently proposed by Jolivet (1978), it is very significant that the defense glands were only found in the subfamilies forming one branch originating from the Aulacoscelinae, which we were unfortunately unable to study (Figure 8). No defense glands were found in some primitive subfamilies nor in other evolved ones belonging to other lines.

A second point is that the two subfamilies Alticinae and Galerucinae are the most evolved in their line. The lack of glands in some species belonging to these subfamilies must therefore be considered as a secondary event. The predominance in both subfamilies of an alternative defensive mechanism should be noted: reflex bleeding in the Galerucinae, the jumping mechanisms in the Alticinae. Even in those species, in which the glands are present, the secretion is never as abundant as in most Chrysomelinae. In this context it is interesting to recall that *Timarcha* is the genus among the observed Chrysomelinae in which glandular defense seems the less effective, judging by the amount of secretion produced. It is also in that genus that reflex bleeding is the most prominent. It therefore seems that protection is ensured by a balance

of mechanisms, which could easily shift in one direction or the other in the course of evolution.

In another line of the phylogenetic tree (Figure 8), including the Hispinae and Cassidinae, alternative defensive mechanisms are obvious: chitinous expansions in the case of the Hispinae (*Hispa*) and flattened shape and cryptic colors for the Cassidinae.

As far as has been determined, no obvious defensive mechanisms is

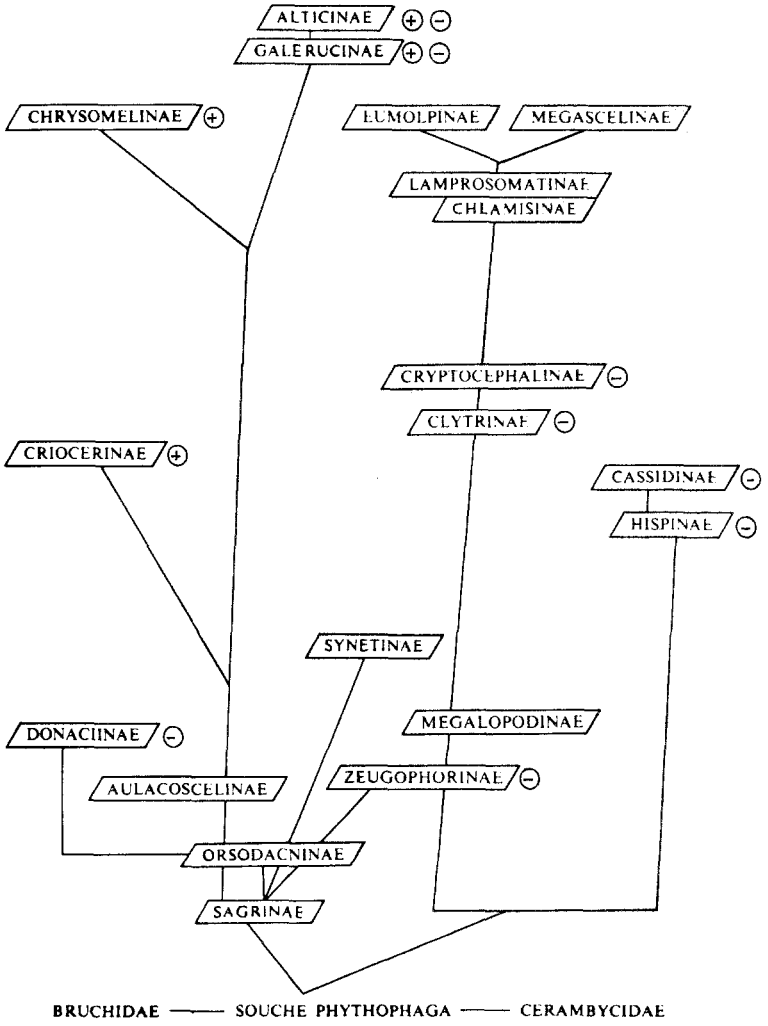

FIG. 8. Phylogenetic tree of the subfamilies of Chrysomelidae according to Jolivet (1978). The signs + or − were added where defensive glands were or were not observed.

known among adults belonging to the lines including the Zeugophorinae, Clytrinae, and Cryptocephalinae. It should be noted that the Clytrinae and to a lesser extent the Cryptocephalinae and Zeugophorinae are essentially polyphagous (Jolivet, 1978). So it could be that spectacular protection has, above all, evolved in strict phytophagous specialists which could be found much more easily by predators which can associate the beetles and their host plants.

If we consider the distribution of the glands within the beetles themselves, some interesting features also emerge. In each subfamily, the glands can be more or less widely distributed on the surface of the beetle, but they are always laterally present in both the elytra and pronotum. Even when they are more largely distributed, the lateral glands are generally more developed. It seems to us that the lateral margins of the beetles are the most efficient locations for defense glands. Small insect predators, for instance ants, will unavoidably contact the secretion which forms a chemical obstacle surrounding the beetles. Moreover, lateral secretions will remain accessible during thanatosis, but the secretion oozing from dorsal glands often will not. Finally, the lateral restriction of the glands would probably not impair the protection of the beetle against birds or other vertebrates. We tentatively conclude that the wide distribution of the glands is a primitive condition and, in the course of evolution, these glands were retained and reinforced only laterally where they are most effective, such evolution having occurred several times independently within the different subfamilies.

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EVIDENCE FOR A MULTICOMPONENT SEX PHEROMONE IN THE YELLOWHEADED SPRUCE SAWFLY

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Abstract—The existence of a female-produced sex pheromone in the yellowheaded spruce sawfly, *Pikonema alaskensis* (Rohwer) (Hymenoptera: Tenthredinidae) was demonstrated by field and greenhouse bioassays. Virgin females, their empty cocoons (with which they were confined during handling procedures), and the hexane extract of these cocoons were attractive in the field. The only Florisil fraction of this extract consistently attractive by itself was that eluted with hexane, but three, more polar fractions (eluted with 5%, 25%, and 50% ether in hexane) each synergized the hexane fraction, increasing bioassay responses 10–30 times. Fractions derived directly from virgin females yielded comparable results. The greenhouse data corroborated the field data, except that the 5% ether–hexane fraction, while very synergistic in the field, was consistently inactive in the greenhouse.

Key Words—Sex pheromone, bioassay, synergism, sawfly, Hymenoptera Tenthredinidae, *Pikonema alaskensis*, experimental design.

INTRODUCTION

The pheromones of various diprionid sawflies in the genera *Diprion* and *Neodiprion* have been studied in depth (e.g., Coppel et al., 1960; Casida et al., 1963, Jewett et al., 1976; Matsumura et al., 1979), but relatively little has been published on pheromones of sawflies in other families. Borden et al. (1978) presented evidence for a sex pheromone in the pamphiliid, *Cephalcia lariciphila* Wachtl. In the Tenthredinidae, Longhurst and Baker (1980) demonstrated the existence of a pheromone in *Nematus ribesii* (Scop.), and Forbes and Daviault (1964) described male behavior in *Pristiphora geniculata*

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(Htg.) which was typical of responses to sex pheromones. No chemical identifications have been reported for these three species. This paper and the following (Bartelt et al., 1982) describe biological and chemical aspects of pheromonal communication in the yellowheaded spruce sawfly, *Pikonema alaskensis* (Rohwer) (Hymenoptera:Tenthredinidae).

P. alaskensis is a significant defoliator of young and/or open-grown trees of various spruce (*Picea*) species in Canada and the northern United States. Its biology has been described by Nash (1939). In Minnesota, the insect is a particular problem in young plantations of white spruce, *P. glauca* (Moench) Voss. It has one generation/year, adults emerging in late May or early June. The flight season coincides with the opening of the spruce buds, and the eggs are inserted in needles of the expanding shoots. The eggs hatch in about a week, and the larvae feed until early July, when they drop to the ground and form cocoons just under the soil surface. They overwinter as prepupal larvae and pupate about 2 weeks prior to adult emergence the following May.

Mating behavior in *P. alaskensis* has not been reported, but it was readily observed in a bright, greenhouse environment when males and virgin females were placed among potted spruce trees. Males hovered around the trees, landed near females, and scrambled over the foliage until locating them. Mating was as in other sawflies, end-to-end with both individuals dorsal side up, and lasted 10–15 sec. The cocoons from which females had emerged, and with which they had been confined in the laboratory handling procedure, were similarly attractive to males when placed on the spruce foliage. Males attempted to mate with the empty cocoons or with other males visiting the same cocoons. Similar hovering and mating behaviors were seen in the field. These observations, and the fact that the species is arrhenotokous (Houseweart and Kulman, 1976), suggested that a female-produced sex pheromone exists and plays a part in the normal life cycle.

METHODS AND MATERIALS

Handling of Insects, Extraction, and Chromatography. The insects used for pheromone collection and laboratory bioassay were collected in late June 1977–1979 as 4th–6th instar larvae in white spruce plantations near Grand Rapids, Minnesota. They were reared on spruce foliage to the cocoon stage and then stored at 0° C. Each winter and spring the previous summer's cocoons were warmed to 15° at the rate of 1000–3000/week, giving a supply of adults from early January into June 1978–1980. During the 4–5 weeks at 15° before adult emergence, the cocoons were placed individually in 1 × 2.5-cm (size 000) gelatin capsules. The isolation prevented mating and also prevented the females, which are rather aggressive, from killing each other.

The males were used within 2 days of emergence for laboratory bioassay. In late spring, some of the females were used for field bioassay; otherwise they were killed by freezing when 3–5 days old, and the insects, cocoons, and capsules were separated and washed with hexane. The extracts were concentrated under vacuum and stored at −70° until used for bioassay or purified. Counts were kept so that the number of "female equivalents" (FE) or "cocoon equivalents" (CE) per milliliter could be calculated.

Crude extracts were subjected to column chromatography on Florisil (2.5% water by weight). The columns were 20 × 3.0 cm, with 2000–6000 equivalents/run. Each column was eluted consecutively with 200-ml volumes of 10 solvents: hexane; then 2.5%, 5%, 7.5%, 10%, 25%, and 50% ether in hexane (by volume); then pure ether; acetone; and finally, methanol. The volume of each solvent was about twice the void volume of the column. The effluent was collected as 10 fractions of 200 ml each. The fractions were stored at −70° until used for bioassay or chemical work.

Field Bioassay. Pheromone preparations were evaluated in the field by catches of males on "sticky" traps. The traps were made from cylindrical, white cardboard ice cream cartons, 9 × 9 cm, open on both ends, and coated on the inside with Tack Trap®. The traps were usually secured to branches of 3 to 5-m tall white spruce trees at a height of 1–2 m. If necessary, foliage was removed from near the trap openings to allow the males clear access. Trees with traps were normally separated by at least 5 m. The studies were done in infested white spruce plantations near Grand Rapids, Minnesota.

Extracts or chromatographic fractions were placed on new 5.0-cm plastic Petri plate bottoms (1978) or 4.0 cm watch glasses (1979–1980) and the solvent allowed to evaporate before setting them in traps. The plates were prepared just before use. Single virgin females used to bait traps were held in brass screen cages 4 cm long × 2 cm in diameter. The studies used 5 FE, 5 CE, or 1 virgin female/trap. The traps were normally baited in early to mid morning, when male sawflies were becoming active.

These studies usually employed two treatment traps/tree. The traps of a pair were separated by ca. 10 cm. In some 1978 studies, an additional, control trap was inserted between the traps of a pair with pheromone treatments. Assignment of treatment pairs to trees and treatments to locations within trees were randomized. Trap catches were recorded at the end of the day. All counts were transformed to $\log (n + 1)$ before analysis to stabilize variance. Trees were treated as "blocks" in statistical analysis, removing the often considerable tree-to-tree variability from residual error. The final study of 1978 (Table 3) employed the balanced incomplete block design, with the five treatments compared in pairs, in all possible combinations. Analysis was done according to Yates (1940).

A serious limitation of the field bioassay was the short duration of the

flight season. In 1978 adult emergence in the test plot was monitored with 12 emergence traps (Thompson and Kulman, 1976). Of the 115 adults captured, 94% emerged within a span of 8 days and 63% within 3 days. This, coupled with the short male life-span, limited the acquisition of meaningful field data to little more than 1 week/year.

Greenhouse Bioassay. Pheromone bioassays during winter and early spring were conducted in a greenhouse cage, 1.5 × 1.5 m at the base and 2 m high. It was screened on front and back and fitted with glass elsewhere. Twelve potted spruce trees ca. 0.5 m tall were placed inside. Air was circulated through the cage by an electric fan set 3 m from the front. About 100 male sawflies were added each day that bioassays were run. (Males are short-lived and rarely lasted more than 2 days in the cage). Pheromone preparations were placed on 6.0-cm watch glasses, at the rate of 2.5 CE or FE/plate. After the solvent evaporated, the plates were positioned in the upwind end of the cage, 10 cm apart, in a row perpendicular to the air flow, and just above the spruce trees. The treated surfaces of the plates were downwind.

The essence of the bioassay is that males ignored clean plates but landed frequently on those treated with pheromone. The visits were usually brief (about 15 sec), and the bioassay was quantified by recording the number of males on each plate every 15 sec for an 8-min period, after which the plates were rotated to different positions and the whole process repeated. The number of runs and the plate placements were chosen, a priori, so that each treatment was assayed once at each cage position. The total counts were then analyzed as a Latin square, the factors being treatment, run, and plate position in the cage. The transformation, $\sqrt{n+1}$, was found to stabilize variance. Data were taken only when at least one male was responding in the cage at virtually every 15-sec check.

Generally, a "low" control and a "high" control were included in each test. The low one was most often a clean plate and the high one an appropriate treatment which the sawflies visited readily in previous tests. The other treatments were then evaluated according to the range of responses shown toward the "controls." Consistent responses toward the high control ensured that inactivity toward any other treatment was due to the treatment itself, rather than the unsuitability of bioassay conditions or other factors. This scheme allowed a clear interpretation of negative results for test treatments.

The intensity of the response varied with environmental factors. Activity was greatest on bright days. Most bioassays were run when the temperature was 20–25°; when the sky was heavily overcast, raising the temperature further would sometimes initiate activity. Bioassays could be run during the morning or afternoon, but the intensity of response usually fell off after 1–3 hr of continuous testing. After several hours of rest the males would usually respond again. The spruce trees in the cage and the air movement caused by the fan were both essential for a reliable, consistent response.

RESULTS AND DISCUSSION

Field Studies. Except where otherwise stated, the studies were performed in late May 1978. As in the greenhouse, the empty cocoons with which females had been confined were attractive to males in the field, compared to controls (Table 1). The hexane extract of these cocoons was also attractive. Of the 10 Florisil fractions, only that eluted with hexane was similar in activity to the crude extract, indicating the pheromone to be quite nonpolar in nature. The recorded cumulative emergence increased from 12% to 84% over the 3 days of the study; thus it was run during the population peak.

In a concurrent test, single, 1 to 2-day-old virgin females were similar in attractiveness to 5 CE of the hexane–Florisil fraction. The mean ($N = 5$) 1-day catches of males (and ranges) were: 81.4 (28–119) for females, 5.4 (2–11) for controls (inserted between the baited traps), and 93.0 (65–152) for the hexane fraction. The low control catches indicated that the two treatments were not acting as a single large trap. Most males flew precisely enough to enter only the attractive traps, even though there was less than 1 cm between trap openings.

Of the remaining Florisil fractions (Table 1), the 5% and 25% ether-hexane fractions (denoted below as just the 5% and 25% fractions) each appeared slightly attractive in one of the six replications, the catches being 11

TABLE 1. MEAN 1-DAY TRAP CATCHES OF *P. alaskenis* MALES FOR EMPTY COCOONS FROM FEMALES, HEXANE EXTRACT OF THESE COCOONS, AND FLORISIL FRACTIONS OF THIS EXTRACT

	Treatment[a]	Control
Cocoons (5/trap)	51.0 ***	2.0
Hexane extract (5 CE/trap)	28.0 ***	1.2
Florisil fractions (5 CE/trap)		
Hexane	62.3 ***	1.2
2.5% ether in hexane	0.5	1.3
5.0% ether in hexane	3.3	2.2
7.5% ether in hexane	0.7	0.3
10.% ether in hexane	0.5	0.3
25.% ether in hexane	2.5 *	0.2
50.% ether in hexane	1.3	0.8
Ether	0.3	0.8
Acetone	0.2	0.7
Methanol	0.3	1.2

[a]Each treatment trap paired with a control; 2 reps/day on 3 consecutive days; * and *** imply significant differences from controls at the 0.05 and 0.001 levels (t tests).

and 14, and their controls, 1 and 0, respectively. These catches accounted for the slightly higher means for these fractions in Table 1.

To test for synergistic effects, the 5% and 25% fractions, alone and in combination with the hexane fraction, were compared to the hexane fraction (Table 2). The combinations of the 5% and/or 25% fractions with the hexane fraction greatly exceeded just the hexane fraction in attractiveness, by factors of 13 to 29. Equally striking, there was no synergism when the hexane fraction and the 5% or 25% fractions were in separate traps ca. 10 cm apart (Table 2, lines 1, 2, and 3). Trap catches on the hexane fraction alone were lower than in previous experiments, probably because the population was past its peak and was declining; the recorded cumulative emergence increased from 84% to 90% during the study. In 1979, three treatments of the study were repeated, using chemicals extracted directly from females rather than their empty cocoons. As in 1978, the 5% and 25% fractions significantly synergized the hexane fraction (Table 2).

The final field study in 1978 was conducted to compare virgin females; the hexane extract; the hexane–Florisil fraction; the combination of 5%, 25%, and hexane fractions; and controls (Table 3). The study was run at the end of the flight season when the population of males was very low (the recorded cumulative emergence increased from 97% to 100% during the study), and the virgin females and the hexane–Florisil fraction were no longer significantly more attractive than controls. The combination of 5%, 25%, and hexane

TABLE 2. MEAN 1-DAY TRAP CATCHES OF *P. alaskenis* MALES ON VARIOUS COMBINATIONS OF 5%, 25%, AND HEXANE FRACTIONS, TESTED AGAINST HEXANE FRACTION (ONE TEST PAIR/TREE, WITH CENTRAL CONTROL IN 1978)

	Test fraction(s)[a]	Control	Hexane fraction	Observations/mean
1978 (5 cocoon equivalents/trap)				
5%:	2.3	1.0	0.7	3
25%:	0.3 *	1.0	5.7	3
5% + 25%:	0.5	1.5	3.5	2
Hexane + 5%:	63.0 ***	1.7	4.7	3
Hexane + 25%:	28.7 ***	0.7	1.0	3
Hexane + 5% + 25%:	98.0 ***	3.3	5.7	3
1979 (5 female equivalents/trap)				
Hexane + 5%:	13.7 **		0.7	3
Hexane + 25%:	39.7 *		4.0	3
Hexane + 5% + 25%:	97.0 ***		4.3	3

[a]Significant differences (t tests) between treatments and the hexane fraction, at the 0.05, 0.01, and 0.001 levels, indicated by *, **, and ***, respectively.

TABLE 3. MEAN 1-DAY CATCHES OF *P. alaskenis* MALES FOR A PAIRED COMPARISON OF 5 TREATMENTS (BALANCED INCOMPLETE BLOCK DESIGN).

Treatment	Mean catch ($N = 8$)[a]
Control	0.63 a
Virgin females (1/trap)	1.13 a
Hexane Florisil fraction (5 CE/trap)	1.25 a
Hexane extract (5 CE/trap)	4.75 b
Hexane + 5% + 25% fractions (5 CE/trap)	39.80 c

[a]Different letters denote significant differences at the 0.05 level by the least significant difference (LSD) method.

fractions, however, was still quite active. The crude hexane extract was between the hexane fraction and the 3-fraction combination in attractiveness. While this extract contained the same chemicals as the more active combination, it apparently had additional masking or inhibitory chemicals as well.

Other Field Results and Observations. Qualitatively, the males were most active around noon, and fewer were caught in the cooler, early morning hours or toward evening. This was the same trend reported by Casida et al. (1963) for *Diprion similis*, the introduced pine sawfly. Males were less active on overcast or cool days than on sunny, warm days. When traps were left in the field overnight, there was no evidence of males being caught during the hours of darkness. There was no indication of females being attracted to the pheromone treatments.

The host tree seems to play an important role in sex attraction. In a 1980 experiment, three traps placed in spruce trees and baited with 2 FE of a potent, purified form of the hexane fraction (the "HPLC fraction," Bartelt et al., 1982) caught 25, 106, and 188 males, while three identical traps attached to poles ca. 2 m from other spruce trees (trap height 1.5 m) caught 0, 0, and 2. On the second day, fresh traps set at the same six locations (but with trap positions, on poles or in trees, reversed) caught 77, 78, and 94 in trees and 0, 1, and 1 on poles. In a subsequent, similar test, traps baited with the purified hexane fraction plus the 5% and 25% fractions gave comparable results: a total of 182 males in three traps in spruce trees and 0 on three poles. Traps in other kinds of trees in the plantation (balsam firs, willows, poplars) caught 0–10 males/day, far below catches in spruce trees. While *Diprion similis* has been reported to fly 30 m or more from the host plant in response to virgin females or pheromone baits (Coppel et al., 1960), male *P. alaskensis* seemed reluctant to respond to the female scent anywhere but in spruce trees.

Greenhouse Bioassay—Florisil Fractions. The active Florisil fractions were used to develop the greenhouse bioassay, the tool to be used to monitor

pheromone purification when adults were not present in the field. The following results illustrate the properties of the bioassay, parallels between field and greenhouse results, and some additional conclusions about the Florisil fractions.

As in the field, hexane-Florisil fractions derived from the empty cocoons of females were active (Table 4), although two such preparations differed somewhat in activity. A hexane-Florisil fraction derived directly from virgin females was superior to the cocoon-derived material, however (Table 4). The hexane fractions were usually in the bioassay cage about an hour before the male response began. The attractiveness of empty cocoons was probably an artifact of the insect-handling procedures and had no biological significance. Extracts of the females' gelatin capsules were also found to be attractive.

Table 4 also shows the variability typically seen between two replications of a treatment (in no case were such differences significant), and it also shows that a "real" difference between treatments (e.g., the 1978 cocoon material and controls) could be obscured by the presence of an even more active treatment (the female-derived fraction). Absolute scores for a treatment tended to vary from experiment to experiment, depending on what other treatments were present, and therefore, only comparisons of scores within an experiment are meaningful.

Also as in the field, the 25% fraction strongly synergized the hexane fraction (Table 5). One hour after the plates were prepared, the combination

TABLE 4. GREENHOUSE BIOASSAY: MEAN SCORES FOR HEXANE-FLORISIL FRACTIONS FROM SEVERAL SOURCES (2.5 CE OR FE/PLATE)[a]

Treatment		Mean ($N = 5$)[b]
Cocoons, 1978	#1	62.3 c
	#2	60.7 c
Control		2.3 a
Cocoons, 1979	#1	40.3 bc
	#2	30.2 b
Cocoons, 1978	#1	4.3 a
	#2	11.4 a
Control		1.5 a
Females, 1979	#1	59.3 b
	#2	76.2 b

[a]There were 5 runs/plate and 2 plates for each treatment except controls.
[b]Plates followed by different letters were significantly different (LSD, 0.05).

TABLE 5. GREENHOUSE BIOASSAY: TEST FOR SYNERGISM BY 5% and 25% FLORISIL FRACTIONS DERIVED FROM COCOONS (2.5 CE/PLATE) AND CHANGES OVER TIME

	1 hour old		4 hours old		1 day old	
Treatment	Mean[a] ($N = 5$)	Ratio to hexane fraction	Mean ($N = 5$)	Ratio to hexane fraction	Mean ($N = 5$)	Ratio to hexane fraction
Hexane	5.6 a	1	4.9 ab	1	40.3 bc	1
Control	0.9 a	0.16	0.2 a	0.04	1.9 a	0.05
Hexane + 5%	7.3 a	1.3	10.6 b	2.2	60.3 c	1.5
Hexane + 25%	122.9 b	22	33.2 c	6.8	33.0 b	0.82
Hexane + 5% + 25%	130.1 b	23	34.7 c	7.1	25.8 b	0.64

[a]In each test, different letters indicate significant differences (LSD, 0.05).

of hexane and 25% fractions had a score about 20 times that of the hexane fraction alone, similar to the ratio of trap catches in the field. After 4 hr, however, the combination exceeded the hexane fraction by only sevenfold, and after one day (males having been added to the cage) there was no difference between these treatments. The effect of the 25% fraction was relatively short-lived. The hexane fraction, by itself, was not significantly more active than the control on the first day but was clearly active on the second day. The 1- and 4-hr data again showed that the activity of the hexane fraction could be obscured by the presence of an even more active treatment (the combination). Curiously, the 5% fraction never did exhibit the ca. 15-fold synergism in the greenhouse that was seen in the field, and the combination of all three fractions behaved much as the combination of 25% and hexane fractions.

In another test of the cocoon-derived Florisil fractions (Table 6), the 25% fraction again strongly synergized the hexane fraction, and again, the 5% fraction did not. In addition, the 50% fraction was similar in synergism to the 25% fraction. (The 50% and hexane fractions had not been tested together in the initial field studies. It is possible for a single compound to occur in two consecutive fractions, but this issue has yet to be resolved for the 25% and 50% fractions). No other fractions gave comparable synergism, although more subtle effects could not be ruled out.

Table 7 shows the results of a test for synergism in female-derived Florisil fractions. The 25% and 50% fractions again showed strong synergism and, as before, the hexane fraction alone had a low score when competing with these combinations. Here too, the 5% fraction did not synergize the hexane fraction, although the same preparation later gave positive synergistic results

TABLE 6. GREENHOUSE BIOASSAY: TESTS FOR SYNERGISM IN FLORISIL FRACTIONS DERIVED FROM COCOONS (2.5 CE/PLATE)[a]

Treatment	Mean ($N = 3$)[b]	Treatment	Mean ($N = 3$)
Hexane + 25%	138.7 b	Hexane + 25%	95.0 b
Hexane	2.7 a	Hexane + 50%	105.3 b
Hexane + 5%	3.3 a	Hexane + ether	2.7 a
Hexane + 25%	125.5 b	Hexane + 25%	106.0 c
Hexane + 2.5%	4.5 a	Hexane + methanol	1.0 a
Hexane + 10%	2.0 a	Hexane + 7.5%	9.7 b

[a] A subsequent, qualitative test found the hexane + 25% fractions to be considerably more active than the hexane + acetone fractions.
[b] In each test, different letters indicate significant differences (LSD, 0.05).

in the field (Table 2). No other fractions showed strong synergism, although some appeared repellent, the combinations with the hexane fraction behaving as controls.

Other Observations. Qualitatively, males responding to the hexane fraction in the greenhouse approached the plates from downwind, hovering and moving toward the plates very slowly and precisely. Upon landing they

TABLE 7. GREENHOUSE BIOASSAY: TESTS FOR SYNERGISM IN FEMALE-DERIVED FLORISIL FRACTIONS (2.5 FE/PLATE)

Treatment	Mean[a]	Ratio to hexane fraction
Hexane	41.4 bc	1
Control	1.9 a	0.05
Hexane + 2.5%	30.6 b	0.74
Hexane + 5.0%	15.8 b	0.38
Hexane + 7.5%	66.2 c	1.6
Hexane + 10%	3.5 a	0.08
Hexane	7.4 a	1
Control	0.5 a	0.07
Hexane + 25%	201.5 b	27
Hexane + 50%	148.8 b	20
Hexane	50.9 c	1
Control	2.2 a	0.04
Hexane + ether	13.6 b	0.27
Hexane + acetone	74.8 c	1.5
Hexane + methanol	5.5 ab	0.11

[a] In each test, treatments followed by different letters were significantly different (LSD, 0.05).

TABLE 8. BIOASSAY CHARACTERISTICS OF VARIOUS FLORISIL FRACTIONS AND COMBINATIONS

Treatment	Field bioassay	Greenhouse bioassay
Hexane	Catches above control levels when population high.	Upwind hovering, alighting on plate, occasional mating attempts.
Hexane + 25% (or + 50%)	Catches far above those for the hexane fraction alone.	As with the hexane fraction, except mating attempts more numerous and vigorous. Also "branch-swarming" behavior.
Hexane + 5%	Catches far above those for hexane fraction alone.	Activity pretty much as with the hexane fraction alone; 5% fraction has little effect.

walked about, investigating the plates with their antennae. Males contacting others on a plate frequently attempted to mate, backing toward each other to engage the claspers while beating their wings. Less often, single males spontaneously showed the same behavior toward the treated plate. Visits typically lasted about 15 sec.

When the 25% (or 50%) fraction was mixed with the hexane fraction, the mating attempts were much more intense and frequent (perhaps because of the larger number of males crowding onto the plates), and in addition, other males congregated on the spruce shoots immediately downwind of the treated plate. These also actively attempted to mate with each other. This "branch swarming" behavior was rarely seen when only the hexane fraction was present. The bioassay characteristics of the active Florisil fractions in the field and greenhouse are summarized in Table 8.

An extract of male sawflies, and empty cocoons and gelatin capsules from males, were tested in the greenhouse but were not attractive to males.

To gauge the polarity of the chemicals in the active fractions, standards were chromatographed under the same conditions. The hydrocarbons eluted with hexane; esters, aldehydes, and ketones of 14–18 carbons eluted with 5% ether in hexane; and alcohols of 12–14 carbons eluted with 25% ether in hexane. The low polarity of the primary component suggested a difference from the diprionid sawflies, which use esters for sex attraction (Jewett et al., 1976). In addition, the existence of powerful synergists and the drastically reduced activity of pheromone away from the host tree are features not reported for diprionid species. Bartelt et al. (1982) deals with the identification of the active material in the hexane fraction. Work on the synergists is still in progress.

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HYDROCARBON COMPONENTS OF THE YELLOWHEADED SPRUCE SAWFLY SEX PHEROMONE: A Series of (*Z, Z*)-9,19 Dienes

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Abstract—The primary sex pheromone of the yellowheaded spruce sawfly, *Pikonema alaskensis* (Rohwer) (Hymenoptera: Tenthredinidae), was found to include a series of straight-chain hydrocarbon dienes, all with the double bonds in the 9 and 19 positions and all with the (*Z,Z*) configuration. The major components, of 29, 31, 33, 35, and 37 carbon atoms, were synthesized. In the field and the greenhouse, the synthetic dienes were far above control levels in activity but, at least during the first hours of bioassay, were somewhat less active than the female-derived materials on a weight basis. In the field, a mixture of all five synthetic dienes, in the proportions found in the females, was more attractive than any single one, on a mole basis. In addition, (*Z,Z*)-9,19 dienes of 28, 30, 32, 34, 36, 38, and 39 carbons have been detected in females in minor amounts. The first five were bioassayed, and each was found to be similar in activity to the 35-carbon component when compared on a weight basis. The synthetic dienes, while active by themselves, were strongly synergized by two, more polar, Florisil fractions derived from females. Experimental design considerations are discussed.

Key Words—Sex pheromone, Tenthredinidae, *Pikonema alaskensis*, hydrocarbons, dienes, synergists, experimental design, ozonolysis, mass spectra, methoxymercuration, Hymenoptera.

INTRODUCTION

The yellowheaded spruce sawfly, *Pikonema alaskensis* (Rohwer) (Hymenoptera: Tenthredinidae) was shown to possess a multicomponent sex pheromone by responses to Florisil fractions derived from females (Bartelt et

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al., 1982). This paper deals with the identity of the only Florisil fraction consistently active by itself, that obtained by eluting with hexane.

METHODS AND MATERIALS

Collection of pheromone, partial purification on Florisil, and general procedures for field and laboratory bioassays were described in Bartelt et al. (1982). A modification of field technique was that the antioxidant, 2,5-di-*tert*-pentylhydroquinone, was added to baits (0.1 mg/trap). The balanced incomplete block design was used for most of the field experiments, the treatments being tested in pairs, in all possible combinations. The paired traps were set ca. 20 cm apart in individual spruce trees, which were regarded as "blocks." This design was used in order to compensate for tree-to-tree variability, which was considerable in some studies. Analysis of these experiments was done according to Yates (1940). Variance-stabilizing transformations were used for all analyses, log $(n + 1)$ for field data and $\sqrt{n + 1}$ for greenhouse data. Additional details of individual experiments are included with the results.

High-pressure liquid chromatography (HPLC) was done with a Waters Associates M6000A pump and R401 differential refractometer detector. The column was 100 × 4 mm 20% $AgNO_3$ on silicic acid, and it was eluted with 5% or 25% toluene in hexane (v/v) at 1.0 ml/min. Injections were 5–50 μl, with a maximum of 200 female equivalents (FE) or 1 mg of synthetic pheromone.

Gas–liquid chromatography (GLC) was done on a Hewlett Packard 5830A, equipped with a flame ionization detector, effluent splitter, and thermal gradient collector (similar to that described by Brownlee and Silverstein, 1968). Columns were all glass, with lengths of 1.9 m and inside diameters of 2 mm (analytical) or 4 mm (preparative). Column packings included 3% Dexsil 300 on 80/100 Gas Chrom Q for hydrocarbon analysis and collection; 5% Silar 5CP on 80/100 Gas Chrom Q for analysis of ozonolysis products; and 5% OV-1 on 80/100 Gas Chrom Q and 5% Silar 10C on 60/80 Gas Chrom Q for hydrocarbon analysis. The carrier was N_2 at 18–20 ml/min for 2-mm columns and 70–80 ml/min for 4-mm columns. Various temperature programs were used and are described with relevant results.

Mass spectra were obtained on an LKB-9000 gas chromatograph–mass spectrometer, using a 0.6-m 3% OV-1 column and electron ionization energies of 20 or 70 eV. The high-resolution mass spectrum was obtained by direct inlet on an AEI MS-30. Infrared spectra were obtained on a Beckman Acculab 3, with the samples (all oils) held between NaCl plates.

Ozonolysis was done as described by Beroza and Bierl (1967). Samples for bioassay were run in 0.5 ml hexane for 2 min at −70°. Samples for double-bond location were run in 50 or 100 μl of CS_2, also at −70° for 2 min,

and the ozonides were reduced with triphenylphosphine. Sample sizes were usually 10–50 μg, although some as small as 0.5 μg were used. Five μl of the reaction mixture were injected in the Silar 5CP column, held at 60° for 4 min, and then temperature programed to 240° at 10°/min. Retention times for aldehyde and dialdehyde standards were determined; the GLC conditions permitted aldehydes from C_6 to C_{22} to be easily recognized. Dialdehydes had retention times similar to those of the simple aldehydes with six additional carbon atoms.

Methoxymercuration–demercuration was carried out as described by Baker et al. (1978), except that the reaction volume was 0.5 ml, containing 1 mg $Hg(CH_3CO_2)_2$ and 5–100 μg of hydrocarbon. The net reaction was to add a methoxy group to one carbon of a double bond and a hydrogen atom to the other. The extracted reaction products were injected directly onto the GLC–mass spectrometer, separation of unreacted hydrocarbons from methoxy derivatives being accomplished by the GLC inlet system.

RESULTS

Purification and Bioassay. The hexane-Florisil fraction derived from the hexane extract of virgin female *P. alaskensis* (Bartelt et al., 1982) was subjected to HPLC on the $AgNO_3$ column, using 5% toluene in hexane as the solvent. Only two peaks, both rather intense, appeared in the chromatograms 1.2–2.8 and 2.8–5.6 ml after injection. By the greenhouse bioassay the two peaks were inactive, but responses to the subsequent "baseline" were strong. For example, in one experiment 4 fractions were tested in the greenhouse and gave the following mean bioassay scores: 0–6 ml, 0.0 males; 6–11 ml, 53.5 males; 11–17 ml, 51.8 males; and 17–40 ml, 125.3 males (bioassay parameters: 6 FE/plate, 3.5 min/run, 4 runs). In a 1979 field test, the 17–40 ml fraction, at ca. 1 FE/trap, was considerably more active than single virgin females, as mean daily trap catches were 44.6 ($N = 16$) and 4.95 ($N = 20$), respectively, (1 baited trap/tree; $P << 0.001$ by t test on transformed data).

Apparently the pheromone eluted as a very broad band because the GLC traces of active HPLC fractions showed the same peaks in similar ratios. The two inactive HPLC peaks, later determined by mass spectrometry to include saturated and monounsaturated hydrocarbons, respectively, accounted for ca. 85% of the material in the hexane–Florisil fraction, and separating the pheromone from these accomplished a considerable purification. Since $AgNO_3$ retards the movement of unsaturated compounds through chromatographic systems, the pheromone was suspected to be a hydrocarbon with more than one double bond.

The active HPLC fraction (17–40 ml) was purified further by GLC. The chromatogram appeared as in Figure 1. Equivalent chain lengths (ECLs),

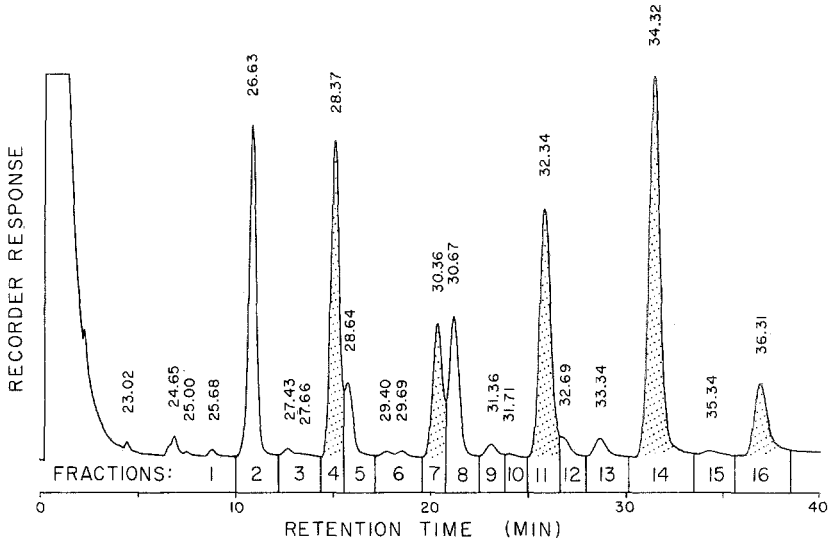

FIG. 1. Gas-liquid chromatogram of the active HPLC fraction (Dexsil 300, 220–300° at 2°/min). Equivalent chain lengths, based on *n*-alkane standards, are shown above the peaks, and boundaries of collected fractions underneath. The peaks initially found to be active are shaded.

based on *n*-alkane standards, are shown above the peaks. Fractions were collected, also as shown in the figure. The fractions were tested with the greenhouse bioassay, one at a time against the control and GLC fraction 4. This fraction was seen to be active in preliminary screening and was used as a "high control" in all tests. The fractions were used at the rate of ca. 0.25 FE/plate. The 3 × 3 Latin-square design was used to allow for position effects in the bioassay cage, and each run lasted 3 min. The data are shown in Table 1.

Five of the fractions, including 4, showed activity significantly above control levels (based on 0.05 *t* tests in the $\sqrt{n+1}$ scale, residual variance pooled over all 15 tests). The consistent response to fraction 4 indicated that the males remained sufficiently active throughout the 4-hr bioassay period. The active peaks are shaded in Figure 1. These had ECLs 0.31–0.37 carbon units greater than even-carbon *n*-alkanes, suggesting the pheromone included a series of related compounds, each member differing from the next by 2 carbons. The active fractions were chromatographed on OV-1 and Silar 10C, but remained as single peaks. The bioassay amounts of the active peaks (Table 1) ranged from 0.12 to 0.84 μg, based on integration of GLC peaks. The five active peaks totaled ca. 10 μg/female.

Fractions 2 and 8 contained peaks as large as the active ones but behaved as controls in the bioassay. They had ECLs 0.63–0.67 greater than *n*-alkanes, so they did not belong to the "active" series. In purified extracts of male

TABLE 1. GREENHOUSE BIOASSAY OF GLC FRACTIONS[a]

Fraction number	Total bioassayed (μg)	Mean scores		
		Test fraction	Control	Fraction 4
1	0.08	0.33	0	18.7 *
2	0.58	0.33	0	21.3 *
3	0.03	0	0	20.0 *
4	0.62	–	–	–
5	0.14	0	0.67	16.3 *
6	0.03	0	0	15.7 *
7	0.31	8.3 *	0	10.0 *
8	0.32	0	0	13.3 *
9	0.03	1.0	0	14.7 *
10	0.01	0.33	0	15.3 *
11	0.61	9.0 *	0.33	15.0 *
12	0.03	0	0.67	23.7 *
13	0.05	0.67	0	19.0 *
14	0.84	48.7 *	0	7.0 *
15	0.004	0.33	0	19.3 *
16	0.12	4.0 *	0	13.0 *

[a]Fractions 1–3 and 5–16 tested individually against fraction 4 and controls (ca. 0.25 FE/plate). Mean scores are given ($N = 3$, 3 min/run). The (*) indicates significant difference from the control at the 0.05 level.

TABLE 2. PAIRED COMPARISON (BALANCED INCOMPLETE BLOCK DESIGN) OF 5 INDIVIDUAL ACTIVE GLC FRACTIONS, A MIXTURE OF ALL 5, AND CONTROLS IN THE FIELD[a]

Fraction	Quantity (μg/trap)	Total ($N = 12$)	Range
4	1.8	76 c	0–30
7	.9	35 bc	0–15
11	1.8	29 bc	0–7
14	2.3	44 c	0–10
16	.4	20 b	0–6
Mixture	7.2	229 d	4–46
Control	–	8 a	0–5

[a]Total 1-day trap catches, fractions used at the rate of ca. 0.7 FE/trap. Treatments followed by different letters are significantly different (LSD 0.05, $\log(n + 1)$ scale).

sawflies, the "active" GLC peaks were not detectable, but there was a large peak corresponding to fraction 2 of the females.

The five individual active fractions, a mixture of the five (all at ca. 0.7 FE/trap), and controls were tested in the field to assess relative activity (balanced incomplete block design, two complete replications, 12 traps/treatment). The 1-day total trap catches are given in Table 2. Each individual fraction was significantly (if not dramatically) more active than the controls. As in the greenhouse, the least abundant (16) was also the least active, but there was no "dominant" fraction. None approached the activity of the whole mixture. Therefore, no single compound was essentially responsible for the attractiveness of the hexane–Florisil fraction. But the study could not determine whether the five fractions were "interchangeable" (the greater activity of the mixture being purely a quantitative effect) or whether the males could differentiate among the five compounds and sought the mixture in preference to any one homolog.

Dose Response to the Active Peaks. The responses over time to different doses of the HPLC fraction (quantitated by GLC) are shown in Table 3. In the greenhouse, doses of 4 and 40 μg of active peaks/plate were consistently

TABLE 3. COMPARISON OF VARIOUS DOSES OF ACTIVE HPLC FRACTION (A) IN GREENHOUSE AND (B) IN THE FIELD (BALANCED INCOMPLETE BLOCK DESIGN), AND CHANGES IN ACTIVITY WITH TIME[a]

	A: Greenhouse bioassay Mean bioassay score (N = 4, 3 min/run)	
Dose (μg)	0–20 min	20–40 min
40 (4 FE)	9.06 b	12.64 c
4 (0.4 FE)	17.22 c	7.70 b
0.4 (0.04 FE)	7.43 b	1.16 a
Control	0.0 a	0.0 a

	B: Field bioassay Total trap catch (N = 3)				
Dose (μg)	0–0.5 hr	0.5–1.5 hr	1.5–2.5 hr	2.5–24 hr	Overall total
200 (20 FE)	0	7	5	87	99
20 (2 FE)	10	19	4	4	37
2 (0.2 FE)	0	3	1	3	7
Control	0	0	1	1	2

[a]Total μg for the 5 active components and approximate FE are given. Significant differences in the greenhouse study denoted by different letters (0.05 level).

active, but the lower dose was visited more often initially, and only after ca. 30 min did the higher dose become dominant. The 0.4-μg dose was initially active but dropped off quickly to control levels, at least when competing with higher doses. In another study (Table 1), 0.62 μg of a single component was consistently active for 4 hr.

The results of a small balanced incomplete block experiment, run in the field near the end of the 1980 flight season, are also shown in Table 3. The final 1-day totals showed the expected relationship to dose, 200 μg being most active and 2 μg being barely different from controls. During the first half hour, however, each of the 20-μg traps, and only these, caught males. One of these (with 4 males during the first half hour) was paired with a 200-μg trap. If the 200-μg dose was initially so high as to be repellent, it did not prevent a trap only 20 cm away from catching males. As in the greenhouse, the highest dose gradually became dominant.

Identification. The combined active GLC fractions were ozonized and tested in the greenhouse against an identical mixture which had not been ozonized and a control. The "intact" mixture had an average score of 78.3 per 4-min run, while the control and ozonized mixture each had scores of 0. This test further supported the presence of at least one double bond.

Mass spectra were obtained for the five active GLC fractions (GLC inlet, 70 eV). The spectra were similar in form; that for fraction 11 is shown in Figure 2, as an example. The spectra gave no evidence of more than one compound per active fraction. Parent ions were observed at m/e 404, 432, 460, 488, and 516, consistent with hydrocarbons of 29, 31, 33, 35, 37 carbon atoms, respectively, each with 2 degrees of unsaturation. The form of the mass spectra suggested straight-chain hydrocarbons, by virtue of the prominent parent ions and the absence of relatively intense odd-mass peaks, which could indicate branching. The straight-chain form was further supported because the compounds readily entered a 5 Å molecular sieve from 2,2,4-trimethylpentane solution (Sonnet el al., 1979).

Although evidence pointed to straight-chain dienes, the mass spectra gave no information about the locations of double bonds. To determine these, methoxy derivatives of the five separate dienes were prepared. The mass spectra of such derivatives are discussed by Baker et al. (1978) and Blomquist et al. (1980). In the mass spectrometer, methoxy derivatives cleave preferentially on either side of the branch point, yielding characteristic oxygen-containing fragment ions which can be used to determine the original double bond positions.

The reaction mixtures were injected into the GLC–mass spectrometer (0.6-m OV-1 column at 280°). In each case three GLC peaks were seen, the first corresponding to unreacted parent compound, and the second and third (with ECLs of ca. 2 and 4 additional carbon units) being mono- and dimethoxy derivatives, respectively. Scans of all peaks were taken at 20 eV.

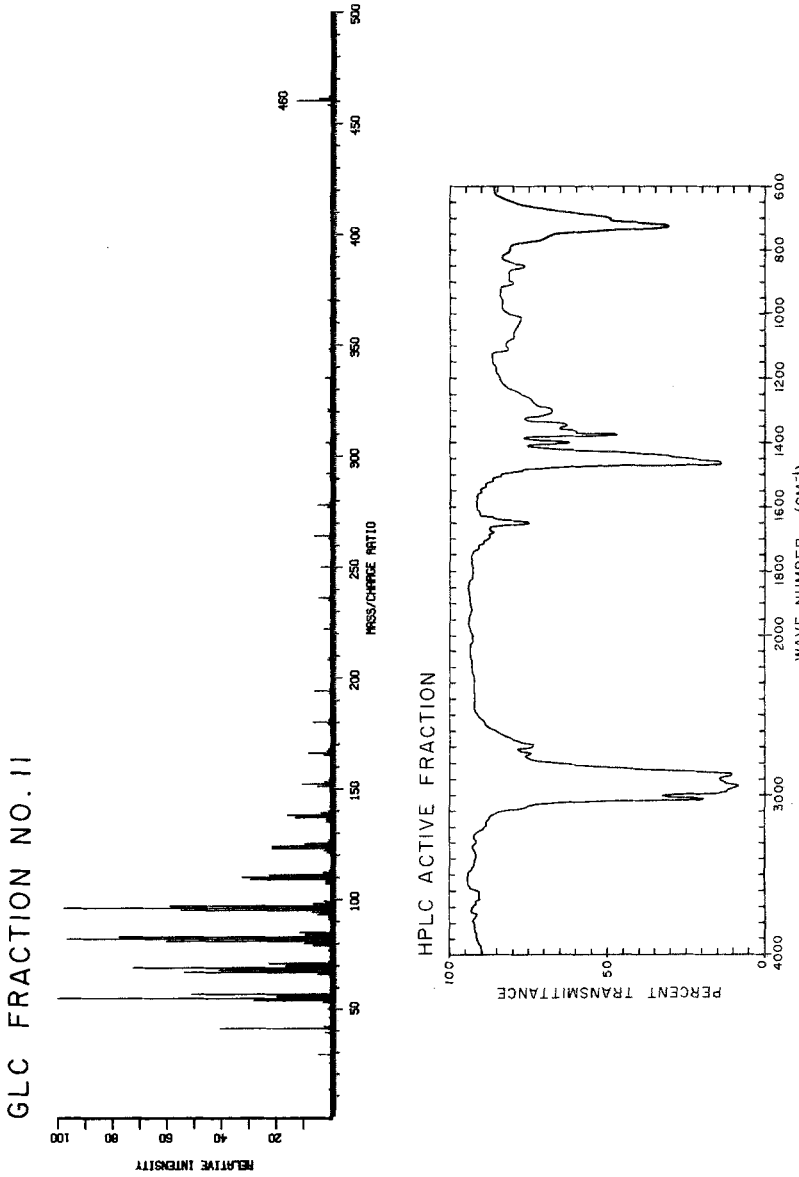

FIG. 2. Mass spectrum (70 eV) of the GLC fraction #11 and infrared spectrum of the active HPLC fraction (neat sample held between NaCl plates).

The spectra of the mono- and dimethoxy derivatives for the C_{33} component are shown in Figure 3. Even though the reaction mixture was completely resolved by the GLC inlet system, there was a strong peak at 460, suggesting the facile loss of methanol from the derivatives in the mass spectrometer. Nevertheless, four pairs of odd-mass cleavage fragments were also observed for the monomethoxy derivative, as postulated for a pure diene. The dimethoxy derivative yielded a similar spectrum except that the same eight fragment peaks were much more intense. Four of these would not be produced by simple cleavage of a dimethoxy derivative, which again suggested the loss of methanol from the compound, but the four simple-cleavage fragments containing two methoxy groups were also detected. Although the apparent competition between cleavage and loss of methanol was unexpected, the spectra did indicate the parent compound to be a 9,19 diene. The fragments are rationalized in Figure 3. The absence of other intense fragments in the dimethoxy spectrum argues for relatively high isomeric purity. The other four active compounds yielded similar spectra, and all were found to be 9,19 dienes. The "key" fragment ions from the mass spectra of the methoxy derivatives are shown in Table 4. A high-resolution mass spectrum was obtained on the reaction mixture for the 35-carbon diene (direct inlet), and it confirmed the C—H—O composition for all 8 major fragment ions and the parent ion of the diene. In addition, two of the four dimethoxy fragments were intense enough to be detected, and their compositions were also confirmed.

Double-bond positions were also checked by ozonolysis, the GLC retention times of the products being compared to those of standard aldehydes and dialdehydes. For each diene, large peaks at the retention times of nonanal and decanedial were seen, as well as decanal, dodecanal, tetradecanal, hexadecanal, or octadecanal, for the appropriate dienes (Table 4). Ozonolysis confirmed the 9,19 double-bond positions for all five dienes and also the relative isomeric purity, with ca. 1–5% of the GLC peak area of the ozonolysis products being inconsistent with 9,19 dienes.

Infrared spectra were obtained for the 35-carbon diene (the most abundant) and also (Figure 2) for the active HPLC fraction. The latter spectrum was of better quality because the sample size was not limited by the preparative GLC procedure, but the features of both spectra were identical. Major peaks included those at 2950, 2880, 1465, 1380, and 720 cm^{-1}, typical of hydrocarbons. The presence of double bonds was confirmed by a peak at 3020 cm^{-1}. The peaks at 1650 and 1400 and the shoulder at 690 cm^{-1} indicated that the *Z* configuration was present, and the absence of any peak at 970 cm^{-1} indicated the *E* configuration was not. (The *E* configuration was not detected in any HPLC fraction). Thus, the configuration of the pheromone dienes was (*Z*,*Z*), and the tentative structure determination was complete.

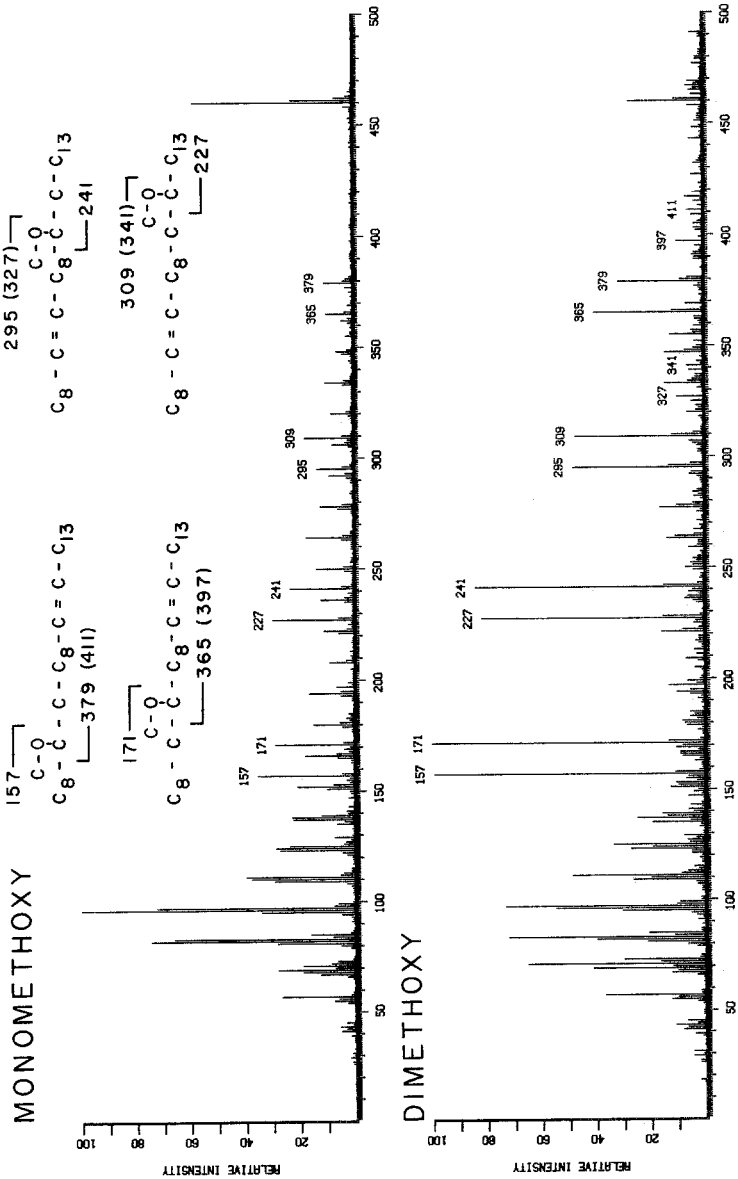

FIG. 3. Mass spectra (20 eV) of the mono- and dimethoxy derivatives of GLC fraction #11. The key fragment peaks in the monomethoxy derivative are rationalized in the inset. Values in parentheses are fragment masses expected when an additional methoxy group is added to the remaining double bond.

TABLE 4. LOCATION OF DOUBLE BONDS IN ACTIVE GLC FRACTIONS

Fraction no.	Molecular weight	No. of carbon atoms	Key fragment masses of methoxy derivatives	Ozonolysis fragments	Double-bond positions
4	404	29	157,171[a],295,309[a], 185,323	Nonanal, decanedial, decanal	9,19
7	432	31	157,171,295,309, 199,213,337,351,	Nonanal, decanedial, dodecanal	9,19
11	460	33	157,171,295,309, 227,241,365,379	Nonanal, decanedial, tetradecanal	9,19
14	488	35	157,171,295,309, 255,269,393,407	Nonanal, decanedial, hexadecanal	9,19
16	516	37	157,171,295,309, 283,297,421,435	Nonanal, decanedial, octadecanal	9,19

[a]The 171 and 309 fragment masses were each produced by two different cleavages in this derivative. Their intensities were correspondingly about twice those of the adjacent key fragments in the mass spectrum.

The large, inactive, GLC peaks (fractions 2, 5, and 8) were analyzed by ozonolysis. A mass spectrum was obtained for fraction 2, and an infrared spectrum was obtained for a mixture of the three fractions. The data indicated the fractions to be (*Z,Z*)-6,9 dienes of 27, 29, and 31 carbons, respectively. The 27-carbon (*Z,Z*)-6,9 diene was also identified in males and is, coincidentally, the major cuticular hydrocarbon in *Periplaneta americana* (L.), the American cockroach (Baker et al., 1963). In preliminary field and greenhouse bioassays, these dienes showed no promise of synergizing the (*Z,Z*)-9,19 dienes.

Synthesis. The synthesis of one (*Z,Z*)-9,19 diene is outlined in Figure 4. The rationale was to convert a commercially available, unsaturated, 18-carbon alcohol to a 19-carbon aldehyde, then to form the second double bond and complete the carbon chain by a Wittig condensation. In this work we were guided by Sonnet (1974), Shani (1979), Hilgetag and Martini (1972), and Maercker (1965). All reactions gave yields in excess of 50%. The products were purified by passing through a column of basic alumina, HPLC on the $AgNO_3$ column, and finally by preparative GLC on Dexsil 300 (isothermally at 280–315°, depending on component). In each case the final product was a colorless oil at room temperature.

Because the pheromone eluted from the HPLC in such a broad band with the 5% toluene in hexane solvent, the conditions were modified before

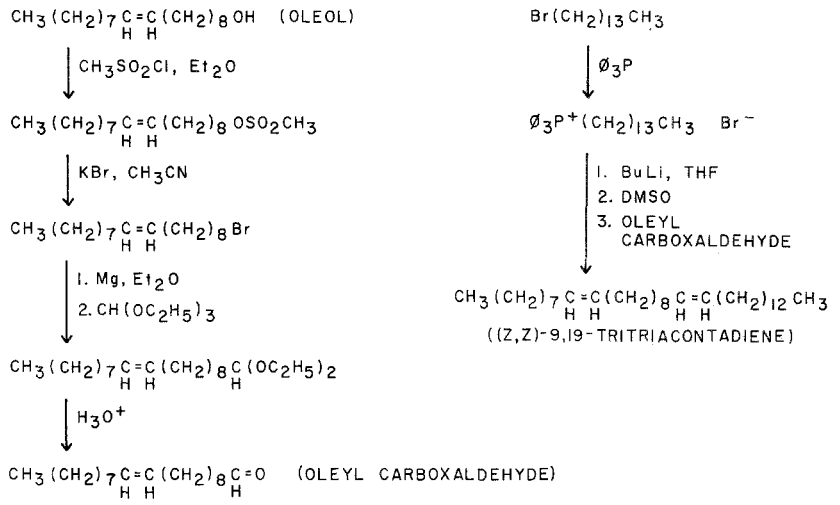

FIG. 4. Synthetic scheme for the 33-carbon pheromone component. Schemes for the other components were identical except that alkyl bromides of 10, 12, 16, or 18 carbons were substituted for the 14-carbon bromide.

purifying the synthetics. With 25% toluene in hexane, the female-derived (*Z,Z*)-9,19 dienes (and the pheromone activity) eluted as a discrete peak 6–10 ml after injection. The synthetic (*Z,Z*)-9,19 dienes produced an identical peak. The (*Z,E*) and/or (*E,Z*) isomers eluted at 3–5.5 ml after injection; so the procedure could be used to obtain geometrical purity in the synthetics. The *E* double bonds were present because technical grade oleol was used as a starting material and because the Wittig reaction produced some *E* product.

The mass spectra, GLC retention times, and major ozonolysis products of the purified synthetic dienes were identical to those of the female-derived dienes. Based on the intensity of the 970 cm^{-1} IR band, compared to the (*E,Z*)/(*Z,E*) product, the synthetic was judged to be >96% geometrically pure. By GLC, the synthetics were each over 98% pure, and the purity with respect to double-bond location was high, with only 1–2% of the total ozonolysis–GLC peak area not being consistent with 9,19 dienes.

Bioassay of Synthetic Dienes. A mixture of synthetic dienes (proportions as in females) was tested in the greenhouse against a similarly prepared mixture of female-derived GLC fractions and also against the HPLC material (25% toluene solvent). Changes in relative activity with time after plate preparation were measured (Table 5A). The female-derived materials were always initially superior to the synthetics, although even the synthetics were consistently above control levels. The differences between synthetic and female-derived dienes diminished rapidly over the first few hours, however, and after a day or two, any differences appeared related only to the amount of

TABLE 5. COMPARISON OF SYNTHETIC DIENES TO FEMALE-DERIVED DIENES IN THE GREENHOUSE (A) AND FIELD (B)[a]

	A: Greenhouse bioassay Mean bioassay score (N = 3)				
Treatment	0–0.5 hr	0.5–1 hr	1–1.5 hr	24 hr	48 hr
1. Mix of 5 active GLC fractions (3 μg)	98.3 c	38.0 b	22.0 b	24.7 b	–
Control	0.0 a	0.0 a	0.7 a	0.0 a	–
Synthetic dienes (3 μg)	22.0 b	22.3 b	16.0 b	25.3 b	–
2. HPLC fraction (18 μg)	63.0 c	44.7 c	–	14.3 b	–
Control	0.0 a	0.3 a	–	0.0 a	–
Synthetic dienes (9 μg)	18.7 b	11.3 b	–	5.7 ab	–
3. HPLC fraction (11 μg)	42.7 b	–	–	37.7 b	19.0 b
Control	0.0 a	–	–	0.3 a	0.3 a
Synthetic dienes (19 μg)	26.0 b	–	–	37.0 b	41.3 c

Treatment	B: Field bioassay Total trap catch (N = 24)	Range
HPLC Fraction (20 μg)	561 c	0–73
HPLC Fraction (4 μg)	79 b	0–14
Control	7 a	0–2
Synthetic dienes (20 μg)	204 b	0–73

[a]Total μg 9,19 dienes in parentheses (quantitated by GLC); proportions of mixtures as in females.

diene presented. The reason for the initial difference in activity remains unknown. Stored at −20° C, all samples appear to retain activity indefinitely.

In the field, the mixture of synthetic dienes (20 μg/trap) was tested against the same HPLC material used in the greenhouse (4 and 20 μg of 9,19 dienes/trap) and controls (balanced incomplete block design, eight complete replications, 24 traps/treatment). The 2-day trap catches (Table 5B) were analyzed, but observations were also made at more frequent intervals. The synthetic mixture was clearly superior to the controls but was not as active as the female material on a weight basis. Qualitatively, as in the greenhouse, the female-derived material was very active immediately, and the synthetic, starting more gradually, never did catch up.

To investigate whether the five dienes could "substitute" for each other in attracting males, each synthetic diene, and a mixture of all five (prepared in

the proportions found in females) were presented at the rate of 0.05 μmol total diene/trap (20–26 μg/trap, depending on molecular weight). Empty, control traps were also included in the study (balanced incomplete block experiment, two complete replications in different plantations, a total of 12 traps/treatment). If dienes were "interchangable" to the males we might expect the individual components with higher molecular weights (and lower volatilities) to be somewhat less active, and the mixture to have an intermediate activity. If the males could distinguish among the dienes, however, each being a "legitimate" pheromone component, we would expect the mixture to be most active.

The data are shown in Table 6, and the latter explanation fits better. The mixture was significantly more attractive than any component. Of the individual components, the C_{37} and C_{31} components were somewhat more active than the others. Curiously, these components were the least abundant of the five in the females, accounting for 5.5% and 11.1% of the total 9,19 dienes, respectively. The three, more-abundant components were only slightly more active than controls, the C_{33} component differing from controls at only the 0.10 level.

Synergism and Experimental Design. The synthetic dienes were tested for synergism by polar, female-derived Florisil fractions. The study was conducted in such a way that two experimental designs, balanced incomplete block and completely randomized, could be compared as well. It was unknown if data from closely paired, "competing" traps could be interpreted the same as data from widely separated traps and if interactions somehow caused by close pairing would nullify the gains in precision obtained by eliminating tree-to-tree variability.

TABLE 6. TOTAL 2-DAY TRAP CATCHES FOR PAIRED COMPARISON OF INDIVIDUAL (*Z*,*Z*)-9,19 DIENES, A MIXTURE OF ALL 5, AND CONTROLS[a]

Treatment	Total (N = 12)	Range
C_{29} (20 μg)	77 b	0–29
C_{31} (22 μg)	213 bc	1–106
C_{33} (23 μg)	43 ab	0–13
C_{35} (24 μg)	63 b	0–24
C_{37} (26 μg)	291 c	1–137
Mixture (23 μg)	588 d	4–164
Control	16 a	0–5

[a]In the mixture the dienes were in the same proportions as in females. All traps, except controls, contained 0.05 μmol total diene (20–26 μg). Significant differences (0.05 level) in the $\log(n + 1)$ scale denoted by different letters.

The treatments, freshly prepared each day, consisted of the synthetic dienes (20 μg/trap, proportions as in females), alone and in combination with the 5% and/or 25% ether–hexane–Florisil fractions (Bartelt et al., 1982). The Florisil fractions were used at the rate of 5 FE/trap and are denoted below as the "5%" and "25%" fractions. The experiment was run on 3 consecutive days, both as a balanced incomplete block (2 traps/tree, 3 replications/treatment) and as a completely randomized design (1 trap/tree, 3 replications/treatment). The test pairs and single traps were randomly assigned to the 18 trap trees employed each day. Trap trees were at least 15 m apart. The traps were baited at 0830 hr, and trap catches were recorded after 1, 2, 3, 4, and 8 hr. Analysis was done separately for both designs and the five trapping intervals, variability due to days being removed. The population of males was near its peak, and the weather was hot, clear, and breezy on all 3 days. The data for the two designs are given in Table 7. Totals over both designs for the first four intervals are shown in Figure 5.

The dienes + 25% traps were initially far more active than the dienes alone (overall, trap catches 16 times higher during the first hour). But after 3 hr there was no hint of synergism. The cumulative difference generated during the first 3 hr was not very evident in the final totals. Chemically, the 25% fraction behaved as though relatively volatile or present in relatively small

TABLE 7. EFFECT OF SYNERGISTS (5% AND 25% ETHER-FLORISIL FRACTIONS, 5 FE/TRAP) ON ACTIVITY OF SYNTHETIC DIENES (20 μg/TRAP)[a]

Treatment	Total (N = 9)					Overall total	Range
	0–1 hr	1–2 hr	2–3 hr	3–4 hr	4–8 hr		
A: Balanced incomplete block design							
Dienes	2 a	19 a	8 a	21 a	54 a	104 a	4–31
Dienes + 5%	0 a	71 b	126 c	137 b	259 b	593 b	26–107
Dienes + 25%	43 b	34 a	31 b	20 a	30 a	158 a	1–57
Dienes + 5% + 25%	95 b	160 b	163 c	166 b	337 b	921 b	34–178
B: Completely randomized design							
Dienes	5 a	9 a	7 a	8 a	31 a	60 a	1–19
Dienes + 5%	10 a	90 c	192 b	137 b	289 b	718 c	16–164
Dienes + 25%	71 b	29 b	22 a	7 a	58 a	187 b	2–51
Dienes + 5% + 25%	86 b	168 c	143 b	194 b	300 b	891 c	21–181

[a](A) Total trap catches over time intervals for balanced incomplete block design (2 traps/tree, 9 replications/treatment), and (B), for completely randomized design (1 trap/tree, 9 replications/treatment). Both designs run concurrently in the same area. Separate analyses were done for both designs and the five intervals, as well as the overall totals. Letters denote differences at the 0.05 level.

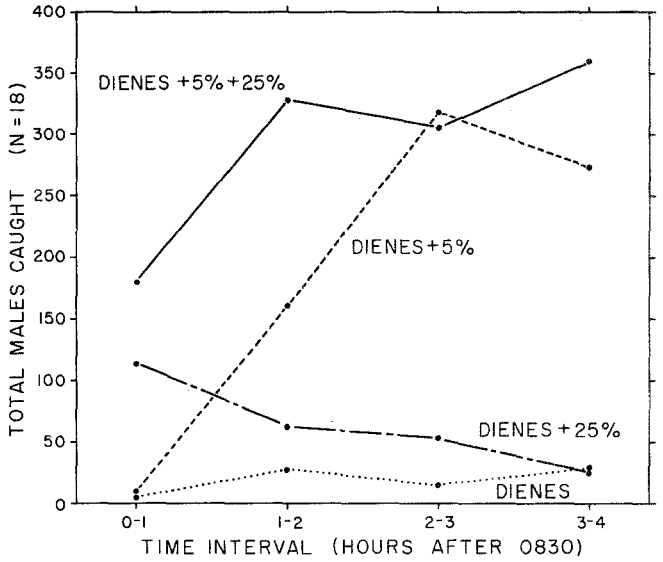

FIG. 5. Catches on traps baited with synthetic dienes, alone or in combination with female-derived Florisil fractions. Each point is a total for 18 traps. The study was initiated at 0830 and catches recorded at hourly intervals.

amounts. Studies still in progress indicate both to be true. Conversely, the 5% fraction was completely inactive during the first hour, but caused an 8-fold increase over the rest of the study. The dienes + 5% + 25% combination showed both positive effects, being overwhelmingly more active than the dienes alone throughout the day. The initial inactivity of the 5% fraction was apparently not due to a repellent effect of too much pheromone or other compounds, because the dienes + 5% + 25% traps were very active from the start. While the study was in progress, four traps baited with only the 5% and 25% fractions were no different from empty controls, in agreement with earlier findings (Bartelt et al., 1982).

The paired and separate designs yielded very similar results, both with respect to trends over time and actual totals. None of the 20 mean catches on separated traps (four treatments, five time intervals) differed from the corresponding means derived from the paired, incomplete block analysis [0.05 t tests, log $(n + 1)$ scale, variances being determined from the separate and paired analyses]. Separating the treatments by 20 cm, rather than 15 m, did not change the nature of the information obtained from the trap catches.

With the log $(n + 1)$ transformation, variances for each design remained quite constant among treatments and also among trapping intervals (whether 1 hr or 4). Pooled over the five trapping intervals, the paired design gave a 35% lower variance than the separate design. When the totals for the whole day

were analyzed, the paired design gave a 17% lower variance. For this study, the paired design yielded a moderate increase in precision. In studies with less potent treatments, such as single diene components, tree-to-tree variability was greater, and pairing the traps was probably of still greater importance to precision.

Other Hydrocarbon Components. The ECLs of certain minor GLC peaks in Figure 2 were consistent with even-carbon members of the pheromone series, and their inactivity in the original greenhouse bioassay might have been due to the small amounts tested. Five GLC fractions, corresponding to the 28-, 30-, 32-, 34-, and 36-carbon members of the pheromone series were collected and tested in the greenhouse against the 35-carbon pheromone diene, each at the rate of 1 μg/plate, rather than in the proportions found in the female. All five were clearly active (Table 8.). (Reinjection of collected fractions in the gas chromatograph confirmed that the active, odd-carbon dienes could be separated completely from these minor peaks). Ozonolysis of each fraction demonstrated the presence of double bonds in the 9 and 19 positions, and the retention time on the $AgNO_3$ column (25% toluene in hexane as solvent) supported the (*Z,Z*) configuration. Mass

TABLE 8. MEAN GREENHOUSE BIOASSAY SCORES AND ANALYTICAL DATA FOR 5 ADDITIONAL GLC FRACTIONS[a]

ECL of peak	No. of carbon atoms	Ozonolysis products	Double-bond positions	Mean bioassay scores		
				Test fraction	Control	C_{35}
27.43	28	Nonanal, decanedial	9,19	16.7 *	1.0	7.0 *
29.40	30	Nonanal, decanedial, undecanal	9,19	11.3 *	0.7	13.0 *
31.36	32	Nonanal, decanedial, tridecanal	9,19	31.0 *	0.0	11.7 *
33.34	34	Nonanal, decanedial, pentadecanal	9,19	17.0 *	0.3	16.0 *
35.34	36	Nonanal, decanedial, heptadecanal	9,19	13.0 *	0.7	12.7 *

[a]Each fraction was tested against the 35-carbon, female-derived 9,19 diene (1 μg/plate for all fractions) and controls. The (*) denotes difference from controls, 0.05 level. Molecular weights for the 31.36 and 33.34 peaks were determined by mass spectrometry to be 446 and 474, respectively.

TABLE 9. SUMMARY OF HYDROCARBONS IN PHEROMONE SERIES[a]

Name	No. of carbon	% of total hydrocarbons in female	% of total 9,19 dienes in female	Bioassayed	Synthesized
(Z,Z)-9,19-Octacosadiene	28	0.06	0.6	Yes	No
(Z,Z)-9,19-Nonacosadiene	29	2.47	22.5	Yes	Yes
(Z,Z)-9,19-Triacontadiene	30	0.04	0.4	Yes	No
(Z,Z)-9,19-Hentriacontadiene	31	1.22	11.1	Yes	Yes
(Z,Z)-9,19-Dotriacontadiene	32	0.12	1.1	Yes	No
(Z,Z)-9,19-Tritriacontadiene	33	2.17	19.7	Yes	Yes
(Z,Z)-9,19-Tetratriacontadiene	34	0.19	1.7	Yes	No
(Z,Z)-9,19-Pentatriacontadiene	35	3.90	35.6	Yes	Yes
(Z,Z)-9,19-Hexatriacontadiene	36	0.05	0.5	Yes	No
(Z,Z)-9,19-Heptatriacontadiene	37	0.60	5.5	Yes	Yes
(Z,Z)-9,19-Octatriacontadiene	38	0.01	0.1	No	No
(Z,Z)-9,19-Nonatriacontadiene	39	0.13	1.2	No	No

[a]Total hydrocarbons extracted averaged ca. 90 μg/female, and 9,19 dienes ca. 11 μg/female.

spectra were obtained for the 2 largest of these peaks, and their molecular weights (446 and 474) indicated them to be dienes of 32 and 34 carbon atoms. Closer examination of the gas chromatograms revealed two additional minor peaks, corresponding to the 38- and 39-carbon dienes. Ozonolysis again confirmed the 9 and 19 double-bond positions. These, however, have not yet been bioassayed individually.

The biological importance of these minor components has yet to be established. In a small, paired, field study in 1980, eight traps with all 12 female-derived dienes, in the same proportions as in females, caught a total of 142 males, while eight containing just the five major components caught 90, but the difference was not significant (all traps contained 6 μg total dienes). The effect of these additional components is probably subtle and needs to be investigated further. A summary of present information about the 9,19 dienes is given in Table 9.

DISCUSSION

By the evidence presented, the (*Z, Z*)-9,19 dienes of 29, 31, 33, 35, and 37 carbon atoms are a major part of the sex pheromone of *P. alaskensis*. These alone elicit responses from males. But the complete system is more complicated. First, there are minor amounts of (*Z, Z*)-9,19 dienes with 28, 30, 32, 34, 36, 38, and 39 carbon atoms, the first five of which have been tested and shown to be active. Second, there are important synergists, in the 25%

ether–Florisil fraction (probably an alcohol) and in the 5% ether–Florisil fraction (perhaps an ester, aldehyde, or ketone), which improve responses of males up to 10 times or more. However, these crude synergists are consistently inactive by themselves. Only when the dienes are present can attraction be demonstrated. Finally, the initial bioassay difference between synthetic and female-derived dienes may involve yet another class of compounds.

The major dienes seem surprisingly active for their very high molecular weights. We suppose that the relatively large amounts found in females and, perhaps, high sensitivity of the males compensate for low volatility.

This sawfly has a pheromone system vastly different from those of the diprionid pine sawflies. Sex attraction in the genera *Diprion* and *Neodiprion* depends to a large extend on esters derived from various optical isomers of 3,7-dimethyl-2-pentadecanol (e.g., Jewett et al., 1976, 1978; Matsumura et al., 1979). Hydrocarbons have not been implicated.

Instead, the results from *P. alaskensis* have paralleled more closely those from the Diptera, where unsaturated hydrocarbons have been shown to have pheromonal activity (e.g., Carlson et al., 1971; Sonnet et al., 1979; Uebel et al., 1978). The system in *P. alaskensis* is novel, however, in that a large series of related hydrocarbons has activity and that the hydrocarbons are strongly synergized by nonhydrocarbon compounds.

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DETECTION OF SECONDARY METABOLITES IN MARINE MACROALGAE USING THE MARSH PERIWINKLE, *Littorina irrorata* SAY, AS AN INDICATOR ORGANISM

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Abstract—Twenty-four chemically profiled organic macroalgal extracts and seven purified natural products were bioassayed using the mesogastropod *Littorina irrorata* Say to determine if the presence of secondary metabolites in crude algal extracts could be correlated with the activity of the snail. Avoidance behavior by snails in the assay was highly correlated with the presence of secondary metabolites in the macrophyte extracts.

Key Words—*Littorina irrorata*, marine natural products, biological activity, bioassay, marine macrophytes.

INTRODUCTION

Many marine organisms produce structurally unusual secondary metabolites (Scheuer, 1973, 1977, 1978: Baker and Murphy, 1976; Faulkner, 1977; Faulkner and Fenical, 1977). These metabolites can be defined as novel organic substances which display structural features differing from those previously encountered in marine and nonmarine sources. Homologs and analogs of previously characterized novel substances may also be defined as unusual secondary metabolites if they are isolated from a new source. Marine macroalgae in particular have yielded and continue to yield a diverse array of novel natural products (Fenical, 1975; Wratten and Faulkner, 1976; Moore, 1977). Methods used to detect the presence of these natural products vary with the investigator, but generally include thin-layer chromatography profiles and spectrometric fingerprinting of the crude extract.

Many isolated natural products are biologically active and are suspected in the mediation of intra- and interspecific biological relationships in the

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marine environment. Therefore, bioassay results can potentially be used to indicate the presence of unusual secondary metabolites. Early workers tested the biological activity of crude marine extracts in terrestrial assay systems (Pratt et al., 1951; Roos, 1957; Welch, 1962; der Marderosian, 1969; Bhakuni and Silva, 1974), but these had little significance in the context of the marine environment. Later workers used nonbehavioral and behavioral laboratory marine based assays on extracts or purified compounds to determine biological activity (Kittredge, 1976; Targett, 1979; Targett and Mitsui, 1979; Gerwick et al., 1979; Müller, 1979; Sun and Fenical, 1979; Paul et al., 1980). Observations on the degree and specificity of herbivory in the natural environment have also been made (Ogden, 1976; Vadas, 1977; Ogden and Loebel, 1978). Most of these studies lacked chemical profiles of the organisms tested or were limited in the range of extracts assayed. No direct correlation was made between the presence of unusual secondary metabolites in extracts and activity in particular marine bioassay systems.

In screening large numbers of marine organisms for the presence of unusual secondary metabolites, the ideal test assay organism should be sensitive, but not indiscriminate, to a broad range of bioactive substances, require small sample size, simple set up, and yield immediate results. Mechanisms for detecting chemical changes in the environment occur in all groups of animals (Kohn, 1961). Assays involving chemosensory behavior are informative because chemosensory cells are extremely sensitive to the presence of low concentrations of chemicals. If these chemicals are toxic at high concentrations, low concentrations may affect the behavior of the organism (Mackie and Grant, 1974), reflecting in part the organism's evolved response to natural "noxious" compounds (Kittredge, 1976).

Gastropod mollusks have a demonstrated sensitivity to chemosensory stimuli, are hardy and ubiquitous, making them an excellent choice for bioassay purposes. Comparison of results from a behavioral chemosensory assay involving the gastropod *Nerita tessellata* Gmelin and more elaborate nonbehavioral bioassays involving other marine organisms (Targett, 1979) suggest that this snail, and perhaps others, react to a broad range of bioactive substances. In a few cases (Kittredge, 1976; Ohta, 1977), snail activity in behavioral bioassay tests was attributable to the presence of an unusual secondary metabolite. However, there is only limited information on how reliably a gastropod assay or any marine chemosensory assay can detect unusual secondary metabolites in crude extracts because no work has matched chemical extract profiles with assay activity. Using a bioassay procedure developed by Ohta (1977) and mesogastropod *Littorina irrorata* Say, we correlated the activity of the snail with the presence of unusual secondary metabolites in 24 algal extracts. Ecological implications and extensions of the assay to other gastropod species are discussed.

METHODS AND RESULTS

Littorinid gastropods are common, herbivorous, intertidal snails with worldwide distribution. Their biology, behavior, and chemosensory ability are well documented (Peters, 1964; Hall, 1973; Hamilton, 1976, 1977a,b, 1978a,b). Tactile and olfactory sensing takes place with the cephalic tentacles.

For this work, the marsh periwinkle, *Littorina irrorata* Say, was collected from the salt marshes near Skidaway Island, Georgia. Test animals were retained no longer than 72 hr in aerated seawater before use.

Macrophyte Samples. Collections of macrophytes[1] from the Florida Keys, Florida Gulf coast, and the Skidway River (Table 1), were prepared by extracting the fresh frozen material MeOH/$CHCl_3$ or IPA/CH_2Cl_2 (1:1). Solvent was removed in vacuo, and the resulting residue was partitioned between water and diethyl ether, ethyl acetate, or methylene chloride. The residue remaining after drying and evaporation of the organic solvent constituted the crude extract. Purified compounds were obtained by chromatographic resolution of the crude extract.

Snail Assay. The assay method is a modification of one described by Ohta (1977). Algal extracts were taken up in ether and applied in a 15-cm^2 circular band to a filter paper disk at concentrations $\leqslant 1 mg/cm^2$. The impregnated filter paper was wet with ~5 ml of seawater in a Petri dish and three snails (0.8–1.5 cm) were placed in the center of the paper. The snails were observed for trail-following behavior (Hamilton, 1977b). In cases where this occurred, experiments were repeated to avoid a bias in the results. Because many species of mollusks, including *L. irrorata*, exhibit circuitous movement upon release (Hamilton, 1977b), it was necessary to establish a test interval of sufficient duration to ensure that this initial response did not affect the overall results. A 10-min test interval was optimal. If a snail was "corraled" within sample boundaries for 10 min after its cephalic tentacles were extended, the response was rated positive (+), indicating avoidance. If the snail crossed the sample band in less than 10 min, the response was rated negative (−), indicating no avoidance. Solvent and blank controls showed no activity.

Crude Extracts. Crude algal extracts were tested at concentrations of 0.8 ± 0.2 mg/cm^2, a threshold level determined from preliminary tests on 13 of the 25 extracts (Table 1). At this concentration, there was high correlation between active extracts and those which, as indicated by TLC and NMR, contained unusual secondary metabolites. Extracts which showed no evidence of unusual secondary metabolites also showed no influence on snail activity. All extracts which contained unusual secondary metabolites gen-

[1]Macrophyte voucher specimens have been deposited with Dr. J. Norris in the Museum of Natural History, Smithsonian Institution.

TABLE 1. ACTIVITY OF CRUDE ALGAL EXTRACTS

Algal species (crude extract)	Collection site[a]	Algal collection date (month-year)	Activity (0.8 ± 0.2 mg/cm^2)[b]
Caulerpa prolifera (Et_2O)	FK	1-80	+++
Dictyota linearis (Et_2O)	FK	1-80	+++
Halimeda incrassata (Et_2O)	FK	1-79	+++
Laurencia poitei (Et_2O)	FGC	2-79	+++
Penicillus dumetosus (Et_2O)	FK	1-80	+++
Sargassum fluitans (EtOAc)	FGC	2-79	+++
Anadyomene stellata (EtOAc)	FK	4-80	-++
Centroceras clavulatum (EtOAc)	FK	4-79	-++
Cymopolia barbata (Et_2O)	FK	1-80	-++
Laurencia obtusa (CH_2Cl_2)	FK	1-79	-++
Polysiphonia echinata (EtOAc)	FGC	3-79	-++
Caulerpa racemosa v. *occidentalis* (Et_2O)	FK	4-79	--+
Dictyota cervicornis (Et_2O)	FK	1-80	--+
Udotea sp. (Et_2O)	FK	1-79	--+
Acanthophora spicifera (EtOAc)	FK	1-80	---
A. stellata (Hex)	FK	4-80	---
Bostrychia binderi (Et_2O)	FGC	3-79	---
Caulerpa mexicana (EtOAc)	FGC	8-79	---
Cladophora sp. (CH_2Cl_2)	FK	1-79	---
Gracilaria foliifera v. *angustissima* (Et_2O)	SKIO	10-80	---
Rhipocephalus phoenix (Et_2O)	FK	1-79	---
Sargassum sp. (Et_2O)	FGC	9-80	---
Ulva lactuca (Et_2O)	SR	5-79	---
Wrangelia penicellata (Et_2O)	FK	1-80	---

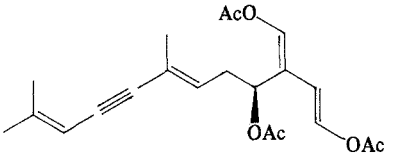
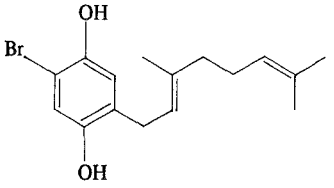
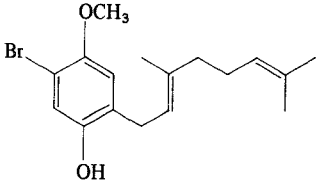
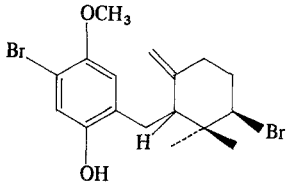
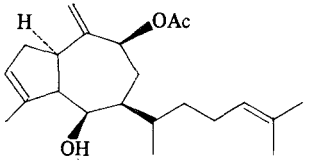
[a]FK = Florida Keys, FGC = Florida Gulf Coast, SKIO = in culture at Skidaway Institute, SR = Skidaway River.
[b]Each + indicates avoidance for a snail, each - indicates no avoidance for a snail (n = 3).

erated activity in the assay. Three extracts, *Anadyomene stellata* (EtOAc), *Centroceras clavulatum*, and *Halimeda incrassata*, which did not evidence unusual secondary metabolites by TLC or 60-MHz NMR showed moderate to strong activity in the assay.

Purified Compounds. Seven purified secondary metabolites from five of the 24 algal species were isolated in ⩾10-mg quantities to test on the 15-cm^2 sample band at 0.6 ± 0.1 mg/cm^2 concentrations (Table 2).

Caulerpenyne was isolated from the Et_2O extract of *Caulerpa prolifera.*

TABLE 2. ACTIVITY OF PURIFIED ALGAL SECONDARY METABOLITES

Alga (compound)	Structure	Activity (0.6 mg/cm^2)
Caulerpa prolifera (caulerpenyne)		+++
Cymopolia barbata (cymopol)		+++
Cymopolia barbata (cymopol monomethylether)		– – –
Cymopolia barbata (cyclocymopol monomethyl ether)		– – –
Dictyota cervicornis *(dictyol B* acetate)		– – –
Penicillus dumetosus (diterpene alcohol)	?	+++
Penicillus dumetosus (diterpene acetate)	?	– – –

Gradient silica gel column chromatography followed by low-pressure silica gel chromatography (Meyers et al., 1979) in 1:1 hexane–$CHCl_3$ of crude fractions containing caulerpenyne yielded the pure product. Caulerpenyne was identified by comparing high-resolution mass spectral, infrared, ultraviolet and [^{1}H]NMR data with literature values (Amico et al., 1978). Caulerpenyne was present at 0.30% of the plant's dry weight.

Cymopol, cymopol monomethyl ether, and a diastereomeric mixture of cyclocymopol monomethyl ether were isolated from the Et_2O extract of *Cymopolia barbata* by silica gel column chromatography and HPLC using μ-Porasil with ethyl actate–hexane mixtures. Cymopol or 2-bromo-5-(3,7-dimethylocta-2,6-dienyl)hydroquinone comprised 0.07% of the plant's fresh weight. Cymopol monomethyl ether comprised 0.01% of the plant's fresh weight. Both compounds exhibited spectral data identical with published values (Högberg et al., 1976). The identification of the diastereomeric mixture of cyclocymopol monomethyl ether [1-bromo-3-(4-bromo-2-hydroxy-5-methoxyphenyl)-2,2-dimethyl-4-methylenecyclohexane] is based on comparison of spectral data with that of the known structure (Högberg et al., 1976). The [^{1}H]NMR data suggest that diastereomers, epimeric at C-3 of the cyclohexane ring, are present. The diastereomeric mixture comprised 0.03% of the plant's fresh weight. Complete structure elucidation is in progress.

Dictyol B acetate was isolated from the EtOAc extract of *Dictyota cervicornis*. Gradient silica gel column chromatography of the crude extract followed by μ-Porasil HPLC in 10:1 hexane–EtOAc of fractions containing the acetate yielded the pure product. Dictyol B acetate was identified by comparing high-resolution mass spectral, infrared, and [^{1}H]NMR data with literature values of the same compound obtained from *Dictyota dichotoma* (Faulkner et al., 1977). In *D. cervicornis* it constitutes 0.04% of the plant's dry weight.

Penicillus diterpene alcohol and diterpene acetate were isolated from the EtOAc extract of *Penicillus dumetosus* by Florisil column chromatography and further purified by reversed-phase (C_{18}) HPLC using mixtures of MeOH and water [100% MeOH to MeOH/H_2O (19:1)]. The alcohol and acetate, present in a 7:3 ratio comprise 1.01% and 0.43% of the plant's dry weight, respectively. The acetate can readily be formed from the alcohol by treatment with acetic anhydride in pyridine. The acetate and alcohol are probably oxygenated diterpenes. By [^{1}H]NMR these two compounds appear to be partially related to geranylgeraniol, but mass spectral data indicates seven degrees of unsaturation and suggest a bi- or tricyclic system. Complete structure eludication is in progress.

Results of tests with pure compounds indicate that the snails did not avoid every unusual secondary metabolite and that they can discriminate between even closely related compounds from the same crude extract.

DISCUSSION

There is at least an 88% correlation between the presence/absence of unusual secondary metabolites in crude extracts and avoidance/no avoidance in this assay. Incongruous results were obtained for only three of the extracts tested, *A. stellata* (EtOAc), *H. incrassata*, and *C. clavulatum* and, for each, the discrepancy may only be apparent rather than real. *A. stellata* (EtOAc) has not yet been shown to contain unusual chemistry. In previous marine assay work (Targett, 1979; Targett and Mitsui, 1979), aqueous extracts of *A. stellata* were highly toxic to fish, induced blood cell hemolysis, negatively affected cell tissue cultures, induced gastropod tentacle withdrawal, and exhibited antibacterial properties. However, no chemical profiles are available for the aqueous extract. Two organic extracts from *A. stellata* were tested with *L. irrorata.* The nonpolar, hexane extract was inactive, while the more polar ethyl acetate extract was active. It is possible that enough of a very polar, primarily water-soluble, material is dissolved in the ethyl acetate extract to effect a biological reaction but to escape routine chemical and spectrometric detection.

C. clavulatum may also contain other undetected secondary metabolites since previous tests on aqueous extracts (*see above*) were toxic to fish, inhibited bacterial growth, inhibited growth of grunt fin cell tissue cultures, and induced gastropod tentacle withdrawal.

Aqueous extracts of *H. incrassata* have shown no activity in a variety of marine-based assays. However, organic extracts for a number of *Halimeda* species probably contain unusual compounds (W. Fenical and J. Norris, personal communications). Because they are extremely labile, the chemistry has remained elusive. *H. incrassata* may contain enough of an unusual material or its byproducts to account for the snail response, even though it is not apparent by TLC or NMR. The presence of an active fatty acid (Kodicek, 1949; Gauthier et al., 1978) would be more difficult to distinguish by TLC or NMR from the ubiquituous fatty acids and triglycerides in most extracts. The response of any of the three disparate species could also reflect this.

The production of novel compounds seems to run along algal family phylogenetic lines. Except for *A. stellata*, all of the extracts which generated a positive response in our assay belong to algal families whose genera produce unusual secondary metabolic chemistry. However, six out of the eleven species which tested inactive also belong to some of these algal families. Therefore, phylogeny can be only a general guideline in the choice of species for chemical and chemical ecological work. The results of our work indicate that a simple marine chemosensory bioassay may give far more accurate information concerning species containing unusual secondary substances.

Tests with pure compounds isolated from the algal extracts indicate that

not every secondary metabolite in an extract is active. For example, cymopol is active, while the closely related cymopol monomethyl ether is not. Similarly the *Penicillus* diterpene alcohol is active while the acetate is not. It is possible that the cymopol monomethyl ether and *Penicillus* diterpene acetate represent protected forms of the active alcohols and are utilized for storage in the plant. Work is underway to determine if this can be generalized to other closely related natural products. The preliminary results, however, suggest that the snail assay may provide general insight into chemical-ecological interactions.

Littorina irrorata is sensitive to a broad range of bioactive substances but is not indiscriminate. This method requires small sample size, involves a simple setup, and yields immediate results. In addition, it is a reliable method for screening crude extracts for unusual natural products, both in the laboratory and in the field. It is particularly valuable in the field where spectrometric fingerprinting is not possible. Preliminary tests on the mud snail, *Ilyanassa obsoleta*, indicate that this species may be as reliable as *L. irrorata* for identifying crude extracts containing unusual chemistry. Work is in progress to determine if the assay can be generalized by testing animal extracts and utilizing other gastropod species, thus broadening the assay's usefulness as a detection tool for unusual secondary metabolites.

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EVALUATION OF TWO ATTEMPTS TO TRAP DEFINED POPULATIONS OF *Scolytus multistriatus*[1]

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Abstract—Two experiments were conducted to investigate the use of a pheromone-based trap-out technique for suppressing populations of *Scolytus multistriatus*. In the first experiment, elm bolts containing the immature stages of *S. multistriatus* were placed in an isolated community that contained elm trees, but which was essentially devoid of a resident beetle population. The infested bolts produced a total of 46,485 adult beetles of which 20% were recovered on traps baited with synthetic pheromone. In the second experiment beetles were released in a desert valley containing no resident elms or beetles. Only 1% of 20,000 released beetles were recaptured on traps erected on vertical cardboard cylinders and on elm logs. These rates of recapture are related to the attraction of beetles to naturally occurring brood sources versus pheromone-baited traps.

Key Words—Aggregation, pheromone, elm, mark-release, multilure, Coleoptera, Scolytidae, *Scolytus multistriatus*, trap-out, *Ulmus*.

INTRODUCTION

The smaller European elm bark beetle, *Scolytus multistriatus* (Marsham), is a vector of *Ceratocystis ulmi* (Buism.) C. Moreau, the causal pathogen of Dutch elm disease (DED). The beetle has been the object of much pest-management-oriented research, including sanitation (Miller et al., 1969; Hart, 1970; Barger, 1977), insecticides (Al-Azawi and Casida, 1958; Williams and Brown, 1969; Cuthbert et al., 1973), natural enemies (Valek, 1967; Truchan, 1970; Kennedy, 1979), trap trees (O'Callaghan et al., 1980), and pheromones (Peacock et al., 1971; Pearce et al., 1975; Gore et al., 1977; Cuthbert and Peacock, 1978).

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Pheromone-based trap-out strategies aimed at reducing the incidence of DED by suppressing populations of *S. multistriatus* have been attempted several times (Cuthbert et al., 1977; Arciero, 1979; Birch, 1979; Cuthbert and Peacock, 1979; Lanier, 1979). Most of these investigations have been conducted in large suburban communities where beetle emigration and immigration could not be controlled (Cuthbert et al., 1977; Lanier, 1978). Thus, the number of beetles captured could not be correlated with a precise estimate of the population size. Given these conditions, Cuthbert et al. (1977) estimated a trapping efficiency between 20% and 35%. In an area without elms, Lanier et al. (1976) estimated a recapture of 10.3% of released beetles. As an alternative to estimating beetle population size, Lanier (1979) used the incidence of DED as an index to evaluate the effect of pheromone-based trapping and found that the incidence of DED was reduced after mass trapping of the beetle. This technique of determining the effect of mass-trapping is justified in those areas where movement of the beetles can be controlled, but clearly the method could not be used in the disease-free study sites in California.

Birch et al. (1977, 1981) trapped thousands of *S. multistriatus* in small, isolated communities in a program designed to determine the effectiveness of mass trapping to reduce beetle populations in an area where emigration and immigration could be discounted. However, it was difficult to develop accurate estimates of beetle populations emerging from wood piles and standing trees to correlate with the numbers caught in pheromone-baited traps. As part of this program, a mark–release–recapture experiment was conducted in Independence, California, in which less than 1% of the released beetles were captured (Birch et al., 1981). This may, however, be a very low estimate of trapping efficiency since the handling procedures for marking beetles with fluorescent marker may have reduced their longevity (Birch, unpublished progress reports). These difficulties encountered in estimating the density of resident beetle populations and in handling beetles for marking suggested that additional research was needed under highly controlled conditions.

The present study was conducted in association with the trap-out program described by Birch et al. (1977, 1981). The objective was to assess the efficiency of pheromone-baited traps in capturing a known number of *S. multistriatus* in an attempt to assess the effectiveness of mass trapping as a means of suppressing natural populations.

METHODS AND MATERIALS

Trapping in the Presence of Elms. This study required a location with elm trees to provide a realistic setting of potential hosts, but with no or very few *S. multistriatus*. The absence of indigenous beetles would maximize the accuracy

in estimating the capture rate of beetles after emergence from introduced sources. An isolated study site was also desirable so that beetle dispersal into and out of the study site could be discounted. Deep Springs, in eastern California, satisfied all of these conditions.

Deep Springs is a private school community lying in a desert valley at 1500 m elevation. This valley is surrounded by the 2000- to 3000-m ridges of the White Mountains. There were approximately 30 full-grown English elms (*Ulmus procera*) in the study area. The only other elm trees in the valley were two Siberian elms (*U. pumila*) 2 km to the north. Two more Siberian elm trees were located beyond a 2000-m summit and 48 km to the west, another Siberian elm 43 km to the south over a 2000-m ridge at Waucoba Springs in Saline Valley, and 14–16 Siberian elms 17 km further to the south in Saline Valley at Willow Springs Camp. Otherwise, the nearest concentrated source of elms was Big Pine, California, located 42 km to the west in the Owen's Valley on the opposite side of the White Mountains. It is unlikely that any significant immigration took place from any of these sources during the test.

Two surveys were conducted to determine if the Deep Springs site contained a resident population of *S. multistriatus* prior to the study. All potential brood sources (dead limbs and woodpiles) were searched for evidence of beetle activity in October 1977. Pheromone-baited traps were placed at three different locations (sites 1, 3 and 4, Figure 1) between May 9 and June 9, 1978. Each trap consisted of a white cardboard sheet, 45 × 60 cm,

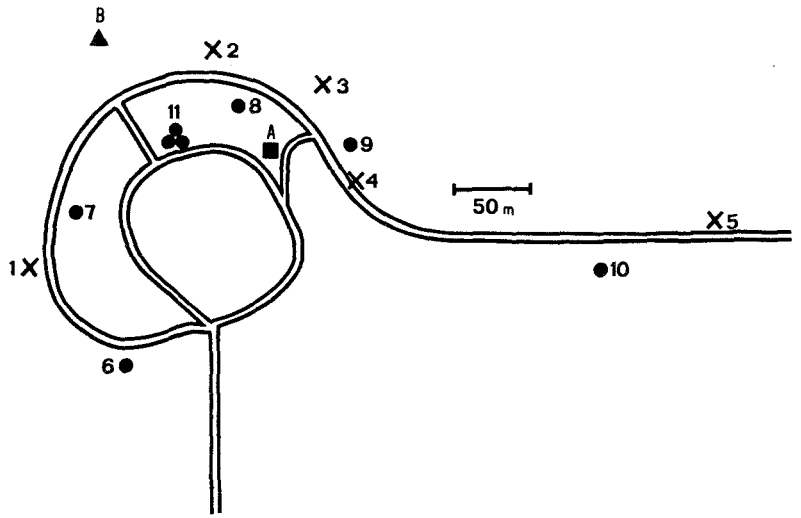

FIG. 1. A map of Deep Springs, California, demonstrating the placement of pheromone-baited traps (X), trap bolts (●), marking container (■), and elm bolt pile (▲).

stapled to a utility pole about 3 m above ground. The cardboard was coated with Stikem Special®, and a "standard" bait containing multilure (cubebene, multistriatin and heptanol) (Pearce et al., 1975) was stapled onto the center of the trap surface (see Birch et al., 1977, 1981). Baits were formulated to be attractive for 90 days.

An English elm was felled out of the area, in Bishop, California, on April 12, 1978, and cut into bolts approximately 1 m long and 8–12 cm in diameter. The bolts were baited with Multilure to initiate colonization by *S. multistriatus* over a short period of time. The development of the brood was monitored after colonization, and the logs were transported to the release site after prepupae and pupae were present. This ensured that the maximum number of beetles would emerge in a short period of time. Each bolt was painted with a 1 : 1 mixture of white latex paint and water prior to the emergence of adult beetles. Beetles emerged through the bark, leaving holes in the white painted surface that were easily counted.

A mark–release system was designed to minimize human contact during the emergence, marking, and dispersal of beetles. Infested elm bolts were placed inside a 1.0 × 0.5-m cardboard drum. The cover of the drum was made of ¼-in. mesh hardware cloth including a 20 × 30-cm area threaded with cotton strands. The cotton strands extended 8–10 cm below the plane of the cover and were coated with magenta fluorescent powder (Dayglo Color Corp, Cleveland, Ohio). The remaining area of the cover was taped to prevent light penetration and to restrict the exit route of the beetles. The only way for beetles to escape from the container was through the "window" of cotton strands coated with powder. The drum containing beetle-infested bolts was transported to Deep Springs on June 9 and removed July 6, giving a total of 27 days during which beetles could emerge (position A, Figure 1).

In addition to releasing marked beetles, a "woodpile" composed of whitewashed elm bolts from Bishop containing pupae and prepupae was constructed in Deep Springs on June 22 (position B, Figure 1). Beetles were allowed to emerge until July 6 when the elm bolts were removed from the study site. There was very little difference in the treatment of beetles emerging from the marking container or woodpile. Both were allowed to emerge normally and great care was taken to avoid manipulating the beetles by allowing them to mark themselves. However, those from the barrel were more protected and had to escape via the strands, and these factors were not controlled.

The number of beetles that had emerged from both sources was determined by counting emergence holes in the bark of the elm bolts. Round holes in the bark of the elm bolts were considered to provide 1 : 1 correlation with the number of beetles that had emerged. This assumption was tested for error by dissecting a subsample area of 100 holes on five different bolts and looking for parasitoid emergence holes, adult beetle reentry holes, emergence

of two or more beetles from the same hole and nontabulated holes which had been overlooked in the initial analysis. The amount of bias in counting due to death of beetles after emergence, but before flight, was also determined by counting the beetles remaining in the marking barrel. The error in analysis of beetle emergence was found to be 5%, and the emergence counts were corrected.

Beetles were recovered by two methods: (1) on pheromone-baited traps, and (2) by their colonization of uninfested elm bolts. Five pheromone traps, identical in construction to the survey traps, were monitored between June 9 and October 25, 1978 (positions 1–5, Figure 1). A sixth trap was placed near the two elm trees 2 km away on August 3 and monitored until October 25. On June 9, an uninfested elm bolt (35 × 8 cm) was placed 3 m above the ground on each of five utility poles located midway between each of the cardboard traps (positions 6–10, Figure 1). Three uninfested elm bolts (200 × 6 cm) were placed in a group on the ground near the center of the study area on August 3 (position 11, Figure 1). The elm bolts were examined every 28 days (i.e., five times) to see if they had been colonized by beetles. The baited traps were removed and replaced every 28 days.

Trapping in the Absence of Elms. This study was conducted in an isolated location at the north end of Eureka Valley, California (U.S.G.S. Soldier Pass Quadrangle, Calif.–Nev. 15-min series). The elevation of the valley floor is 1100 m, and the valley is surrounded by mountains of 3000 m elevation. Vegetation on the valley floor is predominantly a creosote bush, scrub plant community. A single elm was located 33 km south of the study site in Saline Valley. The Siberian elms in Deep Springs Valley were approximately 14 km to the northwest. All elms were separated from the study site by barren mountain ridges.

The experimental design consisted of a 1-km grid of traps (Figure 2), modeled on the grid of traps in Independence (Birch et al., 1977), with traps on 150-m centers. Paper traps precoated with Tangle Trap® were stapled near the upper end of Sonotube® cardboard construction tubes, 2 m long and 0.25 m in diameter. These tubes were guyed in an upright position to provide a vertical visual "silhouette" for flying beetles. All traps were stapled so that they faced the center of the grid and were baited with multilure baits. Sonotubes with baited traps were also placed at sites that were 1 and 5 km from the north and south edges of the grid (Figure 2). Eight elm logs, each 2 m long and 10–20 m diameter were wrapped in fine-mesh metal screening to prevent beetle attack, and placed in the grid as indicated in Figure 2, with traps and baits attached. The logs were heavily scored (the bark cut with a chain saw to expose the phloem) before screening to increase their potential attractiveness.

The grid was established July 3–5, 1979. Elm logs containing pupae or callow adults were placed at the center of the grid in an emergence box (Browne, 1972). The box was painted white and covered with fiberglass

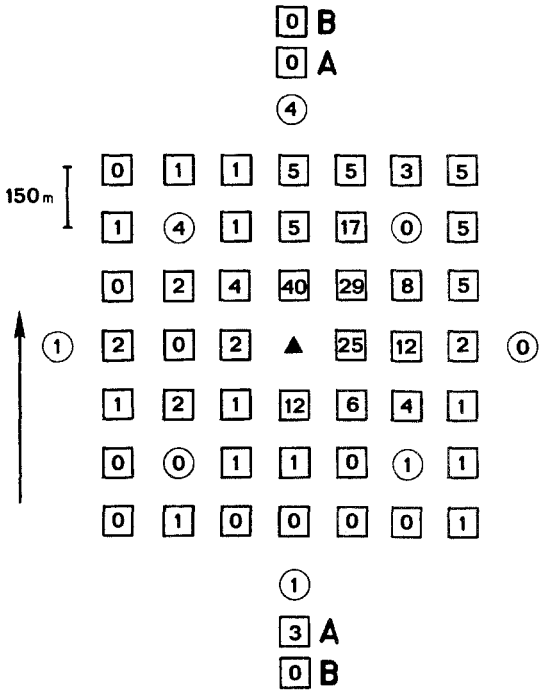

FIG. 2. Trapping grid, Eureka Valley, California (July 1979). Numbers refer to total beetles captured at traps (□) and logs (○). Release site in center of grid (▲). Arrow shows direction of prevailing wind. Traps A and B are 1 km and 5 km, respectively, from the grid.

insulation to reduce the heating effect of the direct and intense insolation in Eureka Valley. Emerging beetles responded to light coming through a long Plexiglas strip window in one side of the box. They fell into a funnel directly below the window and emerged from the stem of the funnel to the external environment.

Beetles were permitted to exit from the box continuously beginning at 11:00 AM on July 6, 1979. Counts were made periodically of beetles falling into a cardboard container placed beneath the funnel for two successive 10-min periods. These counts were extrapolated to determine the number of beetles emerging during each time interval. Emergence from sunrise to sunset was estimated from histograms plotted from hourly counts.

Traps were counted at varying intervals, at which time all *S. multistriatus* were removed from the traps and either discarded or placed in vials with kerosene and returned to the laboratory. During the period prior to the 9 AM trap count on July 9, 1979, a plastic garbage can containing elm branches and leaves was placed beneath the outlet from the funnel. This was done in an

attempt to determine whether the provision of a shelter or feeding sites for the beetles might increase subsequent trap catches. Gut analyses of beetles trapped during this period were later made to determine if those beetles had in fact fed in the elm provided.

Temperature, humidity, and wind (speed and direction) were recorded at intervals which coincided with the timing of respective trap counts.

RESULTS AND DISCUSSION

Trapping in the Presence of Elms. No *S. multistriatus* were found during the survey of dead limbs and logs in October 1977. However, the pheromone-baited survey traps captured a total of 8 beetles over 31 days in the spring of 1978. It is likely that these beetles represented an extremely low resident beetle population since beetles had emerged and were flying at the same time in the Owen's Valley towns. These towns are similar in temperature and elevation to Deep Springs. The numbers would contribute only a slight bias in determining the trap-out efficacy of pheromone-baited traps.

The technique of marking beetles by placing infested bolts inside the cardboard container was successful. A preliminary trial conducted by placing 25 beetles inside the container and awaiting their emergence demonstrated a 100% marking rate. Also, during the field release all beetles observed dispersing from the barrel were marked, and freshly marked beetles were observed on the traps. Unfortunately, the accuracy of detecting marked beetles on the traps decreased over time so estimates of population size based on a mark–recapture index could not be calculated. The reason for eventual loss of the ability to detect the fluorescent powder is unknown.

Beetle emergence from the infested bolts in Deep Springs totaled 46,485. Approximately 22% (10,274) of the beetles originated from bolts placed into the mark–release container, while 78% (36,211) emerged from the "woodpile."

The efficacy of the trap-out technique was assessed from the ratio of released to recaptured beetles. A total of 9219 beetles were captured on the pheromone-baited traps (Table 1), i.e., only 20% of the total number of beetles released. If our assumption that the resident population of beetles was nonexistent or very low was incorrect, then the difference between the number of beetles present and the number trapped would have been even larger indicating a lower capture efficiency. The trapping efficiency of 20% closely corresponds with the results of other studies on larger and less well-defined populations of *S. multistriatus*. Wollerman (1979a) estimated that traps collected 30% of beetles released into a radial pattern of baited traps, while Cuthbert et al. (1977), working with a natural population, estimated trapping 35% and 20% of spring and summer generations, respectively.

The patterns exhibited by the trap catches in space and time suggest that

TABLE 1. NUMBER OF *S. multistriatus* CAPTURED ON PHEROMONE-BAITED TRAPS, DEEP SPRINGS, CALIFORNIA, 1978

	Trap sites (as in Figure 1)						
Date[a]	1	2	3	4	5	Total	%
May	3		5	0		8	
June	1462	2427	868	1806	558	7121	77
July	153	297	175	525	583	1733	19
August	15	7	17	101	13	153	2
September	16	10	21	120	27	194	2
October	0	1	1	14	2	18	<1
Totals	1646	2742	1082	2566	1183	9219	
%	18	30	12	28	13		100

[a]Beetles trapped in May were trapped before the intentional introduction initiated on June 9.

the controlled release did indeed act as the major, if not sole, source of beetles. Two of the traps, those nearest the release points (traps 2 and 4, Figure 1), captured approximately twice as many beetles as the other three traps which were further from the release points. Wollerman (1979a) also collected more beetles on traps located close to a beetle source. A comparison of beetles trapped through time demonstrated that 77% of the total number trapped were captured within the first 28-day interval after introducing the infested elm bolts. Beetles caught during the first and second trapping periods accounted for 95% of the total number caught.

The five single elm bolts located midway between each pheromone trap only attracted a total of four beetles. It is possible that the total area of the bolt and emission of host volatiles was too small and too dilute for beetle colonization or that it was insignificant relative to the pheromone traps. The small grouping of three elm bolts (position 11, Figure 1), which provided a greater concentration of a "natural" resource, between the release points, was colonized by 900 beetles during a time when only 365 beetles were captured on pheromone-baited traps. Thus, it appears that a natural source of beetle pheromone (including elm wood volatiles) may attract a large proportion of a beetle population even in the presence of synthetic pheromone baits. If the controlled placement of elm bolts attracted beetles while in the presence of baited traps, then certainly naturally occurring wood sources would be highly attractive too.

Trapping in the Absence of Elms. A total of 231 *S. multistriatus* were captured on the traps in the Eureka Valley experiment during the test period July 6–12, 1979. Multilure-baited traps caught 212 beetles, while traps on elm logs caught 19 beetles (Table 2; Figure 2). During the same time period

TABLE 2. NUMBER OF *S. multistriatus* RELEASED AND RECAPTURED, EUREKA VALLEY, CALIFORNIA, JULY 1979

Date	Estimated numbers released	Number recaptured	
		Traps	Elm Logs
July 6	5000	2[a]	4
July 7	5512	25	11
July 8	5618	26	0
July 9	3294	158	4
July 10	1500	7	0
Total	20,924	231	19

[a]Traps on Sonotubes unbaited prior to this count.

approximately 20,000 beetles emerged from the elm logs and were released into the environment (Table 2). Daily emergence started abruptly with sunrise, peaked at about 0730 hr and gradually tapered off to stop when it became dark (Table 2).

Most of the beetles (56%) were trapped on the eight traps adjacent to the release point. Traps in the northeast quadrant of the grid caught 72% of the total, and those traps in the northern half of the grid caught 78% of the beetles (Figure 2). This result is consistent with that of Wollerman (1979b), who found that most of the beetles released into the center of a grid of traps were captured close to the release point. However, Lanier et al. (1976) found that 38% of beetles captured in the absence of elms were caught on traps closest to the release site, while 52% were caught on traps 400 m or 600 m from the source of beetles.

Beetle survival studies were run concurrently with the release of beetles. Beetles survived less than 30 min if kept in cages in direct sunlight, but survived for about 12 hr if placed in the shade without water or elm twigs. Holding cages used in these survival studies allowed air movement and apparently did not contribute to overheating of the beetles. Temperatures during the test were lower than normal for the valley. Daytime temperature maxima were about 95° F with low humidities of about 10% relative humidity. Winds were predominantly south to north at less than 6 m/sec.

No beetles were caught on the traps in Saline Valley either before or after the test. Beetles apparently did not fly from the other valleys into Eureka Valley or from the test site into adjacent areas with elms.

The highest number of beetles trapped in any one period, 51% of the total catch, were trapped after the elm branches were placed beneath the emergence funnel. The branches may have provided shelter for the beetles from the harsh

environmental conditions between emergence and the normal flight time of early evening, or they may have provided an opportunity for emerging beetles to feed in the twig crotches. Even though some beetles were observed feeding after emergence, gut analyses of the beetles trapped after supplying elm twigs showed that these responding beetles had not fed, since they had only residual material in the lower mid-gut and hind-gut, and an empty crop. This condition was similar to that of beetles which were not allowed any opportunity to feed.

The very low recapture rate of about 1% of the released beetles could be due to a number of factors: (1) The beetles may have flown from the emergence box and left the area before they could be influenced by the traps. (2) They may also have incurred a large mortality on emergence due to the very adverse climatic conditions and to the lack of suitable refuges or feeding sites. The increase in catch after the provision of a refuge site would lend support to this hypothesis. (3) Mortality may have occurred not only from adverse climatic conditions in the absence of refugia, but also from predation. Large numbers of the dragonflies, *Tarnetrum corruptum* (Hagen) (Odonata: Libellulidae) were present in the Valley and patrolled the study area using the high points of the release box and Sonotubes as rest sites. They were frequently observed taking *S. multistriatus* on the wing as they flew from the box, and subsequent dissection of two *T. corruptum* confirmed that they had been consuming *S. multistriatus*.

Beetles responded to the stimuli provided by the elm logs throughout the experiment, even though these logs were interspaced with multilure-baited traps. This suggests that beetles may locate suitable host material (elm logs) in spite of the presence of large amounts of multilure in the surrounding environment. It is possible that other attractive stimuli are released by freshly cut elm logs besides the cubebene included in the bait and that a small percentage of emerging beetles (equivalent to the "pioneer" beetles in pine scolytids) respond primarily to host odors on emergence.

The recapture rate of beetles released in the presence of elms was approximately 20% of the total number that emerged. This rate is in line with the maximum capture efficiency of multilure-baited traps in other work conducted in California and is similar to recapture rates obtained in other studies in North America. The recapture rate of 1% recorded in the absence of elms in a very unnatural environment is probably a minimal capture efficiency. Very little is currently known about postemergence mortality (including predation and dispersal) of *S. multistriatus*, and it is not known if a 20% capture rate would influence beetle populations at all over several generations. In both tests, elm wood attracted beetles despite being surrounded by pheromone-baited traps. Thus, the deployment of baited traps alone will probably not result in the suppression of beetles where competing elm wood and large beetle populations are present. However, when conducted

in conjunction with a rigorous sanitation program, e.g., tree pruning and woodpile destruction to remove suitable elm wood, mass trapping (and perhaps the use of trap trees) might be a useful tool in the pest management of *S. multistriatus*.

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THEOPHYLLINE INDUCES DIAPAUSE-LIKE STATE IN SOCIAL WASPS

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Abstract—Feeding of theophylline to hornet workers from their moment of eclosion during the summer results in the following effects on the hornets: loss of appetite for proteins, inhibited ovarial development, no cell-building activity, negative phototaxis, gradual slow-down of spontaneous motor activity, difficulty in flight and orientation, and an overall behavior pattern reminiscent of hornet queens in nature when they are in a state of winter diapause. These findings suggest that theophylline exerts anti-juvenile-hormone-like effects on hornets and raise the possibility of utilizing theophylline baits to suppress pest hornet populations.

Key Words—Theophylline, wasps, Oriental hornet, *Vespa orientalis*, Hymenoptera, Vespidae.

INTRODUCTION

The Oriental hornet *Vespa orientalis* is a harmful insect in Israel as well as throughout its distribution area (the Mediterranean basin, Southeast Asia and East Africa); (Bodenheimer, 1933). It preys on honey bees, damages summer fruits, and inflicts painful and occasionally fatal stings on man and his livestock. Several attempts to eradicate hornets or at least to limit the damage caused by them have so far failed, but in the course of such attempts, the baiting of hornets with various xanthines has shown that caffeine exerts physiological and toxic effects on hornets (Ishay and Paniry, 1979; Ishay and Shimony, 1980). Unfortunately, the hornets do not avidly imbibe saturated sugar solutions containing caffeine in concentrations of 0.5 mg/ml (Ishay, unpublished observations). They do, however, imbibe similar concentrations of theophylline and, although these do not produce greater hornet mortality than do control solutions of sugar only, they nevertheless exert interesting

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effects when offered to the hornets continuously and over long periods of time (Ishay, unpublished observations). Consequently, we deemed it worthwhile to investigate in greater detail the effects produced by theophylline on hornets.

V. orientalis workers are active both in nature and in the laboratory during the period of May–December. They do not enter a state of diapause under natural conditions and ordinarily do not survive past the autumn season (Spradbery, 1973). In the natural nest they engage in all activities necessary for construction and maintenance of the nest but not in oviposition—a task usually relegated only to the nest-founding queen. Under laboratory conditions, in the absence of a queen, the workers develop ovaries on the second day of life, build a comb, and commence ovipositing in the cells. Subsequently, they nurse the brood to maturation but, since the eggs laid by workers are not fertilized, they give rise to male progeny only (Ishay et al., 1967; Ishay, 1976).

In the present study, we found that the addition of theophylline to the food offered to workers caused them to behave like diapausing queens in the fall and winter. In other adult insects such behavior has been associated with a marked reduction in the level of juvenile hormone, occurring either naturally or following allatectomy (Novak, 1966; Wigglesworth, 1970). Our paper details the long-range effects on hornets of theophylline as compared to the normal activities of a control group of hornets.

METHODS AND MATERIALS

Hornet workers were reared from eclosion in a vesparium within artificial breeding boxes (ABBs) under conditions of daylight illumination, constant artificial illumination, or constant darkness. Under each of these conditions the hornets were kept in groups of 10, 20, or 40 individuals as follows: 4 ABBs with 40 individuals each, 4 with 20 and 6 with 10, for a total of 14 ABBs and 300 test hornets per condition. Control groups of same size and distribution were also used. For further details consult earlier publications by Ishay (1964) and Ishay et al. (1980). Theophylline (Sigma) in a concentration of 0.5 mg/ml of saturated sucrose solution was offered daily to test hornets ad libitum, with morsels of rabbit or fish meat, honey bees, or other insect pupae as additional food. In the Tel-Aviv area and elsewhere, the latter serve as protein sources for hornets (Ishay et al., 1967). Control hornets received the same diet but without theophylline. Counts were made daily to determine hornet survival and longevity. Concurrently, observations were made on the manner and nature of feeding, building, oviposition, brood nursing (when extant), and the response to optic (illumination) and mechanical (slight tapping on the glass wall of the ABB) stimulation. Several hornets were sacrificed intermittently by ether anesthesia and their abdomens dissected to ascertain the extent of ovarial development.

In addition to the aformentioned groups, six entire colonies were offered sugar solution with theophylline (in parallel to a similar number of colonies which served as controls) after their workers had previously spent several days in foraging flights to the field. Following the feeding on 0.25 mg theophylline/ml saturated sucrose solution, test workers (and controls) were transported to the field where they were released at spaced intervals from the nest, then evaluated for return time to the vesparium, flight navigation capability, success in finding the vesparium and, finally, accuracy of entry into their respective ABBs.

Ether Anesthesia. Hornets fed for 4–6 weeks on theophylline and a control group were exposed to a plug of absorbent cotton soaked in ethyl ether and introduced into their ABBs. Determinations were then made on the length of time required for the hornets to become anesthetized (and lose the righting reflex) and also on the length of time they remained dormant.

Electron Microscopy. Hornets fed for two months on theophylline were killed by ether, and pieces of cuticle from the brown and yellow regions were removed and inspected by scanning electron microscope to ascertain possible structural changes in comparison to a control group. The cuticle preparations were examined in a Jeol SEM-35 at 25 kV. Prior to examination, the preparations were coated with a layer of gold of approximately 300–400 Å in thickness by a Polaron SEM coating unit E 5100 (for details see Shimony and Ishay, 1981).

RESULTS

Longevity. In Table 1, the data on the mean longevity (in days) of theophylline-treated versus control hornets under various illumination conditions are summarized. Theophylline as such does not affect the longevity of hornets since there is no significant difference between the test and control groups taken as a whole, but there is a significant difference under continuous artificial light ($P<0.005$), with the theophylline-treated hornets being shorter lived. Incidentally, hornets fed on protein sources and water (instead of sugar solution) remained alive for only 3–5 days.

TABLE 1. LONGEVITY (IN DAYS)

	Light conditions			
Hornet group	Daylight	Continuous artificial light	Continuous dark	Overall longevity
Controls	43.6	64.1	56.6	54.6
Theophylline treated	47.2	35.1	55.6	46.1

Cell Building. In Table 2 are given the numbers of cells built in each one of the ABBs by theophylline-treated versus control hornets. As can be seen there is a highly significant difference between the theophylline-treated and the control hornets ($t = 10.98$, $p < 0.001$).

Ferility. In Table 3 the differences in fertility between theophylline-treated and control hornets are summarized. The fertility was assessed three weeks after the start of the experiment by egg-laying and subsequent brood development. As can be seen in Table 3 the differences in fertility between the two groups are very significant ($P < 0.0005$).

Behavior. The hornets failed to show any preference for sugar solution alone over the sugar solution with theophylline, so there was no rejection of the theophylline. Behavior of control and test hornets was as follows: On the first day of the theophylline diet the test hornets still behaved ordinarily, but by day 2 they ceased feeding on proteins, although they handled these as well as any other easily chewable material, crumbling it to pieces while constantly moving their antennae, mandibles, legs, and wings. The drug-fed hornets mostly did not engage in building activity (Table 2) (control hornets built within 2 days of introduction into the ABB) nor did they usually oviposit or tend larvae (Table 3), and upon dissection were found to have undeveloped ovaries, no larger than those of newly eclosed individuals (control hornets in contrast developed ovaries within 2–3 days and commenced ovipositing). There was no evidence for the establishment of a hierarchy, and there were no noticeably dominant hornets or hornets which remained restful underneath the roof of the ABB (as among control hornets). Test hornets tended to

TABLE 2. CELL BUILDING

	No. of cells							
Hornet group	0–5	6–10	11–15	16–20	21–25	26–30	31–50	50+
Control	2	4	6	10	6	5	8	1
Theophylline	13	4	–	–	–	–	–	–

TABLE 3. FERTILITY

	Brood instar		
Hornet group	No brood	Egg(s)	Larva(e)
Control	9	14	19
Theophylline	40	2	–

aggregate in a dark corner of the ABB and, from time to time, dropped off the walls, usually falling on their backs and having difficulty in turning over, having apparently lost their righting reflex. Ultimately, they regained their balance and climbed back up to their congregated group. Flight within the ABB was erratic and for short distances only. The hornets departed the crowd only to imbibe sugar solution; that is, they did not engage in any directed motor activity (like food sharing, ventilation, cleaning, or thermoregulation).

After several weeks, they displayed increasing difficulty in moving their bodies, and this paralysis increased to the point of death. For a number of days after the start of theophylline feeding, the test hornets were docile, and their stance on one of the walls was fully reminiscent of queens in their winter diapause.

Response to Stimuli. At the beginning, test hornets were hyperreactive to light or to visual and acoustic stimulation, but gradually they tended not to respond to any stimulus and began showing negative phototaxis.

Ether Anesthesia. The test hornets became somnolent within 3 min (control hornets required 7 min) and remained anesthetized for a mean of 152 min (control hornets awakened after about 56 min). More detailed data are to be published elsewhere.

Electron Microscopy. Under SEM, changes were detectable in the yellow but not in the brown areas of the hornet cuticle. The yellow pigment granules in hornets fed theophylline were of the same shape and size as in hornets several days of age (Figure 1). In contrast, the yellow pigment granules in control hornets increased in size with time and gradually also underwent degeneration (Figure 2).

Effects on Adult Hornets. Adult hornets (more than 7 days old at the start of the experiment) fed on theophylline showed greater sensitivity to the substance, which in high concentrations appeared to be toxic for them. At 0.25 mg/ml the theophylline was not toxic, but the adult hornets failed to attend the queen or the brood and made only rare foraging flights to the field to gather protein or building materials. (These observations were made in six colonies where theophylline was introduced with the sugar solution and a similar number of colonies fed on sugar solution alone.) When the field workers (i.e., those which were observed earlier to fly outside the nest) were removed from the ABB and then released at spaced distances from it, we noticed: (1) that they did not attain proper flying altitude, showing distinct motor difficulties (26 out of 31 workers released in the same day); (2) that they probably suffered visual impairment in that they bumped into large objects during brief flights (24 out of 31); (3) that they had orientation difficulties in that they failed to return to the ABB even when released at a distance of only 100 m from it [only one out of 31 released hornets succeeded in doing so, whereas, 19 out of 24 control hornets found the ABB even from 500 m away, and all of them (16 out of 16) returned after less than 15 min from a distance of

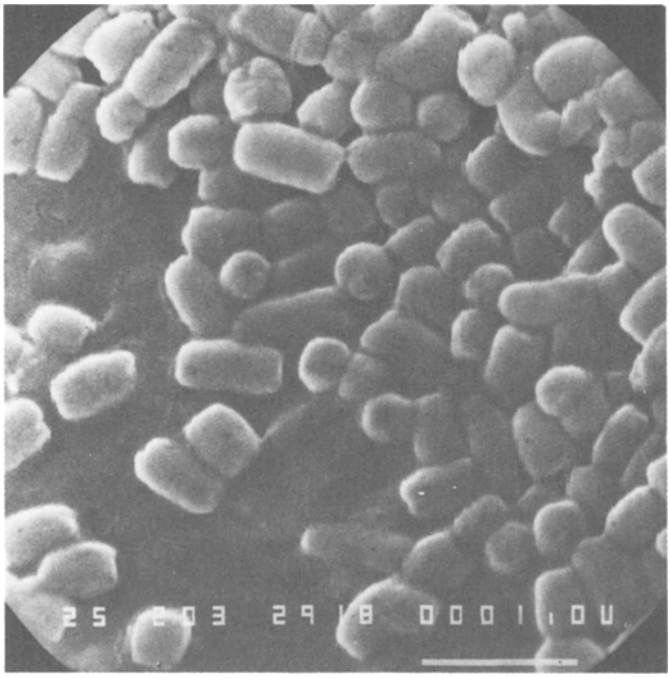

FIG. 1. Yellow pigment granules of *Vespa orientalis* 60-day-old workers fed with theophylline. Note that the average diameter is 0.3 μm, much the same as the size of the pigment granules of control young hornets (2–3 days old). Bar = 1 μm, × 20.000. Usually the granules are also longer in the young hornets or in those fed with theophylline than in old hornets (see Figure 2).

100 m]; and (4) they did not locate the entrance of their own ABB even if released in proximity to the ABB (13 out of 17 workers released in the same day).

All these behavioral patterns were, however, found to be reversible so that hornets taken off the theophylline for 3 days in a row reverted to correct navigation and flight orientation as well as to normal nest activities.

DISCUSSION

Results of the present study indicate that several days following the feeding of test hornets on theophylline they display behavior typically associated with a drop in the level of juvenile hormone in adult insects (Engelman, 1970; Wigglesworth, 1970; Gilberg and King, 1973; Judy, 1974). The behavioral phenomena are characteristic of the stage of diapause in

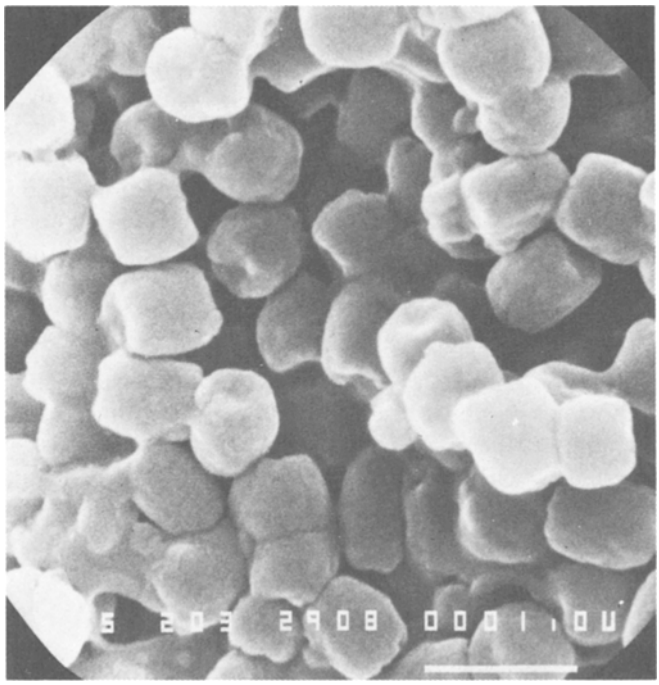

FIG. 2. Yellow pigment granules of *Vespa orientalis* 60-day-old workers fed a regular diet (control group). Note that the average diameter of the pigment granules is 0.6 μm, i.e., twice that of those fed with theophylline (or of young controls) Bar = 1 μm, × 20.000.

various insects, including that in hornet queens in the winter. In hornets, diapause is associated with a shortening of the day and a drop in temperature. The theophylline-fed hornets entered this stage in the middle of the active season, when days are long and temperatures are high. In wasps of the genus *Polistes*, topical application of synthetic juvenile hormone to diapausing individuals causes ovarian development, which suggests that the diapause is reversible by adjusting juvenile hormone levels (Strambi, 1967; Bohm, 1972).

In the present case, inasmuch as the recorded phenomena appeared also under total darkness, it seems that there is no direct connection between the effects of theophylline and the length of the day. In mammals (rats), feeding on theophylline (and other methylated xanthines) induced severe bilateral testicular atrophy with aspermatogenesis or oligospermatogenesis in 85–100% of the test animals (Weinberger et al., 1978). In rats, theophylline also induces a hyperplastic alteration of the small intestinal mucosa, which is similar to that observed in diabetes mellitus (Meier-Hoberg et al., 1979).

Theophylline is metabolized via the P448 microsmal enzyme system in the liver and smoking induces this enzyme system (Greenberger, 1980). Enzyme induction would probably explain why hornets fed on the drug since eclosion tolerate a higher dose than those who were fed on it only as adults. Theophylline inhibits phosphodiesterase, the enzyme required for conversion of 3′,5′-cyclic AMP to 5′-AMP, and thereby increases the amount of 3′,5′-cyclic AMP. Although theophylline induces an initial elevation of intracellular cAMP levels, long-term treatment with theophylline produces a significant decrease in cAMP content as shown by Steinberger and Whittaker (1978). These authors also claimed that cAMP levels were low through cell divisions and that increased cAMP levels inhibited division.

This may explain the biphasic reaction of hornets to theophylline in that during the first period, when they show hyperactivity, they probably have high cAMP levels which inhibit cell division and oviposition, preventing them from cell building (which appears always prior to egg-laying and nursing); after that period, when the cAMP level is low, the hornets are too old to develop ovaries and show only depression of activity.

The negative phototactic reaction of hornets to theophylline is probably similar to the inhibitory effects of theophylline and cAMP on the light response of the dermal melanophores of xiphophorian fish (Wakamatsu et al., 1980).

The alkaloid theophylline is produced by plants of the genus *Coffea* (Coffeideae, Rubiaceae) as well as the genus *Thea* (Theaceae), which are prevalent in tropical and subtropical regions of the Old World—the same regions that abound in species of Vespinae (Vecht, 1957). In these regions, the wasps and hornets tend to masticate the bark of trees to prepare a dough for building purposes, and apparently the presence of the alkaloid in bark has a protective value against these insects.

The mode of action of theophylline in hornets is as yet unclear. In addition to the above-mentioned phenomena associated with elevated cAMP levels, it is possible that the stereoelectronic structure of the metabolized methylated alkaloid partially resembles that of juvenile hormone (as in the distance between the two methyl groups and the keto group on the pyrimidine ring). It might therefore act to block the receptor to juvenile hormone.

The elevation of the cAMP level inhibits the accumulation of proteins and lipids necessary for the formation of eggs and boosts the concentration of glucose in the hemolymph, thus rendering infertile any eggs that are formed. It should be pointed out also that in the natural situation, the queen depresses workers' ovarial development by the secretion of pheromones which the workers lick up (Ishay et al., 1965), but the other activities of workers remain unaffected.

There are other examples among insects where some substances present in the food are capable of inducing diapause; for example, diapause can be

induced in *Leptinotarsa* sp. (Coleoptera, Crysomelidae), by feeding them aged potato foliage (Wilde and Ferket, 1967). There are also juvenile hormone mimetics, such as the "paper factor" juvabione (Slama and Williams, 1966), which induce sterility when applied to adult females (Wilde and Loof, 1973). In this connection, it seems to us that the use of baits containing theophylline in sugar solution may possibly prove an effective means of eradicating hornets in their distribution areas without seriously harming man or other animals in the same area. As is known, diapause entails a diminution in metabolic activities, and this is adaptive in hornets since it occurs in the winter, when the temperatures are low. Feeding theophylline to workers results in a diapause-like syndrome during the summer, with a drop in metabolic activity. This is seemingly accompanied by an inhibition of aging inasmuch as the yellow pigment granules in "old" hornets fed on theophylline remain of smallish diameter and without any faults, much the same as in young hornets.

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CHEMICAL DEFENSE SECRETIONS OF THE TERMITE SOLDIERS OF *Acorhinotermes* AND *Rhinotermes* (ISOPTERA, RHINOTERMITINAE): Ketones, Vinyl Ketones, and β-Ketoaldehydes Derived from Fatty Acids

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Abstract—The defense secretions of advanced "nasutoid" rhinotermitine soldiers from the New World contain enolic β-ketoaldehydes as the major components. The secretions of minor soldiers of *Rhinotermes hispidus* (Emerson) and *R. marginalis* (Emerson) consist primarily of 3-keto-13-tetradecenal and 3-ketotetradecanal, but possess in addition C_{13}, C_{14}, C_{15}, and C_{17} saturated and unsaturated ketones. Major soldiers lacked these compounds and in fact had virtually no frontal gland secretion. The defense secretion of the monomorphic soldiers of *Acorhinotermes subfusciceps* (Emerson) contains mostly 3-keto-(*Z*)-9-hexadecenal and (*Z*)-8-pentadecen-2-one. Biosynthetic origins and interrelationships are postulated for these compounds, and the concomitant evolution of chemical weaponry and the modified labral brush is discussed.

Key Words—Termites, defense secretion, *Acorhinotermes*, *Rhinotermes*, Isoptera, Rhinotermitinae, ketones, vinyl ketones, β-ketoaldehydes, 3-keto-(*Z*)-9-hexadecenal, (*Z*)-8-pentadecen-2-one.

INTRODUCTION

Soldier termites of the advanced subfamilies Prorhinotermitinae and Rhinotermitinae (Isoptera, Rhinotermitidae) employ a labral "daubing brush" in colony defense, applying a lipophilic mixture of electrophilic compounds

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which act as contact insecticides (Prestwich, 1979a,b; Quennedey and Deligne, 1975). These fatty-acid-derived compounds are presumably biosynthesized in the cephalic gland and stored in hypertrophied reservoirs which extend into the abdomen (Quennedey, 1978). This highly effective "frontal weapon" of the rhinotermitine soldiers has undergone considerable evolutionary modification, with the primary trend being the development of "nasutoid" minor soldiers (Quennedey and Deligne, 1975) (Figure 1).

The structures of 2- and 3-alkanones, vinyl ketones (Prestwich et al., 1975; Quennedey et al., 1973), nitroolefins (Vrkoč and Ubik, 1974), and β-ketoaldehydes (Prestwich and Collins, 1980, 1981) have been described as defense secretion components for a variety of rhinotermitids (Figure 2). We now present a more complete analysis of the defense secretions of the highly advanced soldiers of the genera *Rhinotermes* and *Acorhinotermes*, and on the basis of these data we postulate biosynthetic and phyletic interrelationships among the chemicals and the termites producing them.

METHODS AND MATERIALS

Collection and Isolation

Colonies of *Acorhinotermes subfusciceps* (Emerson), *Rhinotermes hispidus* (Emerson), and *Rhinotermes marginalis* were obtained from rotten woody litter in the rain forest at Kartabo Point, Guyana (Emerson, 1925) and were hand-carried to Stony Brook. Our experiences over the past two years had shown that removal of the termite soldiers in the field, extraction at the field site, and transportation of the secretion to New York resulted in complete decomposition of the major secretion components. Therefore, living soldiers were removed from the colony, cooled at −5° C for 1 hr, and crushed in hexane (Fisher HPLC grade). The crude extract was filtered through glass wool to remove termite parts and the solvent was removed in vacuo. The secretion could not be chromatographed on silica gel or alumina without complete disappearance of the major component. Analysis of the secretion by capillary GC allowed only the stable compounds to be observed (Figure 3); the unstable species could be observed only on packed columns at short elution times. Thus, the crude secretion was used directly for [^{1}H]NMR, [^{13}C]NMR, MS, UV, IR, and chemical derivatization without further purification. *A. subfusciceps* soldiers afforded 10 mg crude secretion from 130 individuals. *R. hispidus* minor soldiers gave 50 mg crude secretion from 75 individuals, but major soldiers provided only traces of relatively nonvolatile material. *R. marginalis* minor soldiers yielded 15 mg from 200 individuals, while major soldiers gave only ~ 2 mg from 70 individuals.

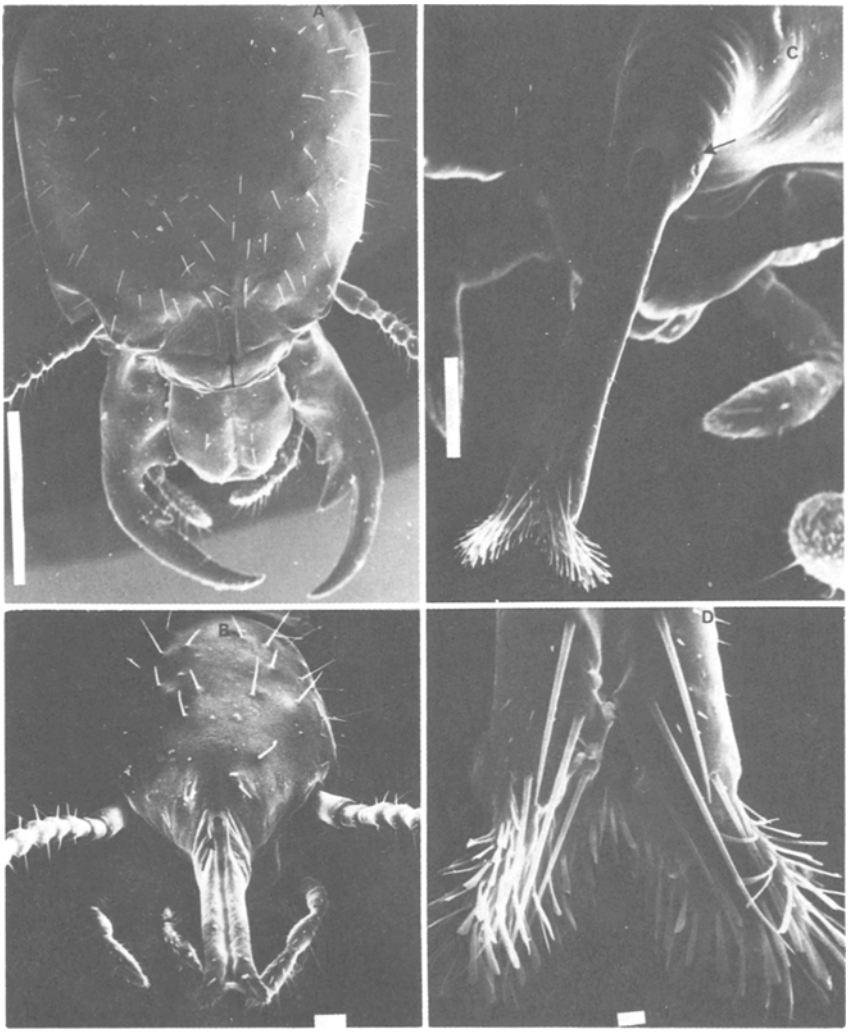

FIG. 1. Scanning electron microscope photographs of termite soldiers heads: (*a*) *Rhinotermes hispidus* major soldier, (*b*) *R. hispidus* minor soldier, (*c*) *Acorhinotermes subfusciceps* soldier, (*d*) *A. subfusciceps* soldier, detail of labral brush. The frontal gland is indicated by an arrow in A–C. Bars represent 1000 μm in (*a*), 100 μm in (*b*) and (*c*), and 10 μm in (*d*). Photographs obtained by Mr. Richard W. Jones and Mr. Charles Bleecher (Stony Brook).

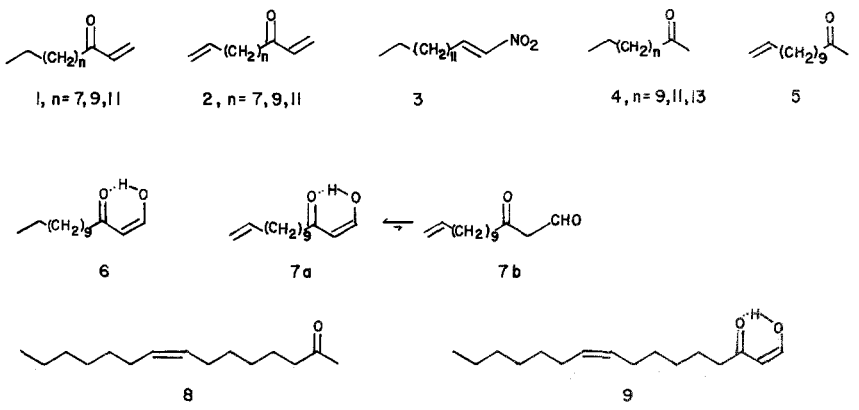

FIG. 2. Defense compounds of the Rhinotermitinae and Prorhinotermitinae. *Schedorhinotermes* spp. contain vinyl ketones **1** and **2** and 2-tridecanone **4a** ($n = 9$) (Quennedey et al., 1973; Prestwich et al., 1975). *Prorhinotermes simplex* contains nitroolefin **3** (Vrkoč and Ubik, 1974). *Rhinotermes* spp. contain traces of **1a** ($n = 9$) and **2a** ($n = 9$), minor amounts of **4a** ($n = 9$), **4b** ($n = 11$), and **4c** ($n = 13$), and major quantities of **6** and **7** (Prestwich and Collins, 1980, and this work). *Acorhinotermes subfusciceps* contains **8** and **9** (Prestwich and Collins, 1981, and this work).

Analytical and Synthetic Methods

NMR spectra were obtained on a Varian Associates CFT-20 NMR operating at 80 MHz for ^1H and 20 MHz for ^{13}C. Shifts are reported for deuteriochloroform solutions in ppm downfield from $(CH_3)_4Si$, using the $CHCl_3$ resonance as the internal standard for ^1H and the $CDCl_3$ resonance for ^{13}C. Microprobe [^{13}C]NMRs were performed in C_6D_6 using a 1.7-mm capillary tube or in a 200-μl, 8-mm-diameter microcell. Gas chromatography was performed on a Varian model 3700 GC equipped with a 19-m × 0.5-mm glass capillary coated with OV-101 and operating at 120° to 200° at 12°/min. Low-resolution mass spectra were obtained on a Hewlett Packard HP5980A mass spectrometer interfaced to an HP 5710A GC equipped with a 2-m × 2-mm ID glass column packed with 3% OV-17 on 100/120 Gas Chrom Q. High-resolution mass spectra were performed on an MS-30 instrument interfaced to a gas chromatograph and a DS-50 data system. Chromatographic solvents were Fisher HPLC grade and were used without further purification. Benzene was dried by azeotropic removal of water and tetrahydrofuran (THF) was distilled under N_2 from benzophenone sodium ketyl.

Synthetic and spectral details for the *Acorhinotermes* compounds **8**, **9**, **15**, and **16** are presented elsewhere (Prestwich and Collins, 1981); we summarize below the experimental details for the *Rhinotermes* compounds

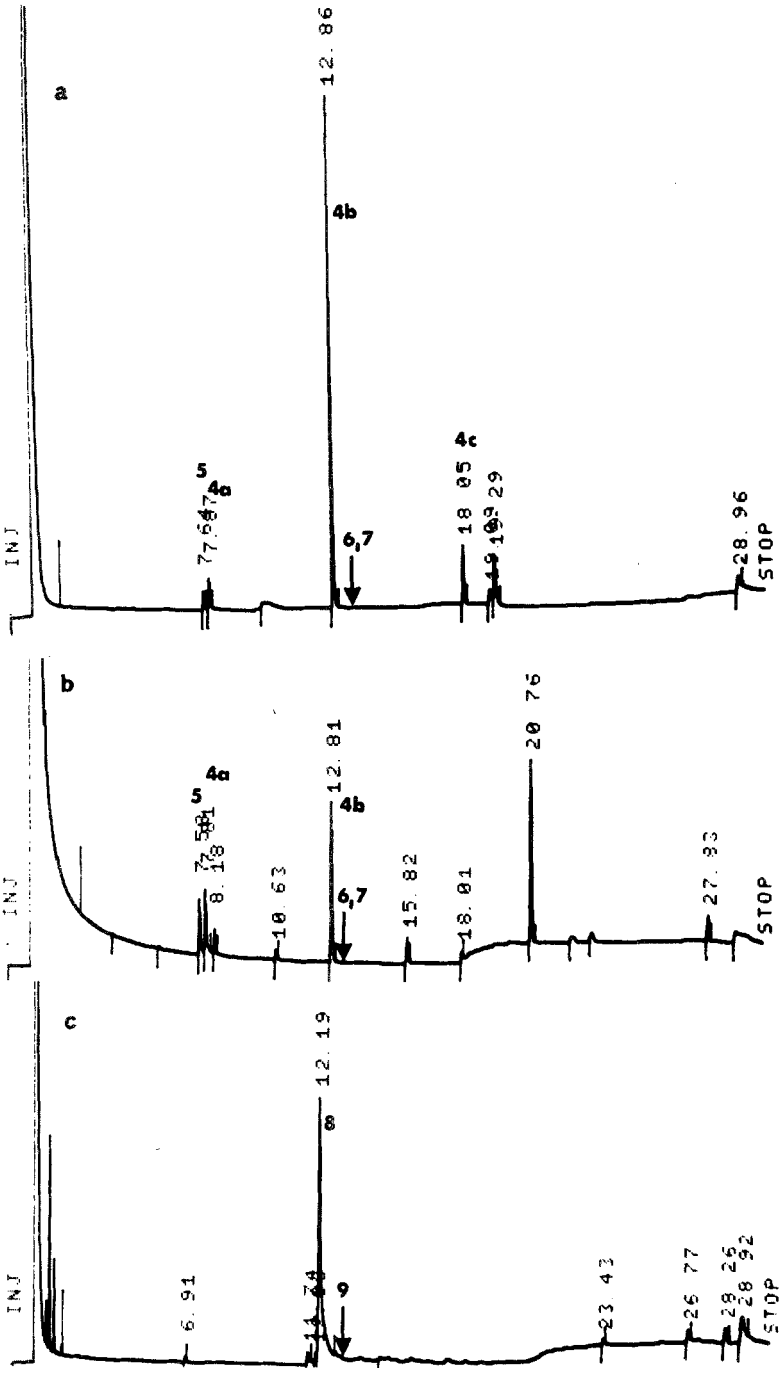

FIG. 3. Capillary gas chromatograms of stable components of "nasutoid" soldier defense secretions of (a) *Rhinotermes hispidus*, (b) *Rhinotermes marginalis*, and (c) *Acorhinotermes subfusciceps*. All chromatograms were obtained on a 19-m × 0.5-mm OV-101-coated glass column operation at $T_i = 120°$ (2 min), $T_p = 4°$ / min and $T_f = 220°$ (10 min). Ketoaldehydes (>80% of each secretion) are not evident in this GC; however, the arrow indicates the position of broadened peaks which may be observed on shorter packed columns. Traces of **1a** and **2a** in (a) appear as poorly resolved peaks between **4a** and **4b**. See Figure 2 for structures.

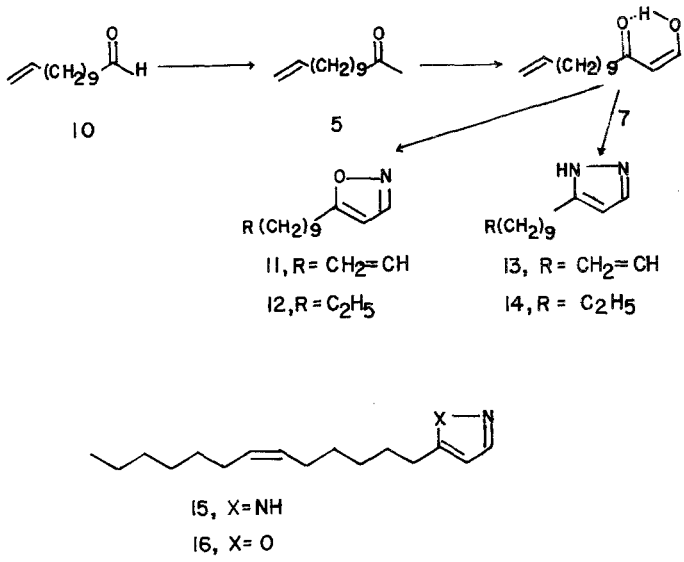

FIG. 4 Synthetic schemes for the preparation of the unsaturated ketones, β-ketoaldehydes, and isoxazole and pyrazole derivatives. Details in text.

which have only been presented in communication form (Prestwich and Collins, 1980). Schemes for these syntheses are shown in Figure 4.

12-tridecen-2-one (**5**). To a solution of 630 mg (3.5 mmol) of crude 11-dodecenal (**10**) (containing 15% of the saturated dodecanal) in THF at 0° C was added 2.8 ml (5 mmol) of 1.8 M ethereal methyllithium. The reaction was stirred 2 hr at 0° to 20° C, quenched with aqueous HCl, and worked up as usual to give 616 mg of the crude methyl carbinol. The crude carbinol in acetone solution was treated with a slight excess of Jones reagent, a few drops of 2-propanol were added, and the mixture was diluted with hexane and filtered through Florisil to give crude methyl ketone **5**. Flash chromatography (5% ethyl acetate–hexane) afforded 215 mg of the TLC homogenous ketone (**5**), which contained 15% of the saturated 2-tridecanone (**4**) by GLC. Both ketones coinjected with the corresponding termite-derived ketones **4** and **5** in the *R. hispidus* and *R. marginalis* secretions. Mass spectra were also congruent. [^{13}C]NMR: δ208.93 (C-2), 139.09 (C-12), 114.07 (C-13), 43.74 (C-3), 33.76 (C-11), 31.57 (C-11, sat'd), 29.70, 29.38, 29.15, 29.08, 28.90 (CH_2), 23.83 (C-1), 22.62 (C-12, sat'd), 14.03 (C-13, sat'd). GC-MS, **5**: *m/z* (rel. int.), 196 (M^+, 0.1), 181 (8), 163 (12), 125 (40), 95 (61), 55 (100), 43 (83); **4**: *m/z* 198 (M^+, 5), 183 (5), 71 (40), 59 (44), 58 (100), 43 (39).

3-Keto-13-tetradecenal (**7**). A solution of 200 mg (1 mmol) of ketone **5** and 0.15 ml distilled ethyl formate in 2 ml of dry benzene was added dropwise to a stirred suspension of 30 mg of finely chopped sodium metal in 5 ml of dry

benzene (Prelog et al., 1947). The mixture was stirred 1.5 hr at room temperature and then 1 hr at reflux. The cooled reaction mixture was decanted into water in a separatory funnel, and the flask containing unreacted sodium was rinsed with ether. The layers were separated, the organic layer washed with 2 N NaOH. The combined basic aqueous layers were washed with 1 : 1 ether–hexane, acidified to pH 3–4 with 4% HCl and β-ketoaldehyde **7** was extracted with ether–hexane. No attempts were made to further purify the chromatographically unstable β-ketoaldehyde (95 mg, 44%), which decomposed to aromatic and aldol products on TLC (Rothman and Moore, 1970) and gave broad peaks on GLC. Spectral data and derivatives (below) indicated the presence of 15% of the 13,14-saturated analog **6** present in the synthetic material (Prestwich and Collins, 1980). Figure 5 shows the [^{1}H]NMR and [^{13}C]NMR spectra of the crude *R. marginalis* and *R. hispidus* secretions, respectively, and these are compared to the corresponding spectra for the synthetic materials.

Isoxazole (**11**). A solution of 26 mg of β-ketoaldehyde **7** containing 15% of **6** in 4 ml of ethanol was stirred with 50 mg of K_2CO_3 and 50 mg of hydroxylamine hydrochloride at 0 to 20° C for 2 hr and at reflux for 6 hr. The reaction was neutralized with 4% HCl, poured into 50 ml of water, and extracted with ether–hexane (1 : 1). The crude isoxazole was purified by pipet flash chromatography (Prestwich and Collins, 1981) by elution with 5% ethyl acetate–hexane to give 15 mg purified isoxazole **11** containing 15% of 13,14-saturated isomer **12**.

Pyrazole (**13**). A solution of 26 mg of β-ketoaldehyde **7** containing 15% of **6** in 4 ml of ethanol was stirred at 0° C during the addition of 4 drops of 2 *N* NaOH followed by 6 drops of hydrazine (95%). The yellow solution was stirred at 0 to 20° C for 1 hr and at 60° for 1 hr. The reaction was neutralized and the product was extracted with 1 : 1 ether–hexane. Pipet flash chromatography of the crude pyrazole with 5, 10, 20, and 40% ethyl actate in hexane gave 13 mg of the purified pyrazole **13** containing 15% of the 13,14-saturated isomer **14**.

RESULTS AND DISCUSSION

The defense secretions of the minor soldiers of *R. hispidus* and *R. marginalis*, and of the monomorphic soldiers of *A. subfusciceps* consist of >85% β-ketoaldehydes which decompose slowly during gas chromatography. The remainder of the secretions are C_{13}, C_{14}, C_{15}, and C_{17} saturated and unsaturated ketones which are biogenetically related to the major secretion components (**6** and **7**) (Figures 2 and 3). Thus, in *A. subfusciceps*, the only identifiable minor component was (*Z*)-8-pentadecen-2-one (**8**), identified by capillary GC and GC-MS comparison to authentic material. In *R. marginalis*

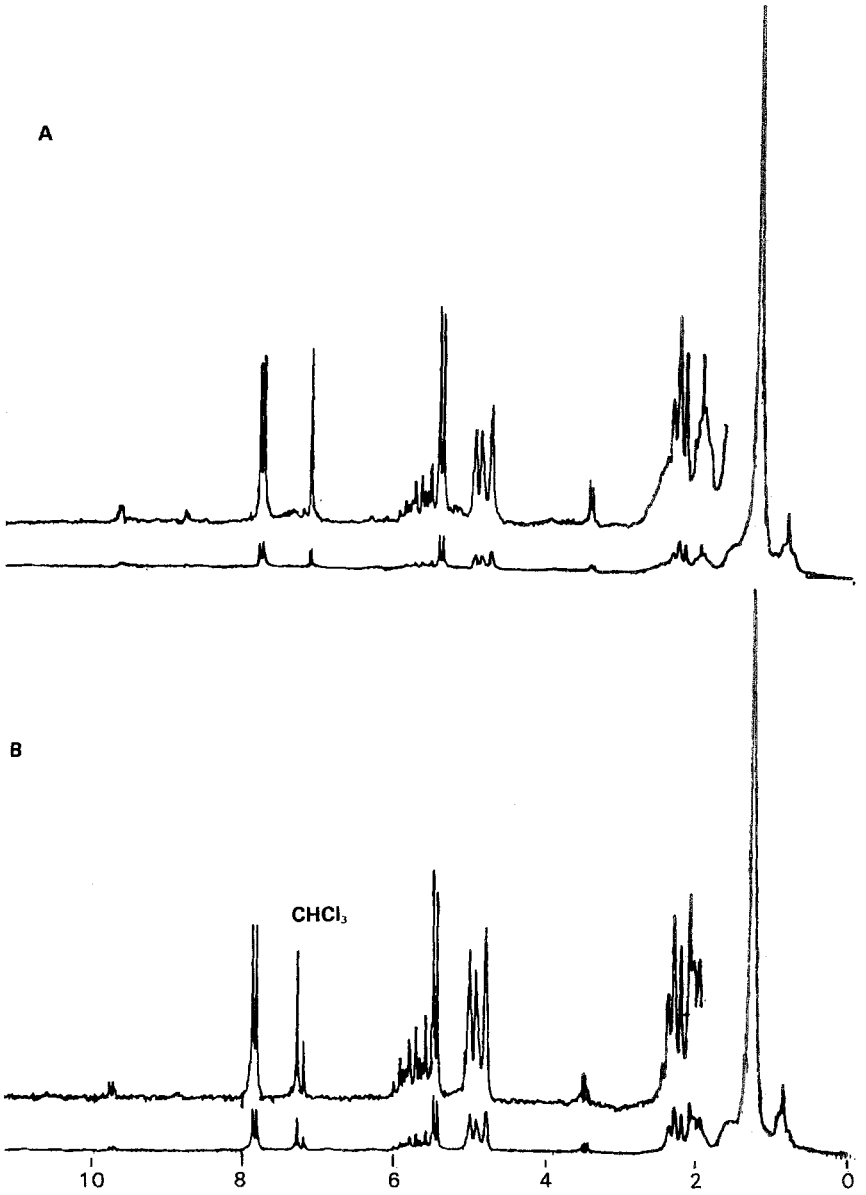

FIG. 5. NMR spectra of crude *Rhinotermes* extracts and synthetic β-ketoaldehydes: (A) [^{1}H]NMR of crude *R. marginalis* extract; (B) [^{1}H]NMR of synthetic **7** (+15% **6**); (C) [^{13}C]NMR of *R. hispidus* secretion; (D) [^{13}C]NMR of synthetic **7** (+15% **6**).

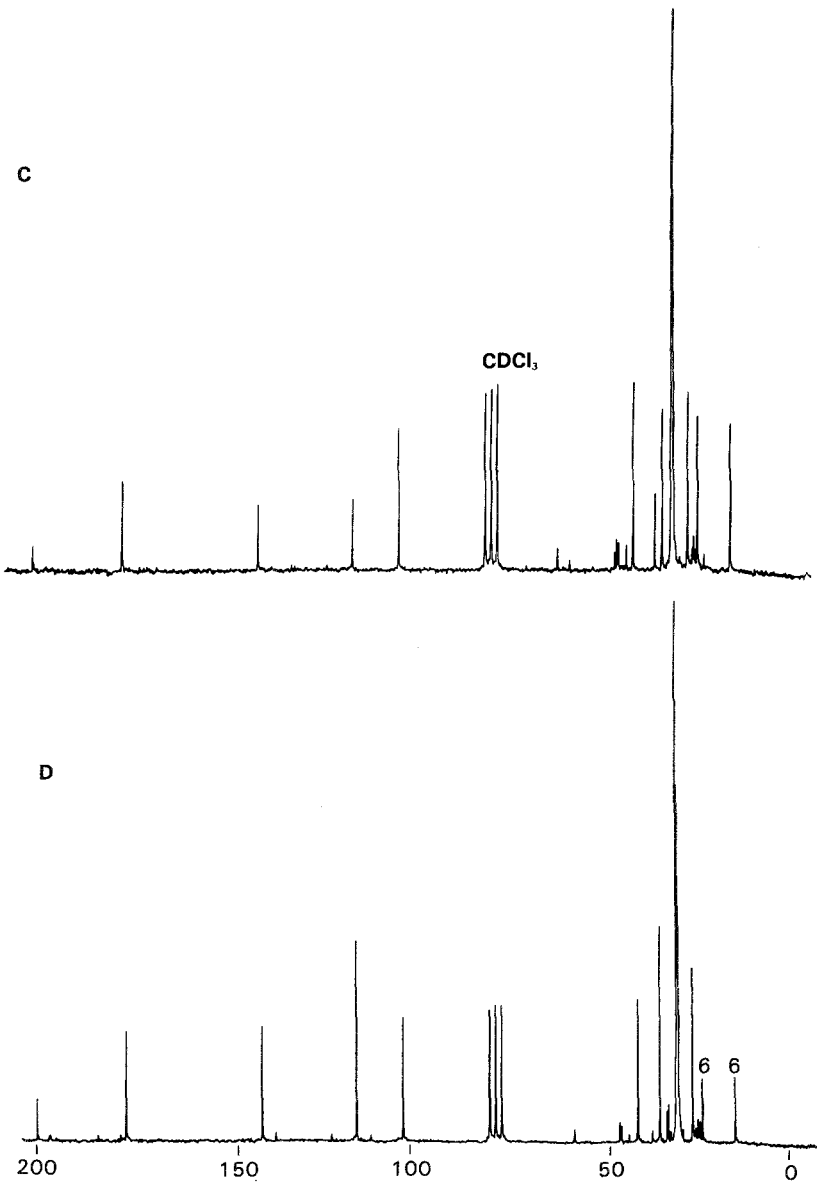

FIG. 5. Continued.

and *R. hispidus*, the primary minor constituents were 2-tridecanone (**4**, $n = 9$), 2-pentadecanone (**4**, $n = 11$), 2-heptadecanone (**4**, $n = 13$), 12-tridecen-2-one (**5**), and traces of 1-tetradecen-3-one (**1**, $n = 9$) and 1,13-tetradecadien-3-one (**2**, $n = 9$).

Each of these constituents was identified by capillary GC coinjection with authentic compounds and by congruence of mass spectra. Comparison vinyl ketones were extracted from *Schedorhinotermes lamanianus* (Prestwich et al., 1975) and homologous 2-alkanones were obtained from *Amitermes unidentatus* (Prestwich, 1979a).

The major soldiers of *R. hispidus* and *R. marginalis* possessed little hexane-extractable material (see also Sanassi, 1969). None of the β-ketoaldehydes, vinyl ketones, or 2-alkanones could be detected in this extract. However, the defense secretion of the nasutoid termites constitutes >10% of the fresh weight of these individuals. The percentage of the β-ketoaldehydes in the minor soldier secretion is so high that the [^{1}H]NMR and [^{13}C]NMR spectra of $CDCl_3$ solutions of the crude hexane extracts are nearly indistinguishable from the corresponding spectra of the synthetic materials (Figure 5, *Rhinotermes* spp.; Figure 6 *Acorhinotermes*).

The β-ketoaldehydes were identified unambiguously as their pyrazole and isoxazole derivatives (Prestwich and Collins, 1980, 1981). The pyrazoles were useful for mass spectral determinations and gave detectable parent peaks; however, the NMR spectra show broadened C-1, C-2, C-3, and H-1 and H-2 resonances due to rapidly equilibrating tautomeric 5- and 3-alkylated forms, and the GC peaks are somewhat broadened. Although isoxazoles did not give satisfactory parent peaks in their mass spectra, sharp NMR and sharp GC peaks were observed for the exclusive 5-alkylated isoxazole nucleus as shown. Thus, analysis of the isoxazole derivatives of the crude *Rhinotermes* spp. secretion showed a ca. 3:4 ratio of unsaturated (**11**) to saturated (**12**) isoxazole, while only a single isoxazole (**16**) was obtained from the *Acorhinotermes* secretion.

The cooccurrence of the C_{14} β-ketoaldehydes with C_{14} vinyl ketones and C_{13}, C_{15}, and C_{17} ketones in *Rhinotermes* species, and the discovery of a C_{16} β-ketoaldehyde with a (*Z*)-9 double bond in *Acorhinotermes* strongly suggests the abundant C_{16} and C_{18} fatty acids (Thompson, 1973) as biogenetic precursors to these compounds. Moreover, the distribution of ketonic components in the secretions provides evidence that the enzymes of the β-oxidation pathway of fatty acyl-CoA derivatives are involved in defense secretion biogenesis. One potential biosynthetic scheme is summarized in Figure 7. For *Acorhinotermes*, palmitoleoyl coenzyme A could enter the β-oxidation sequence, which in worker termites would result in the release of 8 mol of acetyl-CoA and energy. In the soldier cephalic exocrine glands, however, β-oxidation could be interrupted by reductive cleavage of the β-

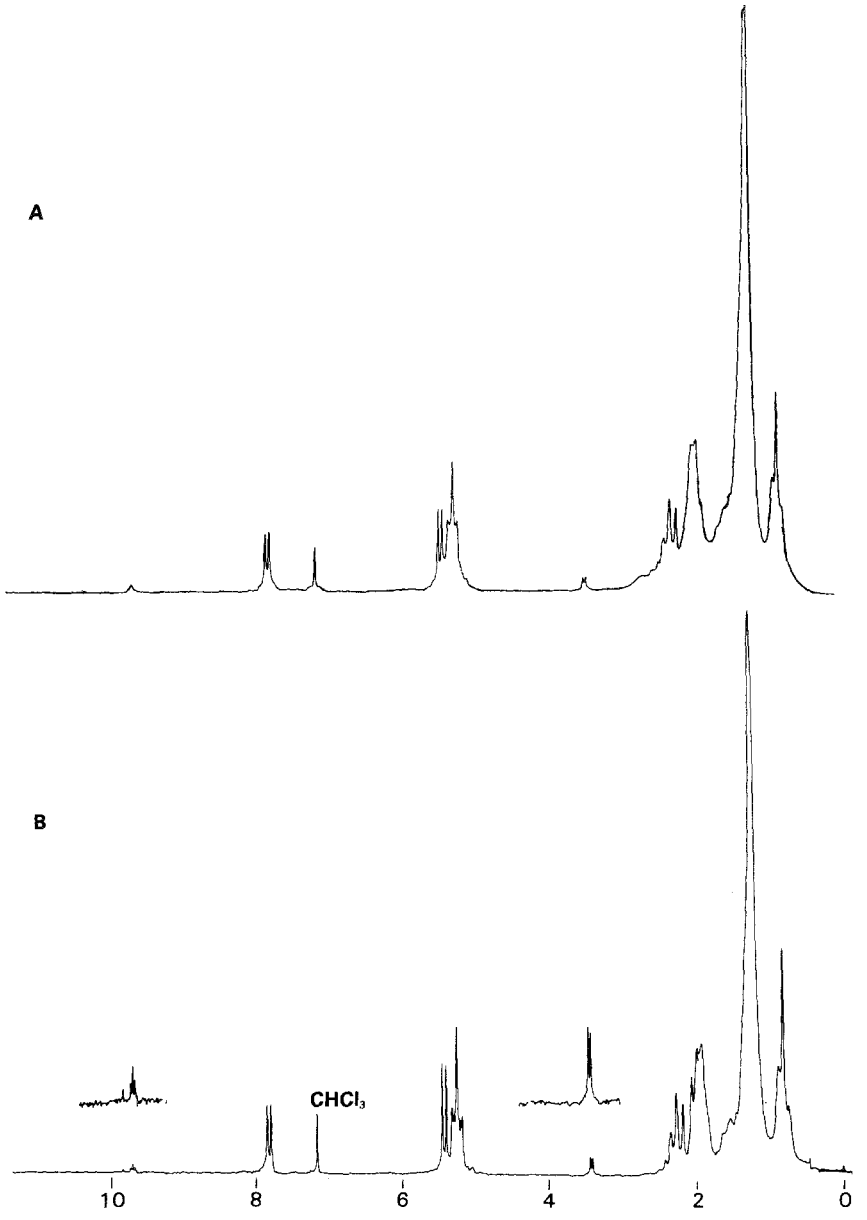

FIG. 6. [^{1}H]NMR spectra of crude *Acorhinotermes* extract (A) and syntehtic β-ketoaldehyde **9** (B).

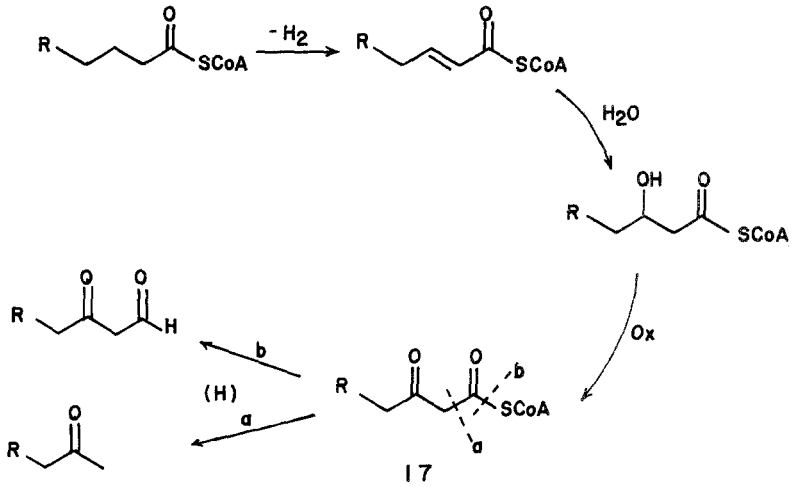

FIG. 7. Proposed biosynthesis of rhinotermitine defense substances.

ketopalmitoleoyl-CoA (**17**). Hydrogenolysis of the bond "b" by a reduced nucleotide cofactor would afford the β-ketoaldehyde **9**, whereas reductive cleavage of bond "a" yields ketone **8**, with the olefinic bond in the (*Z*)-8 position.

In *Rhinotermes* spp. we suggest that palmitic acid is degraded first to myristic acid, which then undergoes partial ω-desaturation to give a mixture of 13-tetradecenoyl and tetradecanoyl (myristyl) CoA thioesters. These may then serve as precursors to the ketones **4** and **5** and to the β-ketoaldehydes **6** and **7**. Not surprisingly, the ratios of ketones **5**:**4**, and the ratio of β-ketoaldehydes **7**:**6** are both ca. 3:4, indicative of a common biological precursor. The C_{14} vinyl ketones **1** ($n = 9$) and **2** ($n = 9$) also occur in approximately 3:4 ratio. We suggest that the vinyl ketones arise via overreduction to the β-ketoalcohol followed by β-elimination to the enone system. In general, the harnessing of the β-oxidation system to the production of defense secretions indeed seems a highly parsimonious utilization of enzymic resources.

The defense secretions of Prorhinotermitinae and Rhinotermitinae are all lipophilic electrophiles, capable of alkylation of biological nucleophiles such as cysteinyl sulfhydryl groups. The phyletic relationships for the family Rhinotermitidae are shown in Figure 8 (Quennedey and Deligne, 1975). Superimposed on the morphological development of the elongate brush-tipped labrum of the minor soldier caste is the evolution of the chemical weaponry. We have hypothesized that the evolution of the nasutoid minor soldier proceeded with concomitant increased toxicity and chemical reactivity of the lipophilic contact poisons used in defense. Our preliminary results with

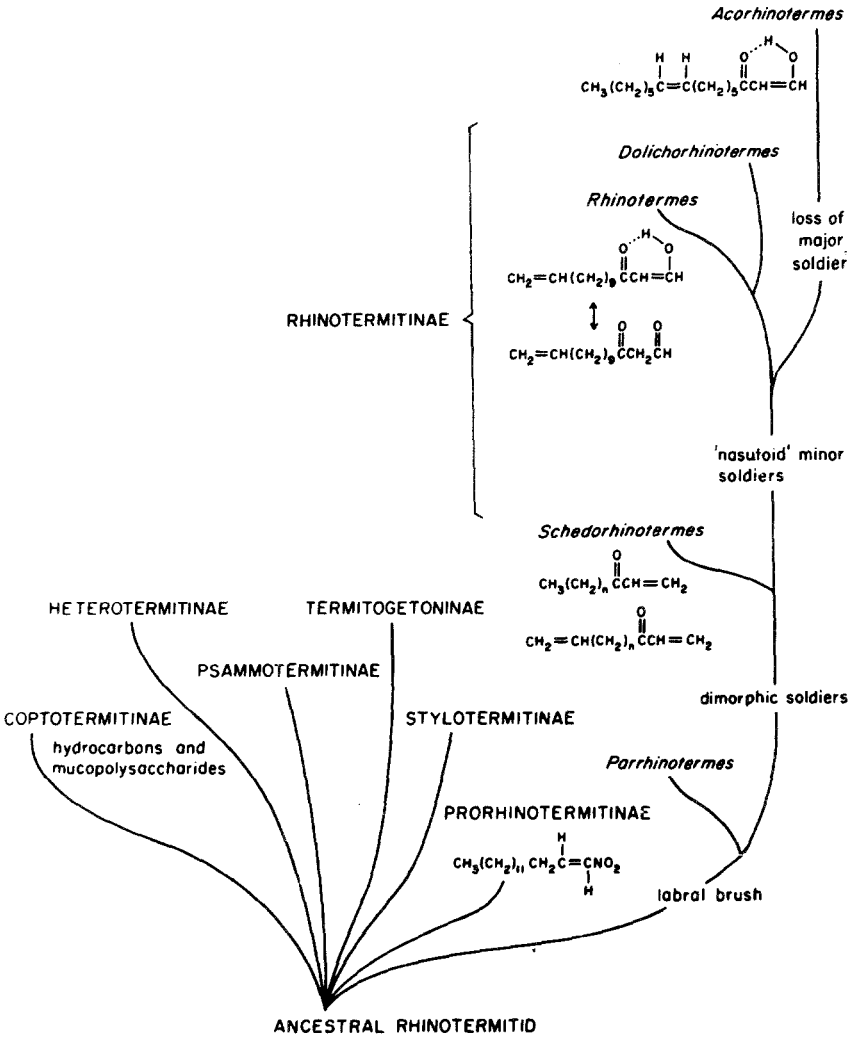

FIG. 8. Evolutionary relationships of the termite family Rhinotermitidae, with emphasis on the chemical evolution in the subfamily Rhinotermitinae (redrawn from Quennedey and Deligne, 1975).

vinyl ketones and nitroolefins have shown this to be a reasonable hypothesis (Spanton and Prestwich, 1981). The high reactivity of the β-ketoaldehydes with respect to self-condensation and to addition of nucleophilic RNH_2 and RSH compounds provides tentative support for the increased toxicity of this defensive secretion.

The major soldier caste has undergone a rapid rise and fall in this

subfamily. In *Schedorhinotermes* spp., major soldiers and minor soldiers are armed with the same chemical defense secretion, with the major soldiers possessing 1000 μg (13% fresh weight) and minor soldiers 200 μg (10.4% fresh weight) of secretion per soldier (Prestwich et al., 1975). In *Rhinotermes* spp., the major soldiers lack the labral brush and secretion, while minor soldiers feature elongate labral brushes and up to 600 μg per soldier. In *Acorhinotermes subfusciceps*, the major soldier caste has been lost entirely.

Soldier termites of *A. subfusciceps* exhibit an alarm behavior which we believe to be unprecedented in termites. During breakup of nest material, about 10% of all individual soldiers collected with forceps were carrying eggs or larvae in their tiny mandibles, thereby providing an effective chemical shield (the labrum) hanging over the surface of their otherwise defenseless charges.

Finally, we feel that the progression from nitroolefins to vinyl ketones to β-ketoaldehydes reflects an evolutionary trend toward increased enzyme parsimony. First, more defense secretion-unique enzymic processes are required in the production of a mixture of vinyl ketones than in the biosynthesis of 3-keto-(*Z*)-9-hexadecenal. Second, the primitive nitroolefin as an electrophilic reagent is wasteful of organic nitrogen, a limiting resource for termites and other xylophagous organisms (Prestwich et al., 1980). The development of lipophilic all-carbon electrophiles would appear to be a key biochemical event which allowed the subsequent diverse evolution of the subfamily Rhinotermitinae.

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(*Z*)-11-EICOSEN-1-OL, AN IMPORTANT NEW PHEROMONAL COMPONENT FROM THE STING OF THE HONEY BEE, *Apis mellifera* L. (HYMENOPTERA, APIDAE.)

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Abstract—(*Z*)-11-Eicosen-1-ol was identified by GC-MS and microchemical methods as a major volatile component, ca. 5 μg per insect, secreted by the sting apparatus of the worker honey bee. When presented on moving lures at the hive entrance, (*Z*)-11-eicosen-1-ol, like isopentyl acetate already known as an alarm pheromone, elicited stinging, and together these two compounds were as active as the natural pheromone from the sting. On stationary lures, (*Z*)-11-eicosen-1-ol prolonged the effectiveness of isopentyl acetate.

Key Words—Honey bee, *Apis mellifera*, Hymenoptera, Apidae, pheromone, (*Z*)-11-eicosen-1-ol, sting, EAG, alarm pheromone, mass spectrometry, epoxides.

INTRODUCTION

A synthetic mixture of the Nasonov pheromone of the honey bee (Pickett et al., 1980) attracts foraging honey bees as well as equivalent amounts of natural pheromone (Williams et al., 1981). However, the electroantennographic (EAG) response to the natural extract is greater than that to an equivalent amount of synthetic pheromone (Williams et al., 1982), suggesting that the natural extract was contaminated by components of other pheromones. The sting is close to the Nasonov gland and contamination of Nasonov secretion extracts by low-molecular-weight components from the sting does occur (Pickett et al., 1980). In this work such components with higher molecular weight were sought, without assuming that they were from the sting.

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METHODS AND MATERIALS

Extraction and Fractionation. Worker honey bees (300) were killed by chilling at −10° C and extracted with hexane (2 × 200 ml) for 3–4 min. The filtered extract was dried ($MgSO_4 \cdot H_2O$), concentrated (20 ml) at the pump, and placed on a column of Florisil (32 × 300 mm) in hexane. The column was eluted with hexane (825 ml), 20% diethyl ether (400 ml), and 50% diethyl ether (400 ml) in hexane, and portions of effluent (25 ml) were monitored by gas chromatography (OV-17). Fractions giving peaks were concentrated (250 μl) under vacuum and stored in glass ampoules under N_2.

Instrumental Analysis. Gas chromatography (GC) was by flame ionization (Pye 104) using glass columns, 5 ft × 0.25 in., 3% OV-17 or 2.5% OV-101, on Diatomite C AW DMCS, 80–100 at 200° C. Approximate amounts of (*Z*)-11-eicosen-1-ol were determined by comparison with peak areas from GC of standard solutions. Gas chromatography coupled with mass spectrometry (GC-MS) employed a glass capillary column, 50 m × 0.25 mm, wall coated with heat-treated Carbowax 20M (PhaseSep) at 200° C linked directly to the source of the mass spectrometer (MM 70-70F + Data System 2025, V.G. Micromass) with electron impact ionization at 70 eV, 200° C.

Chemical Analysis. Portions (10 μl) of the concentrated column effluent were treated with (1) acetic anhydride (2 μl), (2) *N*-trimethylsilylimidazole (Tri-Sil Z, Pierce, 2 μl), and (3) *m*-chloroperbenzoic acid (1 mg) in chloroform (30 μl) followed by evaporation to dryness under vacuum and extraction with hexane (10 μl). The products were analyzed by GC and GC-MS. Ozonolysis was by the method of Beroza and Bierl (1967) on concentrated total extract (20 honey bee equivalents) and analysis by GC (OV-101, 50° 10 min, 4°/min to 200° C).

Synthetic Compounds. Heneicosane, tricosane, pentacosane, (*Z*)-11-eicosen-1-ol, (*Z*)-9-octadecen-1-ol (oleyl alcohol), and (*E*)-9-octadecen-1-ol (elaidyl alcohol) were obtained from commercial sources and epoxides were prepared conventionally using *m*-chloroperbenzoic acid. Structures of alcohols and epoxides were confirmed by ^{13}C nuclear magnetic resonance (NMR) spectroscopy (Jeol-PFT-100), in $CDCl_3$, Me_4Si as standard ($\delta = 0.00$), or MS and purity was established (99+%) by GC. Isopentyl acetate (3-methylbutyl ethanoate 99.9%) was purchased uncontaminated with the 2-methyl isomer (Cambrian Chemicals).

Dissection of Honey Bees. Worker honey bees (20), killed by chilling at −10° C, were dissected into head, thorax, and abdomen, the legs were removed from the thorax, and the abdomen separated into the sting apparatus, gut, and anterior and posterior abdominal halves. Parts were placed in seven vials and crushed under hexane (400 μl). A portion (2 μl) of each extract was analyzed by GC for the presence of (*Z*)-11-eicosen-1-ol.

Stings pulled from the abdomens of freshly killed worker honey bees were dissected into two parts. Each part was crushed under hexane (20 μl) and a portion (2 μl) analyzed by GC for (*Z*)-11-eicosen-1-ol and, on occasion, for isopentyl acetate.

Electroantennography (EAG). The right antenna of a worker honey bee was excised at the proximal end of the scape and laid on a block of plasticine. A glass capillary Ag–AgCl microelectrode, filled with Pringle's saline (Pringle, 1938), was inserted into the cut end of the scape and earthed. A similar electrode, carried on a Bioelectricss PAD 1 probe, was inserted into the outer side of the second distal segment. With electrodes in position the total resistance at the input was 6–19 MΩ.

The test chemical was applied to the glass inside a Pasteur pipet positioned so that it terminated approximately 10 mm from the second segment of the antenna. The other end of the pipet was connected to a glass syringe (2 ml). To treat the antenna with the test chemical, air (1 ml) was ejected from the glass syringe into the Pasteur pipet after the solvent had evaporated. A standard chemical (geraniol 100 μg in 10 μl hexane) and a solvent blank (hexane 10 μl) were included in each test. Fractions of bee extract and synthetic chemicals were tested in ascending concentrations to minimize adaptation. Odors were extracted from the vicinity of the preparation by a fan.

The output from the probe and control unit was monitored with a preamplifier (Palmer, 8121) linked to an oscilloscope (Devices, 3121) and pen recorder (Devices, M2).

Mechanical and chemical stimulation resulted in a negative-going EAG; the activity of a compound was assessed on the amplitude (in mV) of the response. Each series of compounds was tested on five antennae.

Bioassay for Alarm Activity. Two moving lures, each a cylinder of cotton wool (dental roll 40 mm long, 10 mm diameter) wrapped in soft leather, were suspended 200 mm apart by nylon line from the cross-bar of a T-piece of wood.

Solutions of (*Z*)-11-eicosen-1-ol, isopentyl acetate, (*E*)-9-octadecen-1-ol, and (*Z*)-9-octadecen-1-ol (5 μg, 50 μg, 500 μg, or 5000 μg, in 50 μl hexane) or hexane alone (50 μl) were applied to the lures. A sting contains about 5 μg isopentyl acetate (Boch and Shearer, 1966) and about 5 μg (*Z*)-11-eicosen-1-ol. Mixtures of solutions of these chemicals were also compared with single stings in lures, obtained by pressing the abdomen by a honey bee, captured at the hive entrance, against a lure. Within 30–40 sec of application of test chemicals or stings to the lures, they were jerked approximately 50 mm from the entrance to a hive, until one of the lures was stung by a honey bee. Two tests were conducted at each hive entrance, reversing the positions of experimental and control lures and six hives were used in turn. A minimum of

10 and a maximum of 30 tests were made for each experiment until a result was significant ($P < 0.05$). Tests were conducted between July and September when bees were foraging.

Three stationary lures, similar to those described above but not wrapped in leather so that stings did not become embedded, were used in each experiment. They were impaled upright in a line on drawing pins (100 mm apart) attached to a rectangular glass plate. Approximately 30 sec after application of chemicals to the lures, the plate with lures was placed on the alighting board at the entrance to a hive so that the lures were 50 mm from the entrance. Bees on lures and within 50 mm of lures were counted each minute for 10 min after presentation. Nine replicates of each test were made, using three colonies of bees. In each of the three tests on a colony, lure positions were varied to avoid bias from preference of the bees for one part of the entrance when leaving and entering. A colony was not used more than once in 30 min.

RESULTS AND DISCUSSION

Fractions of honey bee extract that gave peaks coincident with those in chromatograms of the total extract were: hexane, 175–200 ml (fraction A), 200–225 ml (fraction B), 225–250 ml (fraction C), and 50% ether in hexane

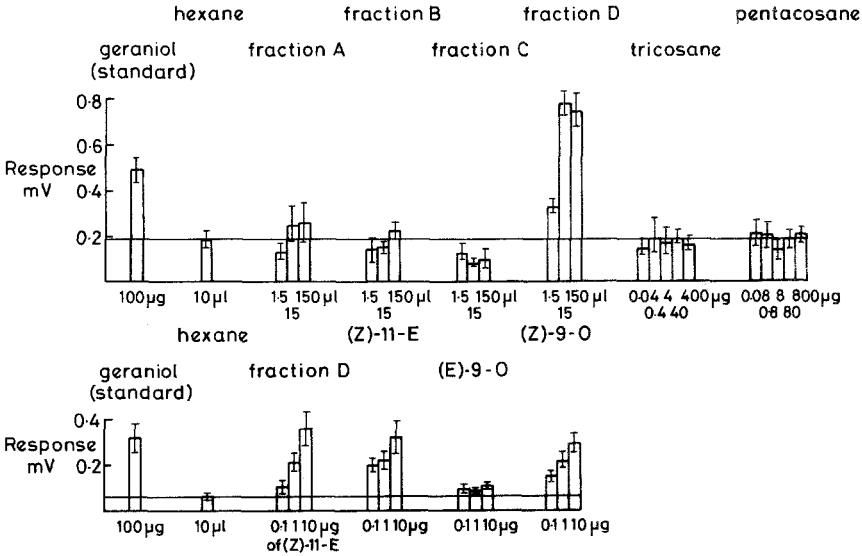
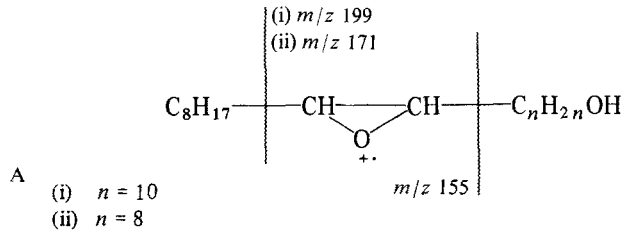


FIG. 1. Antennal (EAG) responses of worker honey bees to fractions of worker bee washings, hydrocarbons, (*Z*)-11-eicosen-1-ol [(*Z*)-11-E] and related alcohols (*Z*)-9-octadecen-1-ol [(*Z*)-9-0] and (*E*)-9-octadecen-1-ol [(*E*)-9-0]. Bars denote SE of means.

(fraction D). Only fraction D gave a significant EAG response (Figure 1). Major peaks in fractions A, B, and C, identified by comparison with published mass spectra (Mass Spectrometry Data Centre, 1975) and by peak enhancement, were from heneicosane, tricosane, and pentacosane. Higher hydrocarbons with C_{27}, C_{29}, and C_{31} were identified by MS only. Synthetic tricosane and pentacosane were also inactive by EAG. Nasonov pheromone components, having relatively low retention times, were detected by GC methods described earlier (Pickett et al., 1980) and shown to elute with 20% ether (aldehydes) and 100% ether (alcohols).

Fraction D gave one major peak on GC-MS (30 min) with mass spectrum; *m/z* 296 (0.2%), 278 (8), 96(63), 83(50), 82(80), 81(46), 69(62), 67(50), 55(B), 41(63). The compound was therefore probably an alcohol, $C_{20}H_{39}OH$ (*m/z* 296, M^+; 278, $M^+ - H_2O$) with a carbon chain probably unbranched, because the spectrum was symmetrical, and with one degree of unsaturation. On treatment with chemical reagents new peaks were obtained from (1) the acetate, $C_{20}H_{39}OCO \cdot CH_3$, *m/z* 338 ($M^+$), (2) the trimethylsilyl ether, $C_{20}H_{39}OSiMe_3$, *m/z* 368 (M^+), and (3) the epoxide, $C_{20}H_{39}OH + O$, *m/z* 312 (M^+, 0.7) 199(12), 155(18), 95(57), 83(63), 82(64), 69(88), 68(44), 67(50), 55(B), 41(66).

The monoalcohol function was thus confirmed by esterification and ether formation, and formation of the epoxide established presence of a double bond in the carbon chain. Bierl-Leonhardt et al. (1980) have suggested that formation of epoxides is useful in locating the double-bond position in long-chain aldehydes and acetates. From the major fragments at *m/z* 199 and 155, the double-bond was tentatively placed at C-11 by mechanism A(i) which



A
(i) $n = 10$
(ii) $n = 8$

is analogous with the fragmentation of other epoxides (Budzikiewicz et al., 1967). Similar fragmentations, A(ii), were observed with epoxides of (*Z*)- and (*E*)-9-octadecen-1-ol (Table 1). It was therefore concluded that the parent compound was 11-eicosen-1-ol, probably (*Z*) from biochemical considerations. Authentic (*Z*)-11-eicosen-1-ol enhanced the peak from fraction D on all three columns when coinjected [(*E*)-11-eicosen-1-ol chromatographed earlier: R_t 29 min, cap. column] and gave a similar mass spectrum (Table 1) to natural material. In addition the two peaks formed on ozonolysis of both the

TABLE 1. NMR, MS, AND GC OF SYNTHETIC COMPOUNDS

	[^{13}C] NMR shift (-ppm)									MS (m/z)	GC capillary, column, R_t (min)
Carbon	1	2	3	4-9, 14-16	10,13	11,12	18	19	20		
(Z)-11-Eicosen-1-ol	62.4	32.7	25.8	29.6	27.2	129.7	31.9	22.6	14.1	296(M^+, 0.2%), 278(M^+-H_2O, 10), 96(68), 83(53), 82(86), 81(49), 69(64), 67(51), 55(B), 41(58).	30
Epoxide	63.0	32.8	25.8	29.5 27.8	26.6	57.3	31.9	22.7	14.1	312(M^+, 0.8%), 199(11), 155(21), 95(55), 83(62), 82(66), 69(78), 68(52), 67(49), 55(B), 41(59).	80
Carbon	1	2	3	4-7, 12-14	8,11	9,10	16	17	18		
(Z)-9-Octadecen-1-ol	62.9	32.8	25.8	29.7	27.2	129.9	32.0	22.8	14.1		15
Epoxide										284(M^+, 0.5%), 171(10), 155(12), 83(50), 82(53), 81(52), 69(86), 67(60), 55(B), 43(53), 41(68).	45
(E)-9-Octadecen-1-ol	63.0	32.8	25.8	29.5	32.6	130.4	31.9	22.7	14.1		14.5
Epoxide										284(M^+, 0.5%), 171(11), 155(13), 83(48), 82(52), 81(54), 69(88), 67(59), 55(B), 43(51), 41(65).	42

natural and synthetic compounds (i.e., nonanal and 11-hydroxyundecanal) were enhanced by coinjection. Epoxides and trimethylsilylethers from natural and synthetic material were enhanced by coinjection on the three columns, and mass spectra of the epoxides from natural and synthetic materials (Table 1) were similar. The major component of the EAG active fraction D is therefore confirmed as (*Z*)-11-eicosen-1-ol.

Figure 1 compares EAG responses to fraction D with those to synthetic (*Z*)-11-eicosen-1-ol [(*Z*)-11-E], and the related alcohols (*Z*)-9-octadecen-1-ol [(*Z*)-9-0] and (*E*)-9-octadecen-1-ol [(*E*)-9-0]. Responses to fraction D and to (*Z*)-11-eicosen-1-ol were similar. The homolog (*Z*)-9-octadecen-1-ol was also very active, but there was no response to the (*E*) isomer. This difference in EAG response to the (*Z*) and (*E*) isomers of 9-octadecen-1-ol and the lack of response to hydrocarbons which were presumably from the cuticle suggested that (*Z*)-11-eicosen-1-ol was acting pheromonally rather than as a structural component despite the large amount present (ca. 5 μg per insect).

Extracts of Nasonov glands isolated by the methods reported earlier (Pickett *et al.*, 1980) contained a smaller proportion of (*Z*)-11-eicosen-1-ol to hydrocarbons than the total honey bee extract, and (*Z*)-11-eicosen-1-ol could therefore not have arisen in the Nasonov gland.

GC analysis of parts of bees to locate the origin of (*Z*)-11-eicosen-1-ol showed that 98% of the total amount present was from the sting apparatus. The remainder was mainly on posterior abdomens (0.5%), legs (0.5%), and thoraces (0.3%) with only traces on heads and anterior abdomens.

Analysis of parts of stings showed that (*Z*)-11-eicosen-1-ol was present in the dorsal anterior parts, associated with the quadrate plates (Table 2). The mean amount was 5.79 ± 0.58 μg/sting ($N = 45$). The traces of (*Z*)-11-eicosen-1-ol found in extracts of Dufours' glands and venom glands and sacs probably resulted from contamination during dissection.

Isopentyl acetate, known to be a major component of the sting alarm pheromone (Boch et al., 1962) was found (GC, OV-101, at 50°) in the dorsal anterior part of the string with (*Z*)-11-eicosen-1-ol (Table 2).

Because (*Z*)-11-eicosen-1-ol was found in the sting, with the already-known alarm pheromone isopentyl acetate, it was tested for alarm activity. Isopentyl acetate does not account for the activity of the natural sting pheromone in eliciting aggression. Although bees are alerted by, attracted to, and congregate around an introduced sting (Ghent and Gary, 1962) or isopentyl acetate on a stationary object (Boch et al., 1962), they used the additional stimulus of movement to attack (Ghent and Gary, 1962; Free and Simpson, 1968). However, Free and Simpson (1968) found that even when moving, objects containing stings were attacked more than objects containing isopentyl acetate, and Boch et al. (1970) found that the capacity of the sting extract to alert and attract bees at the hive entrance was 1.32 times greater

TABLE 2. AMOUNTS (APPROXIMATE) OF (*Z*)-11-EICOSEN-1-OL [(*Z*)-11-E] AND (*Z*)-11-E AND ISOPENTYL ACETATE (IPA) IN PARTS OF STING APPARATUS OF WORKER HONEY BEES

Part of sting	No. stings analyzed	(*Z*)-11-E (μg)	No. stings analyzed	(*Z*)-11-E (μg)	IPA (+ = present)
Dufour's gland	3	0.1	2	0.1	–
Rest of sting		4.5		7.5	+
Venom gland	3	0.0	–		
Rest of sting		8.6			
Venom gland and sac	3	0.1	2	0.0	–
Rest of sting		2.8		6.6	+
Koshewnikow glands	5	0.0	3	0.0	–
Rest of sting		6.3		10.4	+
Quadrate plates and associated glands	6	0.9	3	1.1	–
Rest of sting		3.5		3.9	+
Ventral half	3	0.3	2	0.3	–
Dorsal half		3.2		8.6	+
Anterior half	3	3.8	2	0.5	–
Posterior half		0.1		4.1	+

than the capacity of identical concentrations of isopentyl acetate. Both concluded that other components of the sting extract apparently contribute to its activity. (*Z*)-11-Eicosen-1-ol and related chemicals were therefore tested on both moving and stationary lures and their activity compared with that of isopentyl acetate and natural strings.

When tested alone on moving lures at the hive entrance, both (*Z*)-11-eicosen-1-ol and isopentyl acetate elicited more stings than hexane control lures, whereas (*Z*)- and (*E*)-9-octadecen-1-ol did not. When tested together, (*Z*)-11-eicosen-1-ol and isopentyl acetate elicited more stings than isopentyl acetate alone but the addition of (*Z*)- or (*E*)-9-octadecen-1-ol to isopentyl acetate did not enhance its activity (Table 3). Thus, although the homolog (*Z*)-9-octodecen-1-ol gave a similar EAG response to (*Z*)-11-eicosen-1-ol, presumably because of its similar structural configuration, it was behaviorally inactive. Moving lures with isopentyl acetate only were stung within 19.1 ± 2.6 sec of presentation, lures with (*Z*)-11-eicosen-1-ol only within 36.8 ± 5.2 sec, and lures with both isopentyl acetate and (*Z*)-11-eicosen-1-ol within 11.0 ± 1.1 sec. Thus the addition of (*Z*)-11-eicosen-1-ol to isopentyl acetate also increased the speed of the aggressive response.

One sting equivalent of a synthetic mixture of (*Z*)-11-eicosen-1-ol and isopentyl acetate (5 μg of each) was as effective as the natural sting in eliciting stings. Ten sting equivalents of the synthetic mixture elicited more stings (although the difference was not statistically significant), and 100 sting equivalents of the synthetic mixture elicited significantly more stings than a natural sting (Table 3). Thus, when presented on moving lures (*Z*)-11-eicosen-1-ol and isopentyl acetate together were as active as the natural pheromone from the sting in eliciting stinging attacks.

When tested alone on stationary lures at the hive entrance (*Z*)-11-eicosen-1-ol, unlike isopentyl acetate, did not attract more bees close to or onto lures than hexane (Figure 2). This is the only qualitative difference observed to be elicited by the two chemicals. However, on moving lures, (*Z*)-11-eicosen-1-ol, like isopentyl acetate, elicited more stings than hexane. Therefore both chemicals appear to need the additional stimulus of movement to elicit attack. Lures with (*Z*)-11-eicosen-1-ol and isopentyl acetate together remained attractive for longer than lures with isopentyl acetate alone (Figure 3). By contrast, (*Z*) or (*E*)-9-octadecen-1-ol did not prolong the attractiveness of isopentyl acetate when added to it (Figure 4). The main effect of (*Z*)-11-eicosen-1-ol was therefore to prolong the activity of the more volatile isopentyl acetate presumably by synergizing the activity of the small amount of isopentyl acetate remaining. Similarly, stings of *Apis florea* and *Apis dorsata* contain, in addition to isopentyl acetate, 2-decen-1-yl acetate, which is less volatile and enables the stung object to be recognized for a longer time (Koeniger et al., 1979).

TABLE 3. EFFECT OF (*Z*)-11-EICOSEN-1-OL [(*Z*)-11-E], ISOPENTYL ACETATE (IPA), (*Z*)-9-OCTADECEN-1-OL [(*Z*)-9-O], (*E*)-9-OCTADECEN-1-OL [(*E*)-9-O], AND MIXTURES, ON MOVING LURES IN ELICITING STINGING.

Test chemicals applied to lures (in 50 μl hexane)		No. of tests in which lure was stung first			
Experimental lure	Control lure	Experimental lure I	Control lure I	Experimental lure II	Control lure II
(*Z*)-11-E (500 μg)	Hexane	20[a]	10	18[a]	7
(*Z*)-11-E (5000 μg)	Hexane	12[a]	3	10[a]	0
IPA (500 μg)	Hexane	10[a]	2	19[a]	8
IPA (5000 μg)	Hexane	15[a]	5	9[a]	1
(*Z*)-9-O (500 μg)	Hexane	16	14		
(*Z*)-9-O (5000 μg)	Hexane	14	16		
(*E*)-9-O (500 μg)	Hexane	15	15		
(*E*)-9-O (5000 μg)	Hexane	12	18		
(*Z*)-11-E (500 μg) + IPA (500 μg)	IPA (500 μg)	9[a]	1	18[a]	7
(*Z*)-11-E (5000 μg) + IPA (5000 μg)	IPA (5000 μg)	10[a]	2	9[a]	1
(*Z*)-9-O (500 μg) + IPA (500 μg)	IPA (500 μg)	18	12		
(*Z*)-9-O (5000 μg) + IPA (5000 μg)	IPA (5000 μg)	17	13		
(*E*)-9-O (500 μg) + IPA (500 μg)	IPA (500 μg)	18	12		
(*E*)-9-O (5000 μg) + IPA (5000 μg)	IPA (5000 μg)	16	14		
None	Single sting	5	16[a]	3	12[a]
(*Z*)-11-E (5 μg) + IPA (5 μg)	Single sting	16	14	15	15
(*Z*)-11-E (50 μg) + IPA (50 μg)	Single sting	17	13	17	13
(*Z*)-11-E (500 μg) + IPA (500 μg)	Single sting	19[a]	9	12[a]	3

[a]Significant at $P < 0.05$.

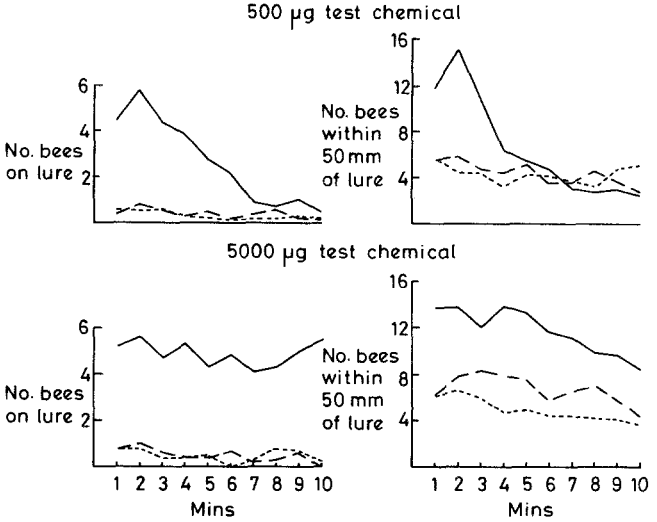


FIG 2. Attraction of honey bees onto and within 50 mm of stationary lures dosed with isopentyl acetate (—), (*Z*)-11-eicosen-1-ol (– –), and hexane (----) during 10 min after presentation at hive entrance (mean of 9 tests).

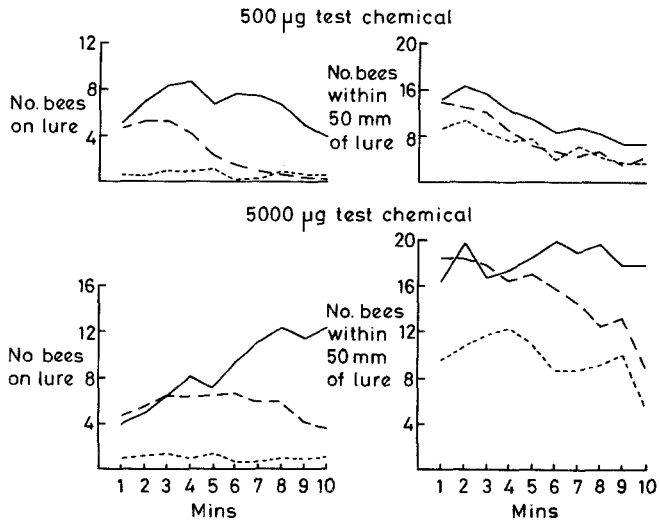


FIG. 3. Attraction of honey bees onto and within 50 mm of stationary lures dosed with isopentyl acetate and (*Z*)-11-eicosen-1-ol together (—), isopentyl acetate (– –), and hexane (----) during 10 min after presentation at hive entrance (mean of 9 tests).

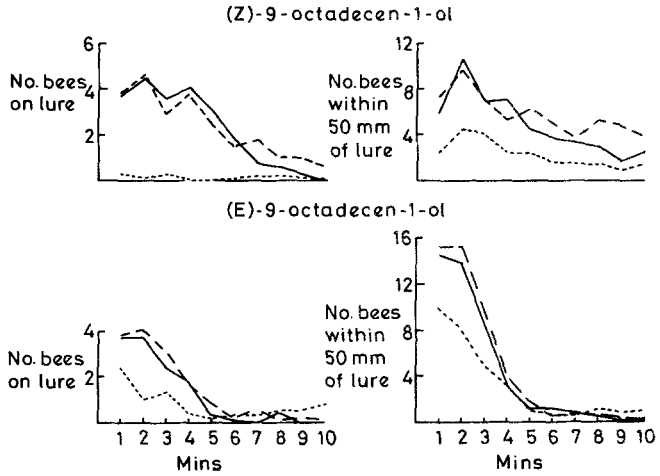


FIG. 4. Attraction of honey bees onto and within 50 mm of stationary lures dosed with 500 μg isopentylacetate and 500 μg (Z)-9-octadecen-1-ol or 500 μg (E)-9-octadecen-1-ol(—), 500 μg isopentylacetate (----) and 10 μl hexane (----) during 10 min after presentation at hive entrance (mean of 9 tests).

Blum et al. (1978) identified a number of other compounds accompanying isopentyl acetate in the sting apparatus, but did not investigate their pheromonal action. They did not identify (Z)-11-eicosen-1-ol as a component, and only lower-molecular weight oxygenated compounds were observed. We believe this to be the first report of (Z)-11-eicosen-1-ol as a pheromone component, although the acetate is a major component of the defensive secretion of larvae of the chrysomelid *Gastrophysa atroceana* Motschulsky (Sugawara et al., 1978) and an unspecified isomer of eicosen-1-ol has been reported in the cephalic marking secretion of the male bumble bee, *Psithyrus campestris* Panzer (Kullenberg et al., 1970).

Although a number of other compounds are known to be present, isopentyl acetate and the newly identified (Z)-11-eicosen-1-ol account fully for the activity of the natural sting pheromone. The roles of the two compounds in the pheromone appear to differ with (Z)-11-eicosen-1-ol responsible for prolonging the activity of isopentyl acetate.

Acknowledgment—The authors thank N.F. Janes for NMR spectra.

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VOLATILE COMPONENTS OF THE OCCIPITAL GLAND SECRETION OF THE BACTRIAN CAMEL (*Camelus bactrianus*)

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Abstract—The male Bactrian camel's occipital scent gland produces a series of steroids including 5α-androst-16-en-3-one in addition to a series of fatty acids and γ-dodecalactone. 3-Methylbutanoic acid is the most volatile of the acids which include hexanoic, a decenoic, and the saturated acids from C_{15} to C_{25} with the exception of C_{24}. The constitution of this secretion changes appreciably with the season.

Key Words—Bactrian camel, *Camelus bactrianus*, occipital gland, 5α-androst-16-en-3-one, γ-dodecalactone, 3-methylbutanoic acid.

INTRODUCTION

The male Bactrian camel (*Camelus bactrianus*) possesses an occipital scent gland which secretes profusely during the winter rutting period. This gland, absent in the female, is a paired macroscopically discrete structure overlying the occipital bone of the skull and is composed of glandular lobules containing tubuloalveolar cells imbedded in a fibrous stroma. Secretory ducts from the gland open into the necks of hair follicles. The follicles are clustered, producing discrete wicklike bundles of guard hairs. The thick wooly undercoat characteristic of Bactrian camels is absent from the occipital gland. The secretion, which has a dark brown color, watery consistency, and heavy, somewhat sweet aroma saturates the long nape hair of sexually mature animals and is used to scent mark the hump and objects in the environment (Wemmer and Murtaugh, 1980). The social significance of this odiferous secretion prompted us to determine its chemical constitution.

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METHODS AND MATERIALS

Hair samples (1 cm^2 from the surface of the gland) were clipped originally from six different animals, two in January of 1978 and the other four in mid-March of 1979 at Front Royal, Virginia. Secretion was later monitored monthly in three animals (1980). The production and total amount of secretion varied among individuals and appeared to vary with the individuals' state of excitement. Individual samples were shipped on ice in sealed containers to Washington, D.C., by truck for analysis.

The hair samples were rinsed several times with Burdick and Jackson methylene chloride and, after concentration to a standard volume, the extracts were analyzed directly by gas chromatography–mass spectroscopy. A Finnigan 3200E computerized gas chromatograph–mass spectrometer utilizing a 1.6 m 3% OV-17 on Supelcoport 60/80 column, temperature programed from 60 to 300°C at 10°/min, was used. Individual compounds were identified by comparison of their mass spectra and retention times with those of standard compounds as well as those of their corresponding methyl esters for the acids.

RESULTS

Even though all samples were obtained from male camels, the composition of volatiles varied widely (Table 1). Samples taken in March of 1979 were much richer in low-molecular-weight compounds while still containing the same high-molecular-weight compounds found in the samples of 1978.

3-Methylbutanoic acid (Figure 1, compound 1) is the most volatile constituent, but it is not consistently present in all samples. No other low-molecular-weight acids were detected even as their methyl esters. Other acids were present including hexanoic (compound 2), a decenoic (compound 3), and the saturated acids from C_{15} to C_{25} (5) with the exception of C_{24}. Only two of the samples contained γ-dodecalactone (compound 4) as the major constituent (Figure 1).

Surprisingly, the samples examined from the winter of 1978 had none of the compounds mentioned above but five different compounds (Figure 2). Closer examination of the 1979 samples indicated these five compounds were present but at low levels compared to the other components when equivalent volumes of extract were examined under identical conditions. The mass spectrum of each of the five compounds was complex, and their molecular ions suggested that these compounds have high indices of hydrogen deficiency. Catalytic hydrogenation of the mixture did not change the spectrum of compound C, the molecular ion remaining at m/z 388. The general appearance of the mass spectrum suggested a polycyclic steroidal skeleton

TABLE 1. OCCIPITAL GLAND COMPONENTS

	Camel (Nat. Zoo ID #)[a]					
	1 (101,451)	2 (101,079)	3 (102,170)	4 (101,171)	5 (101,619)	6 (101,456)
Compound						
3-Methylbutanoic acid				x		x
Hexanoic acid				x	x	x
Decenoic acid				x	x	x
Pentadecanoic acid			x			x
Hexadecanoic acid			x			x
Heptadecanoic acid						x
Octadecanoic acid			x		x	x
Nonadecanoic acid						x
Eicosanoic acid						x
Heneicosanoic acid						x
Docosanoic acid						x
Tricosanoic acid						x
Pentacosanoic acid						x
γ-Dodecalactone				x		x
5α-Androst-16-en-3-one (compound A)	x	x	x		x	x
Cholestene (compound B)	x	x	x	x	x	x
Cholestadiene			x	x	x	x
Dihydrocholesterol (compound C)	x	x	x	x	x	x
386 Steroid (compound D)	x	x	x	x	x	x
412 Steroid (compound E)	x	x				

[a]Collection dates: camels 1 and 2, January 7, 1978; Camels 3–6, March 16, 1979.

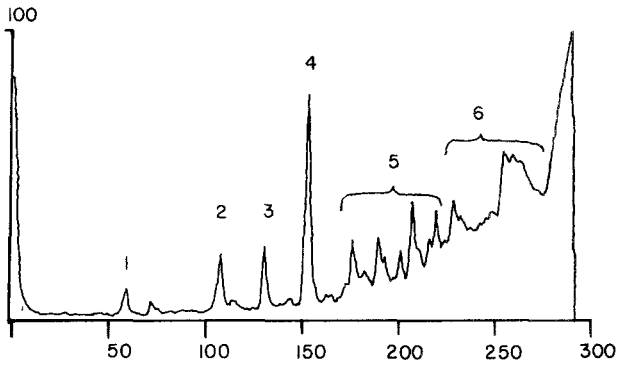


FIG. 1. Gas chromatogram of occipital gland secretion of March 1979. Compounds indicated are: 1 = 3-methylbutanoic acid, 2 = hexanoic acid, 3 = decenoic acid, 4 = γ-dodecalactone, 5 = acids C_{15}–C_{25} (excluding C_{24}), 6 = steroids. (Drawing from the original.)

with a molecular weight two mass units higher than cholesterol. The abundance of m/z 149 over m/z 151 is diagnostic of a 5α steroid having a side chain at C-17 (Tokes and Amos, 1972). Comparison of retention times (peak enhancement) and mass spectra with an authentic sample established the identity of compound C as dihydrocholesterol.

Compound A also appeared to be steroidal, but had a molecular weight of 272. The androstane skeleton (mol wt 260) will accommodate this with two sites of unsaturation and an oxygen. The absence of abundant ions at m/z 215 and 216 in compound A suggested (Mammato and Eadon, 1975) that no oxygenated function was present in ring D, and a peak at m/z 163 indicated a carbonyl function in either ring A or B.

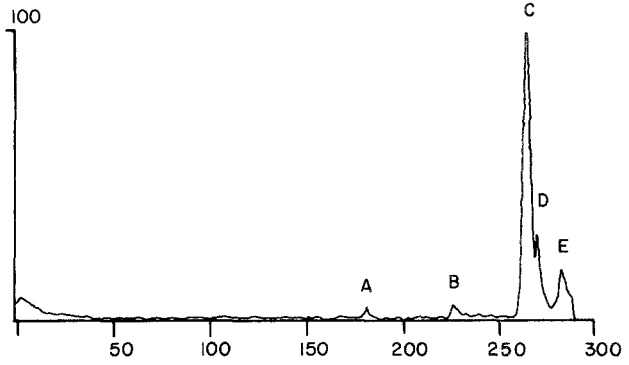
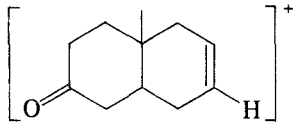
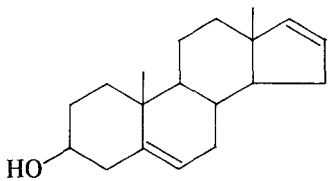


FIG. 2. Gas chromatogram of occipital gland secretion of January 1978. Compounds indicated are: A = 5α-androst-16-en-3-one, B = cholestene, C = dihydrocholesterol, D = 386-mol-wt steroid, E = 412-mol-wt steroid. (Drawing from the original.)

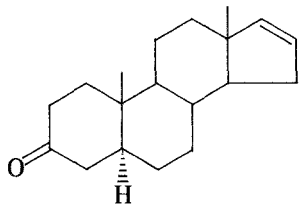


m/z 163

A carbonyl group in either ring A or B requires that a double bond be placed in ring C or D. Significant loss of methyl to *m/z* 257 indicated a possible facile allylic methyl cleavage. The mass spectrum (Spiteller, 1969) of androst-16-en-3-β-ol has an abundant *m*-15 supporting this contention. Loss of water and a methyl group results in an abundant ion at *m/z* 241 (70%) in this alcohol. Compound A has a corresponding ion at *m/z* 239 of much lower intensity (10%), further suggesting the possibility of a carbonyl group in ring A. The mass spectrum of the readily available dienol 7 lent further support to these arguments.



7



A

Comparison of retention times (peak enhancement) and mass spectra of commercially available 5α-androst-16-en-3-one with those of our compound A showed that they were identical. This compound and related alcohols have been isolated previously from urine and fat specimens of swine and boar (Albone, 1977; Sink, 1967) and are associated with sex odors in these animals. The ketone has recently been isolated from human sweat (Claus and Alsing, 1976).

The other three compounds are also steroidal, but their specific structures are unknown. Compound B (mol wt 370) is a cholestene, but the position of the double bond is unknown. Compound D (mol wt 386) is possibly an isomer of cholesterol (but not cholesterol itself) and the structure of compound E (mol wt 412) is unknown. Cholestadiene was detected in the March 1979 samples but was absent in the 1978 samples. Extracts of hair samples from other parts of the body run as controls showed neither these steroids nor the acidic components mentioned earlier. Androgens have recently been measured in *Camelus dromedarius* secretions from the back of the neck (Yagil and Etzion, 1980), but the specific steroids have not been identified.

TABLE 2. VARIATION OF OCCIPITAL GLAND COMPONENTS WITH SEASON

	Camel 1					Tex					Alfred				
Compound	Jan	Feb	Mar	Apr	May	Jan	Feb	Mar	Apr	May	Jan	Feb	Mar	Apr	May
3-Methylbutanoic acid			x			x	x					x	x		
Hexanoic acid			x			x	x					x	x		x
Hexadecanoic acid			x				x					x	x	x	x
Octadecanoic acid			x				x					x	x	x	x
5α-Androst-16-en-3-one						x		x				x	?		
Cholestene (compound B)		x			x			x		x		x			x
Dihydrocholesterol (compound C)			x		x	x		x		x	x	x	x		x

The composition and relative amount of secretion varies greatly as seen in Table 2. The amount of 5α-androst-16-en-3-one varied, but was as high as 100 μg for some camel samples. Further studies are in progress to determine how the production of steroids and/or acids changes in the course of the rutting season and the role of these chemicals in the behavior and reproduction of the camel. We are also examining the saliva and urine to determine whether these same steroids are present there.

Acknowledgments—The dienol 7 and 5α-androst-16-en-3-one were obtained from Steraloids Inc., Wilton, New York 03086. We (F.A., J.W.W.) thank the National Science Foundation (7514564) for their support of this research.

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POSSIBLE DUAL ROLES OF AN ALLELOPATHIC COMPOUND, *cis*-DEHYDROMATRICARIA ESTER[1]

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Abstract—The aqueous extract of the root of *Solidago altissima* was lethal to nematodes. The major compound isolated from the toxic fraction was *cis*-dehydromatricaria ester (methyl 2-decene-4,6,8-triynoate), which is already known as an allelopathic compound of a composite showing growth-inhibition to a rice seedling. This compound showed high toxicity to plant-parasite nematodes. A composite exudes one defending substance against both competitive plants and plant-parasite nematodes.

Key Words—Allelopathy, *cis*-dehydromatricaria ester, allomone, dual roles, nematicide, selective toxicity, plant-parasite nematode, free-living nematode.

INTRODUCTION

A goldenrod, *Solidago altissima* L. is an introduced plant in Japan and has been one of the dominant species in plant communities of Japan. It had spread from southern to northern Japan along the railroads since about 1950. Many elements contribute its dominance, i.e., climate, temperature, humidity, soil properties, propagating power of the plant, and allelopathic effects. Above all, the allelopathic effects have gotten much attention for their aggressiveness, and the cause of dominance of *S. altissima* is well investigated. Kawazu et al. (1969) reported that *cis*-dehydromatricaria ester (methyl 2-decene-4,6,8-triynoate) obtained from the root showed a growth-inhibitory effect to rice

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seedlings, and its effect on other plants was confirmed by Kobayashi et al. (1980). A similar effect was observed with dehydromatricaria lactone and methyl-10-(2-methyl-2-butenoyloxy)-*cis*-2,*cis*-8-decadiene-4,6-diynoate obtained from the stem (Ichihara et al., 1976, 1978). The existence of kolavenic acid in *S. altissima* was confirmed by Tsuji et al. (1977). On the other hand, Ueno and Iyatomi (1978) reported that the aqueous solution of root exudate of *S. altissima* showed nematicidal activity. The last report suggested that other compounds might be responsible for nematicidal activity or an allelopathic compound may show dual activities. Other compounds show plural activities, i.e., juglone for growth-inhibition and pesticidal properties (Bode, 1958; Gilbert and Norris, 1968) and solanine (Kuhn et al., 1955) and tomatine (Buhr et al., 1947; Kuhn et al., 1957) for predator-inhibition and antibiotic effects. However, few studies have been conducted from an ecological viewpoint.

In this study we investigated the nematicidal component of aqueous extract of root of *S. altissima* and showed that an allelopathic compound has possible dual roles thought to be important in ecosystems.

METHODS AND MATERIALS

Isolation of Nematicidal Compound. Wilted *S. altissima*, complete with roots, were collected at the campus of the University of Tsukuba, Sakura-mura, Ibaraki-ken in February 1979. Stems were removed. Roots (1 kg, wet weight) were washed, ground up, and soaked in 6 liters of water for 12 hr at room temperature (20° C). The root solution was then filtered and condensed to 1 liter in vacuum at 70° C and extracted three times with 4 liters of ether. The etheral solution was dried and evaporated. The residue (3.3 g) was separated into three fractions by silica gel column chromatography (Wakogel Q12). The first fraction (0.2 g) was obtained in an eluant of petroleum ether, the second fraction (2.0 g) in ether, and the third fraction (0.8 g) in methanol. Further purification was carried out by thin layer chromatography. Two grams of the second fraction were separated on a 0.5-mm thickness of silica gel thin layer (Merck 60 F254) which was developed in benzene. Five fractions were obtained in R_f 0.95 (54 mg), R_f 0.50 (120 mg), R_f 0.40 (98 mg), R_f 0.38 (94 mg), and R_f 0.05–0.0 (870 mg). The second fraction became a crystal at room temperature and was recrystallized with *n*-hexane to give 45 mg of yellow needles (mp 112° C).

Nematode Collections. A pine wilt nematode, *Bursaphelenchus lignicolus* Mamiya & Kiyohara, was obtained from a wilted pine tree by the method reported by Mamiya and Kiyohara (1972). Pine wood was collected in the pine forest near the university in December, 1979, where severe damage was observed that year by *B. lignicolus*. The nematode was extracted with water from the shavings of pine wood by the Baerman technique. The nematode was

reared on fungus culture, *Botrytis cinerea* Pers. *ex* Fr. The cultivated nematode included all stages from larva to adult. This nematode was also obtained from a Japanese pine sawyer, *Monochamus alternatus* Hops., the vector of *B. lignicolus*, as a dauer larva (wintering larva) by the same method.

A free-living nematode, *Diprogaster* sp. was obtained from fallen dead pine trees. This nematode collection also included all stages.

One sweet potato (*Ipomoea Batatas* Lam.) parasitized by a root-knot nematode, *Meloidogyne incognita* (Koflid & White) Chitwood was supplied by Mr. Tanaka of the Agricultural and Forestry Research Center of this university. The egg mass of the nematode was soaked in water to force hatching by the day preceding the bioassay. Second-stage larvae from the hatched egg-mass, collected by the Baerman technique, were used in the bioassay.

Bioassay. About a hundred nematodes were placed in a test tube containing 3 ml of sample solution. A sample solution was made up in two ways: crude extracts were dissolved in distilled water at 200 ppm; solutions of pure extracts were prepared at various concentrations for more specific tests. The former was used for all fractions to test whether they had nematicidal activity. The latter was used to decide mortality of nematodes. When materials were insoluble in water, another reagent (Tween 80) was added to disperse them. This reagent had no affect on nematodes even at a concentration of 14,000 ppm. After soaking nematodes in a sample solution, the test tube was left for 12 hr at 25° C. Then the nematodes were transferred to the glass slide with 0.3 ml of water. Mortality was measured by counting dead and living nematodes through a binocular microscope (40×). Whether a nematode was dead or alive was judged by visual observation, i.e., when physical stimuli did not induce movement of extended nematodes, we regarded them as dead.

RESULTS

Isolation and Structure of Nematicidal Compound. The isolation methods are summarized in Figure 1. The ether extract of the root showed nematicidal activity. It was separated into three fractions by column chromatography. The fraction eluted with ether was active (100% mortality at 200 ppm), and the other fractions were all inert (0% mortality at 200 ppm). The ether fraction was separated to five fractions by thin-layer column chromatography. Only the fraction in R_f 0.5 was active; the others were inert. This fraction was insoluble but dispersed in water by adding Tween-80 (materials were previously dispersed in 80 times their weight of Tween-80 and then dissolved in water). This fraction was almost pure and became a solid at room temperature. The recrystallization by *n*-hexane gave yellow needles (mp 112° C). The structure of this compound was confirmed as *cis*-dehydromatricaria ester by the spectral data listed in Table 1 and by referring to

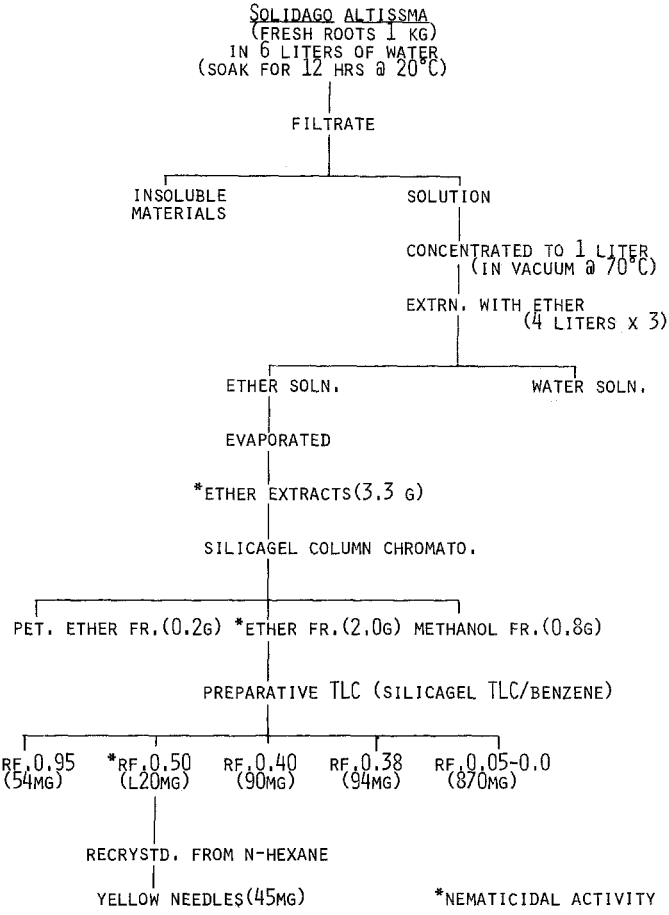


FIG. 1. Scheme of isolating nematicidal compounds.

TABLE 1. SPECTRAL DATA OF *cis*-DEHYDROMATICARIA ESTER[a]

	Value
Melting point	112°C
IR, ν cm (KBr tablet)	2210, 2160, 1707, 1592, 1435, 1235, 1192, 995, 810
NMR, δ ppm ($CDCl_3$)	2.04 (singlet, 3H) 3.82 (singlet, 3H) 6.16 (doublet, J = 12Hz, 1H) 6.32 (doublet, J = 12, 1H)
UV, λ nm (*n*-hexane)	245 (ϵ: 1.16 × 10^5), 256 (1.32 × 10^5), 286 (1.60 × 10^4), 304 (3.38 × 10^4) 324 (4.96 × 10^4), 348 (4.35 × 10^4),
Mass	172 (M^+, 100%), 141, 113, 101

[a] $CH_3-(C\equiv C)_3-C=C(H)-COOCH_3$
cis-dehydromatricaria ester

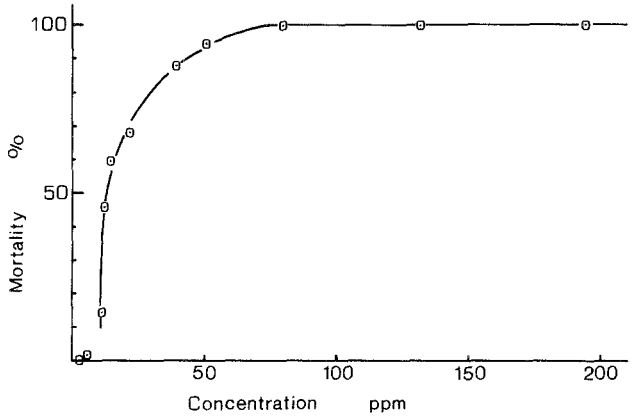


FIG. 2. Nematicidal activity of DME to *B. lignicolus*.

reported data (Stavholt and Sorensen, 1950; Kobayashi et al., 1980). In all tests for isolating the nematicidal component, only *B. lignicolus* was used for bioassay.

Nematicidal Activity. The nematicidal activity of *cis*-dehydromatricaria ester (DME) was measured as shown in Figure 2, and the value of LC_{50} (lethal concentration at 50% mortality) was obtained at 11 ppm. The activity of DME was compared with the general nematicides by the values of LC_{50} which were obtained for three species of nematodes. The results are summarized on Table 2. *B. lignicolus* and *M. incognita* are plant-parasite nematodes and *Diprogaster* sp. is a free-living nematode.

TABLE 2. NEMATICIDAL ACTIVITY OF DME AND SYNTHETIC PESTICIDES[a]

	LC_{50} (ppm)				
	DME	D–D	DCIP	MEP	NH_3
Bursaphelenchus lignicolus	11	250	320	200	80
Meloidogyne incognita	10	360	450	85	95
Diprogaster	70	60	550	60	18

[a]D–D: a mixture of 1,3-dichloropropene and 1,3-dichloropropane. DCIP: di-3-chloroisopropyl ether. MEP: *O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) thiophosphate.

DISCUSSION

The nematicidal activity of DME was determined as the value of LC_{50} at 11 ppm (Figure 2). However, the net LC_{50} of DME was considered to be less and the net nematicidal activity to be stronger than observed in this assay, because the values were obtained in the dispersed conditions and not in the dissolved ones.

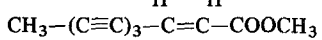
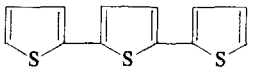
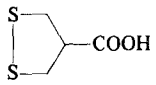
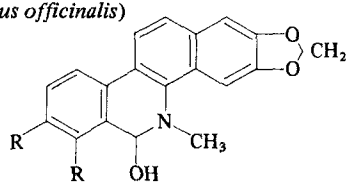
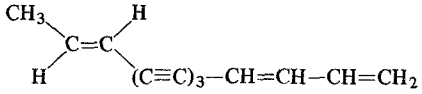
The strong activity of DME was observed by comparison with synthetic nematicides in the soaked system. DME affected plant-parasite nematodes (*B. lignicolus* and *M. incognita*) at the low concentration of 11 ppm, and little difference was observed between *B. lignicolus* and *M. incognita*. A sevenfold lower toxicity by DME was observed with free-living nematodes (*Diprogaster* sp.). On the other hand, synthetic nematicides (D—D, DCIP) were more lethal to the free-living than to the plant-parasite nematodes. DME had highly selective toxicity to the nematodes compared with synthetic ones; it showed stronger activity (8–45 times greater) on plant-parasite nematodes than the synthetic nematicides.

The present results were compared with those of other naturally occurring compounds derived from plants (Table 3). The compounds could not be compared directly by the values of LC_{50}, as the values were obtained under different conditions, i.e., different nematode species, soaking periods, and bioassay temperatures. Even considering these differences, DME can be regarded as a strong nematicide, comparable with tert-thienyl.

In this work we demonstrated in vitro the possible ecological roles of DME. The selective toxicity of DME would benefit *S. altissima* in two ways. First, plant-parasite nematodes infect many types of plants, and DME has a strong lethal effect on them. Second, free-living nematodes are considered to play a role of improving soil properties for higher plants (Ishibashi, 1978), and DME shows weak lethal effects toward them. Regarding the first point, the French marigold, *Tagetes patula* L. (Uhlenbroek and Bijloo, 1975), for example, has been known to reduce nematode populations, presumably due to tert-thienyl's defending nature of mother plants from nematodes. Similar effects can be expected with DME and benefits are quite possible, even though the relationship between the three species of nematodes employed in the present work and *S. altissima* remains obscure in natural ecosystems.

Two reports concerned with the allelopathic effects of DME interested us in view of intra- and interspecific effects. Kawazu et al. (1969) reported that DME showed a growth-inhibitory effect to rice seedlings, which is an interspecific effect. Kobayashi et al. (1974, 1980) reported that DME was germination-inhibitory to their competitive plants and also to *S. altissima* itself. This self-inhibitory effect can be considered beneficial in intraspecific interactions, i.e., preventing overcrowding and the resulting weakness

TABLE 3. NATURALLY OCCURRING NEMATICIDAL COMPOUNDS DERIVED FROM PLANTS

Nematicidal components in plants	LC_{50} (ppm)	Nematodes	Conditions	Source
cis-Dehydromatricaria ester  (*Solidago altissima*)	11	*Bursaphelenchus lignicolus*	20°C 24 hr	
a-Terthienyl  (*Tagetes patula*)	<12.5	*Anguina tritici*	6 days	Uhlenbroek and Bijloo (1958)
Asparagusic acid  (*Asparagus officinalis*)	<50	*Pratylenchus curvitatus*	25°C 145 hr	Takasugi et al (1975)
Chelerythrine Sanguinarine Bocconine  (*Macleya cordata*)	<50 100-150 100-150	*Rhabditis* sp.	27°C 24 hr	Onda et al. (1965)
3-*cis*,11-*trans*-trideca-1,3,11-triene-5,7,9-triyne 3-*trans*, –  (*Carthamus tinctorius*)	10 1.5	*Aphelenchoides besseyi*	25°C 24 hr	Kogiso et al. (1976)

induced by the lack of nutrients. In this way, DME helps maintain the competitive advantage of a stronger mother plant of *S. altissima* in both intra- and interspecific interactions.

The effects of allelopathic compounds are well known to be inhibitory to growth of plants, antibiotic, nematicidal, and inhibitory to predation by hervibores. However, the roles of allelopathic compounds in ecosystems are still obscure as research has progressed with only isolated components of the biotic communities. As a result, only one role has often been identified for one allelopathic compound, although other roles might exist. The present work made it clear that one allelopathic compound plays possible dual roles for one species of plant. DME affects both intraspecific competitors of *S. altissima* and an interspecific competitor, more specifically a plant-parasitic nematode. We fully realize that in order to clarify the net effects of an allelopathic compound further research has to be conducted and approached from an interdisciplinary viewpoint.

Acknowledgments—We thank Associate Professor Kuwahara and Associate Professor Fujii of this university for their pertinent discussions. We also thank the late Professor Tatsumi for his encouragement.

The nematicidal activity of DME was reported by Kawazu et al. (1979, 1980) and Kimura et al. (1979) at the conference of the Agricultural Chemical Society of Japan, but this research was carried out independently of them and provides additional specific information on the action of the compound.

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SEX PHEROMONE OF THE EUROPEAN GRAPEVINE MOTH, *Lobesia botrana* Schiff. (Lepidoptera: Tortricidae): Synthesis and Effect of Isomeric Purity on Biological Activity

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Abstract—A short synthesis of (*E,Z*)-7,9-dodecadien-1-yl acetate from propargyl alcohol and 6-bromohexanol via acetylenic-allenic isomerization of the resulting bis-THP-1,9-non-2-yn-diol is described. The field test of several preparations showed that the *E,E* isomer does not interfere with the biological activity of the pheromone. It was found that the "crude" preparation has higher activity than purified pheromone or virgin females.

Key Words—(*E,Z*)-7,9-Dodecadien-1-yl acetate, European grapevine moth, *Lobesia botrana*, Lepidoptera; Tortricidae, sex pheromone, purification, biological activity, (*E,E*)-7,9-dodecadien-1-yl acetate.

INTRODUCTION

The European grapevine moth *Lobesia botrana* Schiff. (EGVM) is a serious pest of vineyards in Europe, Asia, and Africa. In Israel, EGVM is the major pest of vine and table grapes, and frequent insecticide treatments are needed throughout the season to control it. The sex pheromone of the EGVM was identified by Roelofs et al. (1973) as (*E,Z*)-7,9-dodecadien-1-yl acetate. It has since been used as bait in monitoring traps for the pest in vineyards. It has also been used in experiments on direct control of the moth with mass trapping and mating disruption techniques (Roehrich et al., 1979; Gurevitz and Gothilf, 1981). In view of the demand for EGVM synthetic pheromone in this country a new procedure for synthesis of this compound was worked out.

Several other multistep syntheses of the pheromone (VI) have already been published (Roelofs et al., 1975; Labovitz et al., 1975; Descoins et al., 1977; Negishi and Abramovitch, 1977; Bestmann et al., 1979; Dressaire and Langlois, 1980; Cassani et al., 1980; see also a review by Henrick, 1977).

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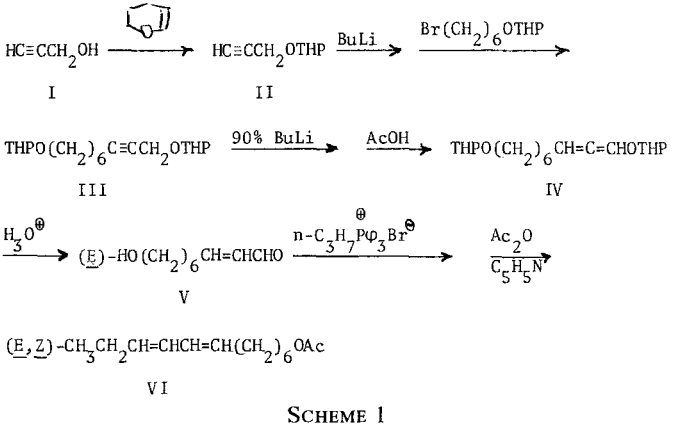
The Wittig reaction, although not completely stereoselective (Bestmann and Vostrovsky, 1979), may be used in the preparation of conjugated dienes. Field testing of the resulting stereoisomeric mixture should enable us to decide upon the required degree of purification of the pheromone for field application.

METHODS AND MATERIALS

General. The crude product, after each chemical transformation, was used without further purification for the next step. IR and NMR spectra were used to check product identity and purity. IR spectra were run on a Perkin-Elmer 377 instrument, either neat or in $CHCl_3$ solution; NMR spectra were recorded on a Varian XL-100 in CCl_4, chemical shifts are in δ, downfield from TMS as internal reference. GLC analyses were obtained on a Packard 417 FID with a 6-ft × 1/8-in. ID, 5% FFAP on Chromosorb W, SS column.

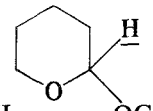
Solvents. Petroleum ether (60–80) and ether were dried over Na and distilled before use. Pyridine was distilled and kept over CaH_2 prior to the reaction. Hexamethylphosphoric triamide (HMPA) was distilled and stored over CaH_2. Tetrahydrofuran (THF) was dried over KOH, distilled, and stored over CaH_2, then distilled over $LiAlH_4$ immediately before use.

Preparation of bis- THP-1,9-non-2-yn-diol (III). In a round-bottom flask equipped with a mechanical stirrer, low-temperature thermometer, N_2 purge, and dropping funnel, 37.8g of 2,3-dihydropyran (0.45 mol) was added at 30–35° to 24.08 g of propargyl alcohol (0.43 mol) and 0.5 g *p*-toluenesulfonic acid (*p*-TsOH). The cooling bath was then removed and the mixture stirred for 1 hr, checked for the disappearance of the OH group in the IR, diluted with 280 ml THF, cooled to −40° and 45 ml of 90% BuLi (in hexane, Ventron) was added within 30 min, from a valve-equipped syringe (Schwartz and Waters,

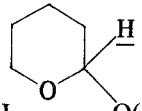


SCHEME 1

1972). The solution was warmed to 0° and treated at 20–30° with 107 g of $Br(CH_2)_6OTHP$ (0.41 mol) (Nesbitt et al., 1977) in 100 ml HMPA and 40 ml THF. The mixture was stirred for 1 hr at room temperature and the volatile materials removed at 30–40°/20mm. The residue was diluted with ether, washed with water, with saturated NaCl solution, and dried over K_2CO_3. Evaporation of the solvent left 127.2 g (96%) of III, IR 2200 cm^{-1}; NMR 4.66



(1H, $OCH_2C{\equiv}C-$, t, poorly resolved), 4.45 (1H,



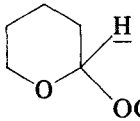
$O(CH_2)_6-$, t, poorly resolved), 4.04 (2H, $-C{\equiv}C\underline{CH_2}OTHP$, m), 3.16–3.84 (6H, $C\underline{H_2}O-$, m), 2.16 (2H, $-\underline{CH_2}C{\equiv}C-$, m), 1.24–1.94 (2OH, $-\underline{CH_2}-$, bs).

Isomerization of III to the Allene, IV. A round-bottom, 4-neck reaction flask with gas-tight mechanical stirrer, N_2 purge, low-temperature thermometer, and septum for adding reagents was connected by a dump tube to a round-bottom quench flask also equipped with mechanical stirrer, low-temperature thermometer, and N_2 purge. The dump tube was an 8-mm tube with a 4-mm stopcock. A ℑ12/5 ball joint in the dump tube allowed for ease of assembly.

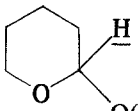
The reaction flask was charged under N_2 with 700 ml 1:19 (v/v) HMPA-THF and 126.4 g (0.39 mol) III and chilled to -78°.

The quench flask was charged with 1.5 liters MeOH and 40 g of acetic acid and similarly chilled. From a valve-equipped syringe 35 ml of *n*-BuLi (90% in hexane, Ventron) were added during 30 min. The reaction mixture was stirred for another 30 min at -78° C and then rapidly pushed through the dump tube (N_2 pressure) into the vigorously stirred quench solution.

The solvents were evaporated, and the residue was dissolved in H_2O and extracted with ether to leave 121 g of crude IV (contained less than 5% of III). IR 1945, 1680 cm^{-1}. NMR 6.38 (1H, $-C{=}C{=}C\underline{H}-O-$, m), 5.58 (1H,



$-CH_2C\underline{H}{=}C{=}C$, m), 4.78 (1H, $OCH{=}C{=}$, m), 4.45 (1H,



$O(CH_2)_6-$, m), 3.2–3.8 (6H, $-C\underline{H_2}O-$, m), 1.26–1.98 (2OH, $-\underline{CH_2}-$, bs).

Hydrolysis of IV to Aldehyde V. A solution of 75 g of allenic-acetylenic mixture (IV) in 9:1 mixture of CH_3OH–H_2O (800 ml) and 0.750 g *p*-TsOH was refluxed for 3 hr. After addition of 10 g solid $NaHCO_3$, the methanol was evaporated, and the residue was extracted with ether, washed with H_2O,

saturated solution of NaCl, and dried over $MgSO_4$ to yield 33.5 g of crude V. IR 3400–3300, 2750, 1670 cm^{-1}, NMR 9.36 (1H, —CHO, d, J = 8 cps), 6.80 (1H, —CH=CH—CHO, dt, J = 15 and 7 cps), 6.04 (1H, —CH=CHCHO, dd, J = 15 and 8 cps), 3.56 (2H, —CH_2OH, t, J = 7 cps). 2.20–2.50 (2H, —CH_2CH=CH, m), 1.2–1.8 (8H, —CH_2—, bs).

Preparation of the Pheromone VI. A suspension of 56.2 g of propyltriphenylphosphonium bromide (0.146 mol) (prepared from propylbromide and triphenylphosphine in 80% yield) in 500 ml THF was prepared in a three-necked round-bottom flask under an argon atmosphere. To this, a solution of 25.1 g of *t*-BuOK (0.224 mol) in 250 ml THF was added dropwise within 30 min at 30°. The deep orange ylide solution was stirred for 1 hr, then cooled to 5°, and 17.4 g of crude aldehyde V (0.112 mol) diluted with 20 ml THF was added dropwise. The temperature was allowed to reach 20–25° (about 1 hr), 6 ml of AcOH were added, and the THF was evaporated. The residue was charged with 30 ml of pyridine and 30 ml of acetic anhydride, and the solution stirred overnight. The mixture was poured into ice and extracted with petroleum ether, washed with a 9:1 DMSO–H_2O solution, H_2O, 5% HCl, saturated $NaHCO_3$ solution, and NaCl solution, and dried over $MgSO_4$. The crude product was distilled at 90°/0.02 mm to yield 12 g of the pheromone (50%) "contaminated" with 15% of the *E,E* isomer. IR 1730, 985, 950 cm^{-1}. NMR 4,98–6.30 (4H, olefinic H, m), 3.94 (2H, —CH_2OC(O), t, J = 7 cps), 2,02–2.32 (4H, allylic H, m), 1.94 (3H, CH_3C(O), s), 1.18–1.82 (8H, —CH_2—, bs), 0.98 (3H, —CH_3CH_2—, t, J = 7 cps).

Purification of the Pheromone to Remove E,E *Isomer.* A solution of 0.7g crude pheromone (VI) and 100 mg tetracyanoethylene (TCNE) in 10 ml THF was stirred at room temperature for 2 hr. After evaporation of the solvent, 20 ml of petroleum ether were added and the mixture left at 5° for 24 hr. The solution was then decanted and evaporated, the crude residue was separated on TLC plates which were eluted twice with 3% ether in petroleum ether (R_f 0.4).

RESULTS AND DISCUSSION

Our approach to the synthesis of the pheromone was to use (*E*)-9-hydroxy-2-nonenal (V) as the key intermediate for the synthesis, applying our experience with acetylenic–allenic conversions with 90% BuLi (Waters et al., 1978) (see Scheme 1). This procedure, although not suitable for industrial preparation (Teich et al., 1979), is a useful synthesis in the laboratory, when a simpler synthesis is not available. We thus prepared the bis-THP-derivative III, and converted it to aldehyde V, via allene IV. By this procedure we proceeded from propargyl alcohol (I) to the Wittig reaction without any

purification of the intermediates. The aldehyde (V) itself was also reacted as a crude material as it is not stable toward distillation. The Wittig reaction proceeded smoothly in THF as solvent with *t*-BuOK as base. Overall yield of the distilled pheromone is ca. 30% based on propargyl alcohol and 6-bromohexanol as starting materials. The crude product could be purified with TCNE to remove the *E,E* isomer, but we found that this isomer does not interfere with biological activity.

The biological activity of the synthetic pheromone preparations was tested in a vineyard by baiting traps (Glass et al., 1970) with a rubber septum (A. Thomas Co.) impregnated with 1 mg of the pheromone dissolved in 200 μl hexane. Three pheromone products were tested: two local preparations included a "crude" synthetic pheromone which contained ca. 15% *E,E* isomer of the EGVM pheromone, the pure pheromone, and a preparation purchased from Institut National de la Recherche Agronomique (INRA), France. These were compared using 2- to 5-day-old virgin females. The females (3–4 females/trap) were confined in a pored plastic cup of 2.5 cm diam. 3 cm long. Each treatment was replicated 4 times. Traps were placed 10 m apart in a randomized complete block arrangement. Twice a week the trapped males were counted and removed, the virgin females were replaced by fresh females, and the treatments were each shifted by one trap position. Results are given in Table 1. The number of males caught in traps baited with the two local preparations were significantly greater than the number caught in traps baited with an INRA preparation or with virgin females.

The results of this study and those obtained previously (Descoins et al., 1974) show that the *E,E* isomer does not interfere with the biological activity of the pheromone, thus simplifying the preparation of the pheromone for field application.

TABLE 1. FIELD CATCH OF MALE EUROPEAN GRAPEVINE MOTH IN TRAPS BAITED WITH VARIOUS PREPARATIONS OF THE PHEROMONE AND WITH VIRGIN FEMALES

Bait[a]	Males/trap[b] 1980	1981
"Crude"	100.2A	13.6A
Pure	81.8A	
INRA	49.3B	3.6B
Virgin females	46.3B	

[a] Explanation of the baits is given in the text.
[b] Average of accumulated catches in 4 traps during 50 nights in July and August 1980 and 5 traps during 15 nights in March and April 1981. Numbers followed by the same letter are not significantly different in 5% level. (Duncan multiple range test).

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A COMPARATIVE CHEMICAL STUDY ON POPULATION-SPECIFIC ODORANTS FROM ATLANTIC SALMON

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Abstract—Samples of skin mucus and intestinal contents were collected from groups of salmon presmolt from three Norwegian rivers. The supernatants obtained after precipitation were gel-filtered on a Sephadex G-25 column, which revealed peaks with absorption at 280 nm. Fractions incorporating a group of retarded peaks were examined by two-dimensional thin-layer chromatography. The spots obtained on the thin-layer plates were sorted out by means of position, intensity, and fluorescence properties. R_f values for the assumed identical spots from each group were subjected to statistical analysis. In one of the chromatography systems, 8 spots of 24 from samples of skin mucus and 6 spots of 27 from intestinal contents show larger standard deviations (SD) of R_f than may be expected when a substance is chromatographed repeatedly. These SDs fit a linear relationship of SD to chromatography length by a probability of less than 0.01. The results indicate a molecular variation between the groups which could be a source for population-specific odorants. If these substances are used in chemical communication, they can provide the specific clues needed for a successful return to the home river.

Key Words—Chromatography, intestinal content, migration, odorants, olfaction, *Salmo salar*, skin mucus, salmon, homing.

INTRODUCTION

The Atlantic salmon returns to the river of its origin during the spawning migration. This casual observation was made by Norwegian fishermen more than 400 years ago, and recorded at the end of the 16th century by the priest Peder Claussøn Friis (Storm, 1881). These observations have later been

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confirmed by several scientific observations, (Calderwood, 1937; Dahl, 1939; Scheer, 1939). Many hypotheses have been presented through the years in an attempt to explain the precise migrations of salmonid fishes in the sea (Harden-Jones, 1968). Since they are all based on sparse observations concerning the total migrating cycle, Neave (1964) characterized them as purely speculative.

The salmonids use their olfactory sense in the detection of their native stream within their home river system. This was shown by Wisby and Hasler (1954), who proposed an imprinting hypothesis in salmon migration. The hypothesis postulates that, because of local differences in soil and vegetation of the drainage basin, each stream has a unique chemical composition. Before juvenile salmon migrate to the sea, they become imprinted to the distinctive odor of their home stream, and as adult salmon they use this information as a cue for homing after reaching the coastal area (Hasler et al., 1978).

Nordeng (1971) provided additional knowledge from nearly 20 years of field work on the total migrating cycle. He introduced a "pheromone" hypothesis for the local orientation of anadromous salmonids. This hypothesis postulates that adult individuals recognize their river by means of population-specific odorants, emanating from the fry in the river. An extended hypothesis for the entire homeward navigation was later proposed by Nordeng (1977). By this, the homeward navigation in the sea was also explained by proposing an inherited response to population-specific pheromone trails released from the descending smolt.

By electrophysiological means, Døving et al. (1974) examined the possibility that various geographic populations of char (*Salmo alpinus* L.) release characteristic odors. Tank water from four separate groups of young char, plus two groups of adults, was used as odorant sources, and single unit activity was recorded from the olfactory bulb of adult char. Differential sensory responses produced by odors from the six groups suggested that populations of char emanate characteristic odors and that the fishes are able to discriminate between them.

Mature anadromous char (*S. alpinus* L.) of a specific population were tested with respect to attraction effects of fish odors by Selset and Døving (1980). The results demonstrated that the fish was attracted to scent of smolts from its own strain. The experiment also provided evidence that the attractive substances are released with the intestinal contents and could be fractionated on a column containing Sephadex G-25 gel.

The aim of this study was to investigate whether chemical variations in the excretion products from different groups of Atlantic salmon could be detected. The present work shows that such variations exist and that they are found both in the intestinal contents and in the fish skin mucus. Preliminary reports have been given by Stabell (1978, 1979).

METHODS AND MATERIALS

Origin of Donor Fishes. Samples were collected from presmolt stage salmon (*Salmo salar* L.) of the three Norwegian rivers Namsen, Driva, and Tafjord. All fishes were artificially hatched and raised at the Research Station for Salmonids, Sunndalsøra Unit,Norway. The parents of the donor groups had been netted during their spawning migration in the three rivers or their estuaries. Since each river may contain more than one stock of salmon, the donor groups may be hybrid ones from stocks within each river. The best approximation to uniform genetics was found by using a pair of salmon as parents for each donor group; i.e., individuals of one group were offspring of one pair only. The material sampled was taken from six presmolt groups, two from each river. In sampling the material, each type collected was mixed within the whole group, each group being viewed as a homogeneous unit. This presupposition was to ensure enough material of each type, within all groups, both for chemical analysis and subsequent electrophysiological testing.

Sampling of Excretion Products. All sampling took place throughout the beginning of May 1977 from 1+-year-old presmolt raised under artificially high temperature conditions. Throughout their life span, all groups of fishes had been given the same type of food. Skin mucus was collected by a "vacuum cleaning" method described by Stabell and Selset (1980). Intestinal contents were collected from the same fishes, again following the description of the above authors. Fish food has been shown as nonattractive in behavior studies with char (Selset and Døving, 1980). Based on these results, the gross amount of fecal residue was eliminated by fasting the fishes for 14 days before the material was collected. The number of donor fishes within each group varied between 2 and 7, and the material within each group was mixed. All material was frozen immediately after collection and stored at −20° C until use.

Chemical Separation Procedure. Both types of samples were handled following the same procedure, using chemical techniques adapted from studies by Selset (1980).

The frozen samples were thawed. Distilled water (2–3 ml) was added to the intestinal contents, which consisted of a relatively thick slurry. After mixing, the samples were centrifuged in a Wifug Doctor 102-06 table centrifuge at 5700 rpm for 15 min. Glacial acetic acid was added to the supernatants to a final concentration of 5%, and the precipitate was centrifuged off at 25,000 *g* for 30 min in a Sorvall Superspeed RC 2-B automatically refrigated centrifuge at 0° C. Absorption spectra of the supernatants, which contained the low-molecular-weight part of the samples, were taken from 240 to 400 nm by using a Gilford 240 spectrophotometer. The supernatants were chromatographed in 1-ml portions at 4° C on a Sephadex G-25 (fine) column (91.5 × 1 cm, elution speed 11–15 ml/hr), eluting with 5%

acetic acid. The effluent was monitored by a Bio-Rad model 1300 UV monitor set at 280 nm, and collected in 1.5-ml portions. The portions were pooled according to the peaks in the elution diagrams, and absorbances were taken from 240 to 400 nm. All fractions were taken to dryness 3 times with addition of distilled water in a rotary evaporator under vacuum at 40°C to remove acetic acid.

Fractions incorporating the retarded peaks, i.e., those peaks appearing after the salt volume (V_s), were examined by two-dimensional thin-layer chromatography. The thin-layer chromatography was carried out on 10 × 10-cm thin-layer silica plates (DC- Alufolien Kiselgel F 254, 0.2 mm, Merck Art. 5554), with L-tyrosine as reference substance in both dimensions. The solvent systems used were: system 1, chloroform–methanol–25% ammonia (volume ratio 2:2:1); system 2, *n*-butanol–acetic acid–water, (volume ratio 3:1:1). After chromatography, the plates were dried for 16 hr at 80–90°C and irradiated with a fluorescent lamp at 254 and 366 nm. At 254 nm, spots appeared as a result of a quenching effect from the fluorescing background. At 366 nm, spots showed up with a blue or yellow color.

The fractions from the gel filtration were tested for possible content of bile salts. A Sterognost-3α Automated test kit (Nyegaard & Co., A/S) was used. Steroids with a 3α-hydroxyl group in the C_{19}, C_{21}, and C_{24} series, including the taurine and glycine conjugates of the last group, were registered by this method. The kit produces the fluorophore resorufin as an end product. Fluorescence was measured in a Perkin-Elmer MPF-3L fluorescence spectrophotometer at exitation wavelength 565 nm and emission wavelength 580 nm.

Statistical Evaluation. The spots which appeared on the thin-layer plates after two-dimensional chromatography were compared among groups within each retarded fraction. The following parameters were used for comparison: position, intensity, and properties of fluorescence at 254 and 366 nm. If a spot was believed to be found in the corresponding plates from at least 4 of the 6 groups, it was given a number within the fraction. R_f values were calculated for the numbered spots in both dimensions in relation to the reference substance L-tyrosine.

A substance chromatographed on multiple thin-layer plates will show small variations in R_f values. This variation will be due to corresponding variations in physical and chemical parameters affecting the chromatography pattern. Mean value ($\bar{X}$) and standard deviation (SD) of R_f were calculated for all the assumed equal spots within the groups. The standard deviation of R_f calculated gives a measure of the variability for each substance in multiple chromatography. The size of this standard deviation must be expected to be a linear function of migrating distance along the chromatography plates. The standard deviation of R_f was therefore plotted against its mean R_f value. This was done separately for each chromatography system, mixing the data from

both samples within each system. Regression analyses were carried out from the plots, expressing the standard deviation of R_f as a linear function of its mean R_f value. Confidence intervals to the regression lines then gave information about the validity of the results, thereby pointing to possible chemical variations between the groups of fishes.

Electrophysiological Correlates. The sampled excretory products, and the fractions from the gel filtration, have been used in electrophysiological studies on the olfactory system of adult Atlantic salmon of the same origin as the samples. A preliminary report on this work has been given by Fisknes (1979).

RESULTS

The absorption spectra of the supernatants, obtained after precipitation with acetic acid, show a uniform pattern within each type of sample. The supernatants from skin mucus show λ_{max} at 268 nm, with an additional and smaller peak at 318 nm. Supernatants from intestinal contents show λ_{max} at 272 nm. These findings are in accord with the results of Stabell and Selset (1980).

Gel filtration of the supernatants on the Sephadex G-25 column shows a uniform pattern within both types of samples. A typical elution diagram of the supernatant from skin mucus is presented in Figure 1A. A similar gel filtration diagram of supernatant from intestinal contents is presented in Figure 1B. In an ordinary gel filtration, the material is eluted between the void volume (V_0) and the salt volume (V_s). Samples from skin mucus as well as intestinal contents contain substances that are retarded in relation to the salt volume. This effect was previously reported by Selset (1980) and Stabell and Selset (1980).

The samples from skin mucus produce five peaks eluted after the salt volume (Figure 1A). The samples from intestinal contents give a similar elution pattern, but in addition a peak is found at a higher elution volume than any of the peaks from skin mucus (Figure 1B). It must be noted that, as for the established fractions, they are not all directly comparable to the peaks in the elution diagrams. Fraction VI from samples of intestinal contents contains two peaks, a result originating from an early standardization.

The reproducibility of the gel filtration pattern might be expressed by partition coefficients (K_{av}) for the individual peak. K_{av} values for the retarded peaks in the chromatograms are presented in Table 1. The results are presented as mean K_{av} value ($\bar{X}$) with standard deviation (SD) for all six groups within both types of samples. Skin mucus gave no retarded peak in fraction VIII in any of the gel filtration profiles. The standard deviations of K_{av} values within both types of excretion products indicates that gel filtration

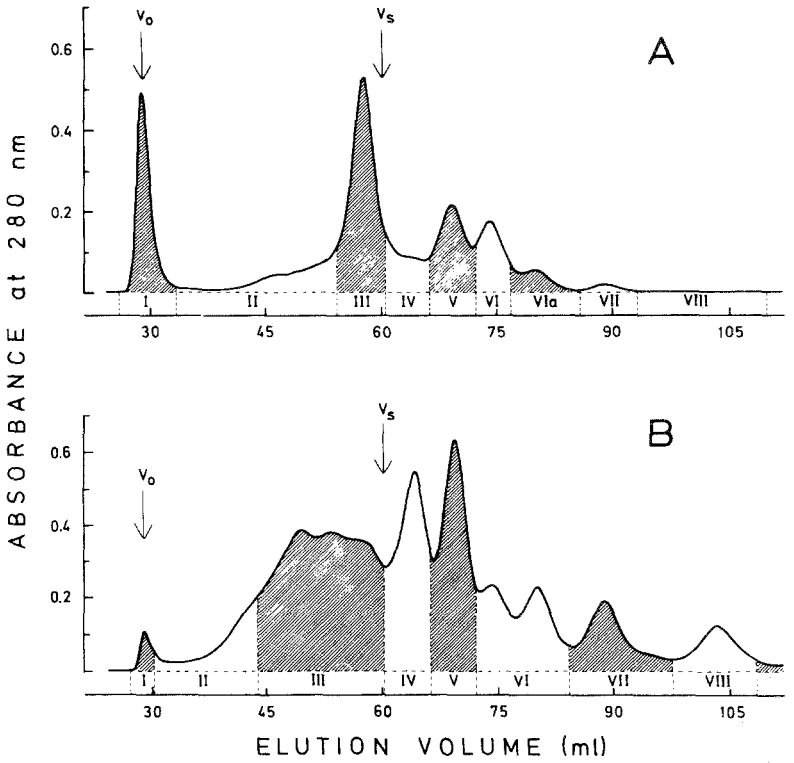

FIG. 1. Elution diagrams from Sephadex G-25 chromatography of the supernatants found after precipitation with acetic acid. (A) skin mucus, (B) intestinal contents. V_o = void volume. V_s = salt volume; 5% acetic acid was used as effluent. The established fractions are numbered with Roman numerals. Note that fraction VI from intestinal contents contains two retarded peaks. The applied samples were derived from multiple fishes within each group.

is very reproducable. K_{av} values from gel filtration of skin mucus as well as intestinal contents show similarities concerning the retarded peaks.

UV scanning (240–400 nm) of the collected fractions were obtained, and λ_{max} for typical UV spectra of the retarded fractions are presented for both types of samples in Table 2. Again it must be stressed that fraction VI from intestinal contents contains two peaks in the elution diagram. Concerning skin mucus, a fraction VIII was also collected. This fraction included the eluted volume portion that might contain a possible retarded peak, 6, from skin mucus. As can be seen in Table 2, fraction VIII from skin mucus does contain substances with low absorbance. Comparing the two types of samples, absorption spectra coincide for at least two fractions. Together with the K_{av} from the gel filtration, the spectra indicate that, within these fractions,

TABLE 1. K_{av} VALUES FOR RETARDED PEAKS FROM GEL FILTRATION WITH SEPHADEX G-25 ELUTED WITH 5% ACETIC ACID[a].

	Peak found in fraction					
	IV	V	VI	VIa	VII	VIII
Skin mucus						
$\bar{X}$	0.812	0.938	1.050	1.192	1.143	–
SD	0.013	0.003	0.008	0.010	0.017	–
Intestinal contents						
$\bar{X}$	0.813	0.932	1.033[b]	1.187[b]	1.375	1.730
SD	0.015	0.029	0.025	0.018	0.029	0.032

[a]The retarded peaks appear after the salt volume (V_s), measured as absorbance at 280 nm. The K_{av} values are given as mean value ($\bar{X}$) and standard deviation (SD) from the six groups examined.
[b]Found in fraction VI. Elution diagrams not divided into subfractions.

substances with mainly similar chemical properties are found to be dominant in both types of samples.

The results from the two-dimensional thin-layer chromatography are presented by representative series of fractions, in Figure 2 from skin mucus and in Figure 3 from intestinal contents. Since only the fractions containing the retarded peaks were subjected to thin-layer investigation, six plates from skin mucus and five plates from intestinal contents are presented. The plates in Figures 2 and 3 are numbered by Roman numerals according to the fraction numbering in the elution diagrams given in Figure 1.

All fractions were first subjected to chromatography in the alkaline system of chloroform–methanol–ammonia (system 1). The reference substance L-tyrosine, marked T, was simultaneously chromatographed on the right side of the plates. After drying and application of L-tyrosine, the plates were chromatographed in the acidic system of butanol–acetic acid–water

TABLE 2. λ_{max} FROM UV ABSORBANCE SPECTRA (240–400 nm) OF FRACTIONS[a]

	Fraction					
	IV	V	VI	VIa	VII	VIII
Skin mucus	258	248	248	252/275	270	275
Intestinal contents	263	248/267	254		270	275

[a]Including the retarded peaks from gel filtration with Sephadex G-25. The fraction numbers are in accordance with Figure 1.

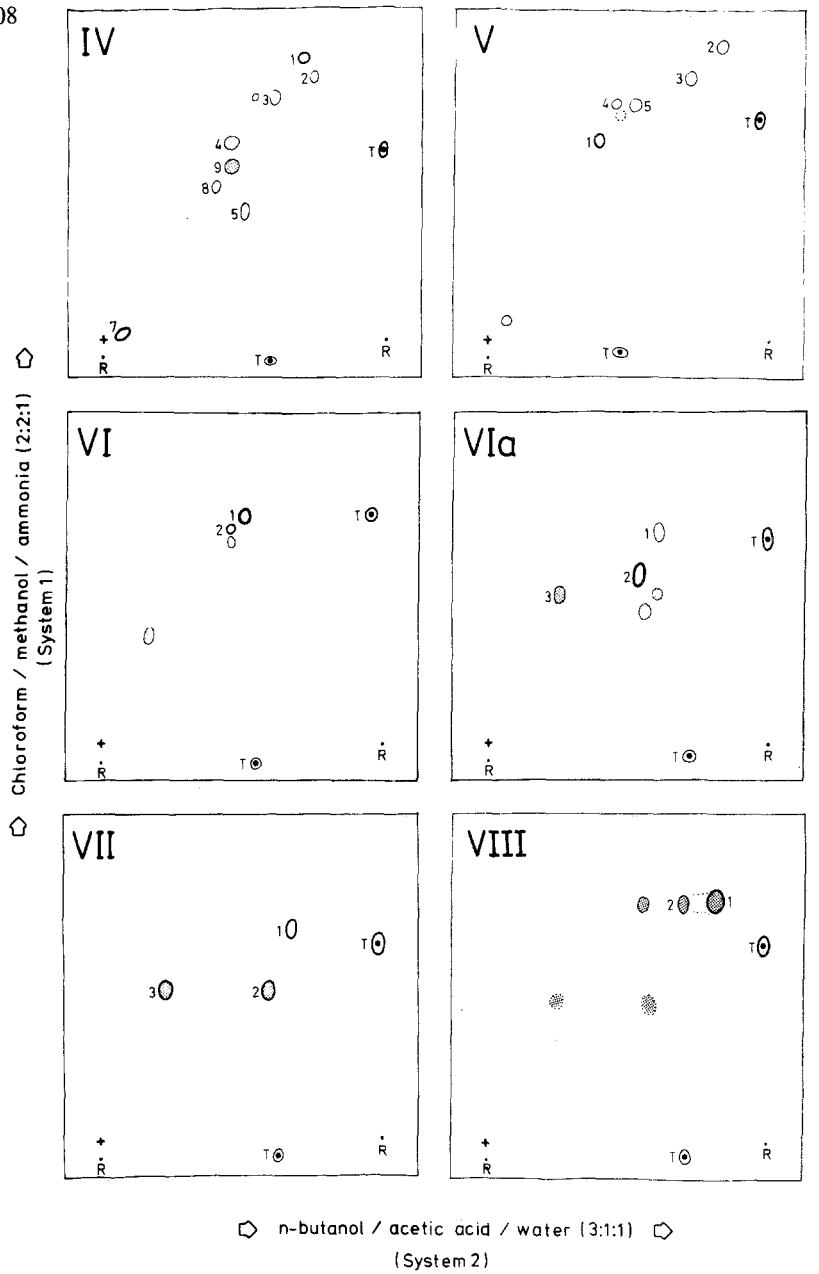

FIG. 2. A representative series of thin-layer chromatography plates from the retarded fractions of skin mucus. The Roman numerals are in accordance with the fraction numbers given in the elution diagram in Figure 1A. The intensities of the spots are indicated by the width of the lines illustrating them. Open spots are identified by a quenching effect from the fluorescing background at 254 nm. Spots filled with dots are identified by a blue color at 366 nm. Spots filled with broken horizontal lines are identified by a yellow color at 366 nm. Spots illustrated by broken frames are found at a higher concentration in neighboring fractions. + = application point of sample; R = application point of L-tyrosine. T = end point of reference substance, L-tyrosine. Arabic numerals indicate the spots found within each fraction.

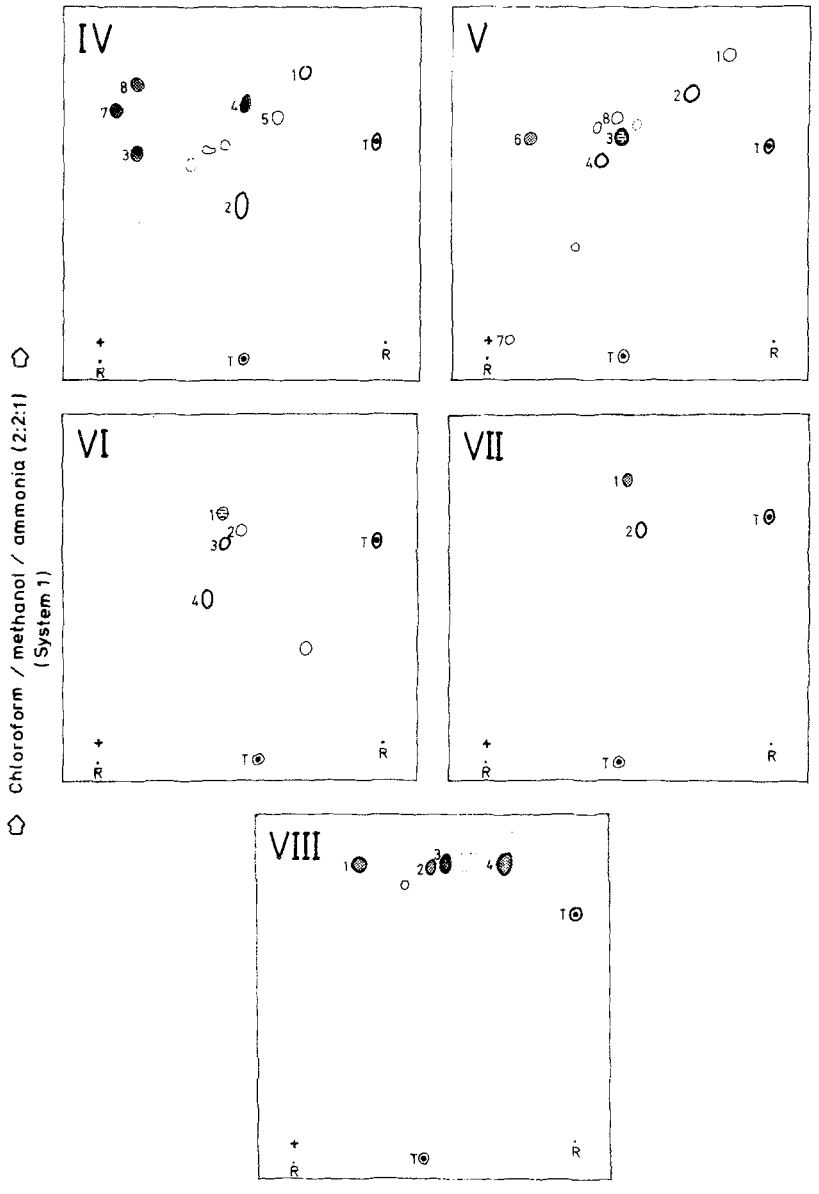

FIG. 3. A representative series of thin-layer chromatography plates from the retarded fractions of intestinal contents. The Roman numerals are in accordance with the fraction numbers given in the elution diagram in Figure 1B. The Figure symbols are the same as those used in Figure 2.

(system 2), i.e., at a right angle to system 1. All spots that appeared on the plates during UV radiation at 254 and 366 nm are drawn in the figures. The intensities of the spots are indicated by the width of their frames. Spots that show blue or yellow fluorescence at 366 nm are filled by dots or broken horizontal lines, respectively, in the figures. Spots illustrated by broken frames are assumed identical to spots found at a higher concentration in a neighboring fraction.

Note that not all the numbered spots are present in the series presented in Figures 2 and 3. Concerning skin mucus (Figure 2), spot 6 from fraction IV is missing. In the fractions from intestinal contents (Figure 3), the following spots are missing: spot 6 in fraction IV; spot 5 in fraction V, and spot 3 in fraction VII. All the missing spots are found, however, in at least four of the remaining groups.

Mean R_f value with standard deviation is presented for all the numbered spots, in both chromatography systems used, from skin mucus in Figure 4, and from intestinal contents in Figure 5. The mean R_fs are calculated on the data from the retarded fractions in all groups of fishes. The fraction numbers on the statistical plates in Figures 4 and 5 are equivalent to the fraction numbers given on the illustrated plates in Figures 2 and 3, respectively. All statistically treated spots show small standard deviations in the alkaline system (system 1), as can be seen in the figures. In the acidic system (system 2), conspicuously large standard deviations are seen within some of the fractions from both types of samples. This large variation in R_f is found for a limited number of statistically treated spots within fractions that mainly contain moderately varying ones.

Figure 6 presents the standard deviation of all series of statistically treated spots, from both types of samples, plotted against their mean R_f values in the two chromatography systems used. Concerning the alkaline system (system 1), the plotted points show a linear relationship with chromatography length. The regression line for the points in system 1 fit the expression:

$$y = 0.024 + 0.025x \tag{1}$$

with a correlation coefficient (r) of 0.35, and a standard error of estimate (S_{yx}) of 0.023. The plot of data in the acidic system (system 2) at first seems to fit a linear regression poorly. Transferring the regression line from the alkaline system into the acidic one, as illustrated by the lowermost broken line, together with the alkaline confidence lines $P = 0.01$ and $P = 0.001$, then gives the impression that most of the points indeed fit this line. Calculating the regression line for the points from the acidic system, satisfying the alkaline regression $P < 0.01$, gives the expression:

$$y = 0.023 + 0.036x,\ r = 0.54 \tag{2}$$

This expression falls inside the standard error of estimate for equation (1).

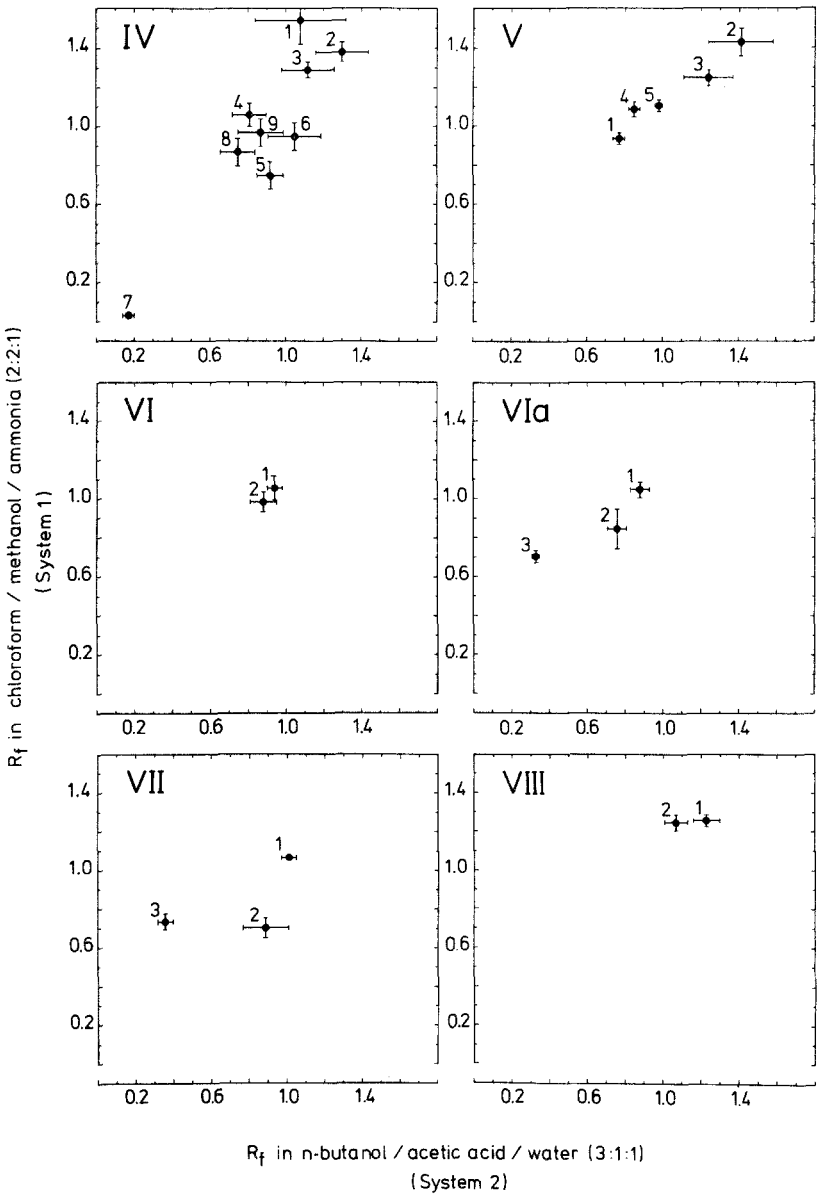

FIG. 4. Statistical representation of the numbered spots on the thin-layer chromatography plates originating from skin mucus. The spots are given by their mean R_f values and standard deviations, from all six groups, in both chromatography systems used. The fraction numbers on the statistical plates are equivalent to the fraction numbers on the illustrated chromatography plates in Figure 2.

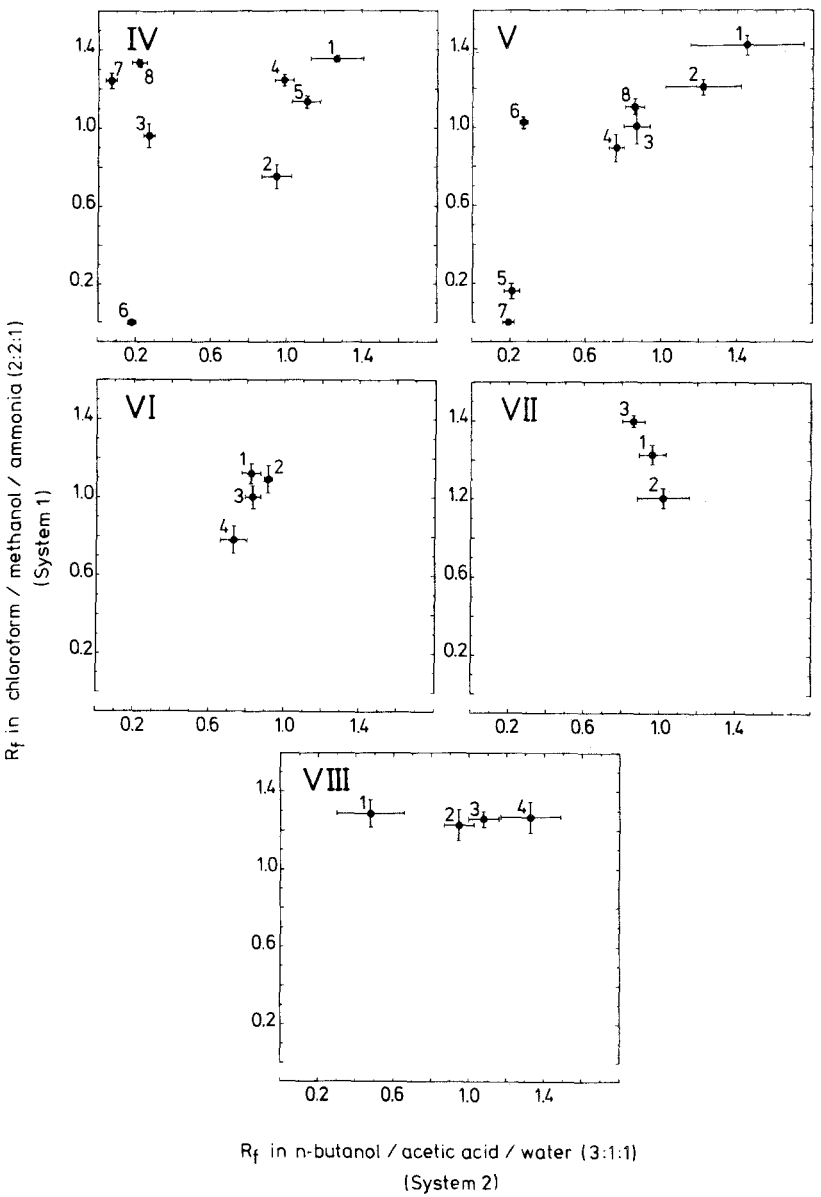

FIG. 5. Statistical representation of the numbered spots on the thin-layer chromatography plates originating from intestinal contents. The spots are given by their mean R_f values and standard deviations, from all six groups, in both chromatography systems used. The fraction numbers on the statistical plates are equivalent to the fraction numbers on the illustrated chromatography plates in Figure 3.

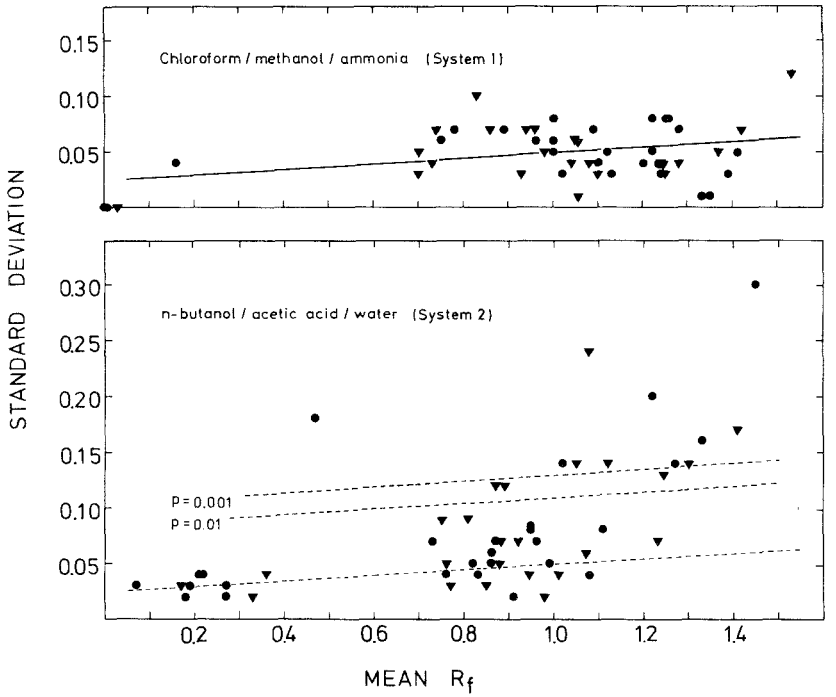

FIG. 6. Evaluation of the statistical data from Figures 4 and 5 in the two thin-layer chromatography systems used. The data are represented by the standard deviation of R_f as a function of mean chromatography length (R_f). ▼-data from skin mucus; ●-data from intestinal contents. The regression line, $y = 0.024 + 0.025x$ from the system of chloroform–methanol–ammonia (upper frame), is plotted as the lowermost broken line in the system of *n*-butanol–acetic acid–water (lower frame). The lines $P = 0.01$ and $P = 0.001$ are also derived from the alkaline data.

Altogether 37 of 51 points show a linear relation of standard deviation to mean R_f in the acidic system. This linear relation is the same as the one shown for all the points in the alkaline system. However, 14 points fall outside the linear regression in the acidic system; most of them are even far outside the probability $P = 0.001$, as can be seen in Figure 6. The substances with such a large standard deviation were chromatographed within the same thin layer plates as the 37 statistically treated spots that follow the linear relationship. The conclusion to be drawn from this must be that the assumed identity between the groups, for the spots that give a large standard deviation of R_f, is not fulfilled.

The statistically treated spots which represent the points falling outside the linear probability $P = 0.01$, are summarized in Table 3. Eight series out of 24 of spots from skin mucus give standard deviations that are found within

TABLE 3. THIN-LAYER CHROMATOGRAPHY SPOTS FROM TWO TYPES OF MATERIAL FALLING OUTSIDE THE LINEAR PROBABILITY OF $P = 0.01$ IN FIGURE 6[a]

	Fraction number			
	IV	V	VII	VIII
Skin mucus	1, 2, 3, 6, (9)	2, (3)	(2)	
Intestinal Contents	1	1, 2	2	1, 4

[a]The spots are given by their numbers within each fraction, according to the numbering in Figures 4 and 5. Spots given in parentheses fall inside the linear probability of $P = 0.001$. Spots assumed identical in the two types of material, according to their R_f values, are presented in the same vertical columns.

this group, five of which are also found outside the probability $P = 0.001$. Six series of spots out of 27 from intestinal contents give standard deviations that are all found outside the linear probability $P = 0.001$. The spots with great variation in R_f values were further compared among the types of samples with regard to R_f values in both chromatography systems. All together three series of spots with large standard deviations are assumed found in both types of samples; these are presented in the same vertical columns in Table 3.

Substances with low absorption at 280 nm, as monitored during the gel filtration, might well be distributed in more than one fraction. According to their R_f values in both chromatography systems, the series IV 2 and V 2, plus IV 3 and V 3 from skin mucus must be assumed identical. The same must be true for the series IV 1 and V 1 from intestinal contents. From this, it is not likely that more than eight series of substances altogether are found to be the varying ones among the groups in the secretions from Atlantic salmon parr.

The 3α-OH steroid test revealed that bile salts are found in fraction IV from intestinal contents. Fraction IV from skin mucus and also fraction VII from intestinal contents may possibly contain steroids. The uncertainty is partly due to the limited amount of material available at this time.

DISCUSSION

In the present experiments, group differences are demonstrated in excretion products from Atlantic salmon. The study was performed by separation with gel filtration and thin-layer chromatography, and statistical analysis of the spots on the thin-layer plates. The assortment of excretion products used in the work, together with separation procedures and statistical evaluation, will be discussed. The term "odorants" used in connection with the current substances will be justified, and the chemical nature of substances will be considered.

Nordeng (1971, 1977) proposed that active odorants could be released from fish skin mucus. Stimulation of the olfactory epithelium with skin mucus was also found to induce a larger EEG activity in the char olfactory bulb than extracts from other organs (Døving et al., 1973). Skin mucus as a principal source of specific odorants was also found in the above-mentioned single unit study on the olfactory bulb in char by Døving et al. (1974). Stabell and Selset (1980), however, showed that the utmost care has to be taken in the collection of skin mucus from fishes. Skin mucus collected in a "customary" way was shown to be contaminated by intestinal juices. This result was supported by behavioral experiments on mature migratory char by Selset and Døving (1980), who found significant attraction to intestinal juices. The above reports pointed, therefore, to intestinal juices plus "pure" skin mucus as the excretion products of interest.

To get a reasonably low number of substances within each portion for thin-layer chromatography, it was necessary to fractionate the material. The fractionation of the material by acetic acid precipitation and gel filtration was introduced by Selset (1980). According to the K_{av} value data in this study, the method is very reproducible. The biological significance in fish migration of the fractions from gel filtration containing the retarded peaks has been clearly demonstrated by Selset and Døving (1980). These experiments make it clear that interest should be focused on the retarded part of the gel filtration product.

The retarded fractions from gel filtration contain substances that were potent olfactory stimulants, according to electrophysiological studies by Fisknes (1979). Fraction V from intestinal contents, containing the most variable group of substances in the present study, gave the lowest thresholds in her measurements. Similar fractions of retarded substances attracted anadromous char in behavioral studies, as demonstrated by Selset and Døving (1980). The above reports therefore indicate that the presently investigated substances may be characterized as odorants.

The thin-layer plates demonstrate a high correlation between the groups both in number of spots within each fraction, and in location of the individual spot. Not all the numbered spots within each fraction were found in all groups. This absence of spots in some groups seems more to be due to concentration phenomena in the samples used than to a total lack of the substance in the group. Some extra spots, showing low intensities, were found within some of the fractions. These unnumbered spots could be contaminants in the system, or they may be present in all groups and found if chromatographed at a reasonably high concentration. It is not likely that these unnumbered spots are potential numbered ones. Such an analysis was, however, beyond the scope of the present study.

Only spots (presumably) found in all groups of fishes were taken into

consideration. Together with the fact that the donor groups contained a variable number of individuals, this shows that individual variations was not studied.

The statistical evaluation is based upon a contradiction principle. If all groups of fishes emanate identical substances, thin-layer chromatography of identical fractions from the groups may be viewed as multiple chromatography of one group. Multiple chromatography includes experimental errors which can be expressed through standard deviation of R_f for the individual substance. The standard deviation of R_f will increase linearly with chromatography length for the different substances. If a collection of spots gives a standard deviation of R_f that does not follow a linear relationship, the presumed identity between the groups for these spots must be wrong.

In the present study, the postulated linearity is fulfilled for all the chromatographed substances in the alkaline system (system 1). In the acidic system (system 2), altogether 14 statistically treated spots, from both types of samples, show standard deviations outside a linear regression. Since the 14 statistically treated spots are chromatographed within the same thin-layer plates as the total 37 which show a linear regression, the presumed identity among the groups for the 14 spots must be wrong. Since no other current spots are to be found on the plates, the results can be summarized as follows:

Atlantic salmon parr emit substances that show small variations in chemical properties between the examined groups of fishes. The chemical variation is not detectable when an alkaline system is used in thin-layer chromatography, pointing to acidic properties for the substances. The group variations demonstrated in excretion products might be sources for mono- or multicomponent systems in chemical communication. The retarded fractions in this study contain 3α-OH steroids. The substances which show variations among the groups might therefore be of steroid type.

Selset (1980) suggests that bile salts might be involved in fish navigation systems. As in his study, the origin of the current substances is uncertain. They might be produced by microorganisms in the intestine, or they might be produced by the fishes themselves. Bile salt derivatives are found to be the most potent odorants ever demonstrated in fishes (Døving et al., 1980). The group variations found in this study are present among substances emanating from both skin mucus and intestinal contents. It is likely that bile salts found in blood plasma may also be found in skin mucus. If produced by microorganisms, they might be absorbed from the intestine. In addition, the salmon skin is able to metabolize steroids, as reported by Hay et al. (1976). The origin of the demonstrated substances are still obscure; future analysis will bring concrete knowledge of their chemical nature and their significance in chemical communication.

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SEX PHEROMONE-INDUCED CHEMOLOCATION IN THE MALE AMERICAN COCKROACH, *Periplaneta americana*

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Abstract—Males of the American cockroach, *Periplaneta americana*, have evolved an efficient search strategy enabling them to locate a source of sex pheromone in the absence of directional information derived from wind. Since these animals are able to utilize chemotaxis to orient almost directly to the source from 45–50 cm away, it is only necessary that they maximize their chances of getting within that range once they perceive the pheromone. They accomplish this by: (1) increasing their overall levels of activity and (2) initiating local search. In this way, they are able to cover a rather large area in a short period of time, i.e., before the source female changes location. If their paths take them to within 45 cm of the source, they are able to locate it soon thereafter. It is probable that very similar strategies are utilized by many other nonflying insects to locate sources of airborne odors.

Key Words—Chemolocation, sex pheromones, *Periplaneta americana*, Orthoptera, Blattidae, orientation.

INTRODUCTION

Females of the American cockroach, *Periplaneta americana*, produce a sex pheromone that attracts conspecific males from moderate distances away. In still air, the diffusion of pheromone molecules results in the formation of a concentration gradient radiating outward from the source in all directions. Since the habitat of *P. americana* is often inside man-made structures, the directional cues from wind are probably minimal. Indeed, many cockroach

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habitats in temperate regions are as close to static as any found in nature. Recent studies of the orientational responses of adult males of *P. americana* to sex pheromone have shown that they are capable of orienting to nearby pheromone sources by true chemotaxis (Rust et al., 1976), as well as to air currents containing sex pheromone by "chemoanemotaxis" (Rust and Bell, 1976; Bell and Kramer, 1980). In the study by Rust et al. (1976) sex pheromone was placed only 20 cm from the cockroach on a Y-maze globe. This study was undertaken to determine how the male gets to within 20 cm of the pheromone source in the first place. In particular, we wanted to determine and elucidate any search strategy a male might utilize to maximize the probability of locating a pheromone source, i.e., a mate, with the least expenditure of time and to determine the role chemotaxis might play in mate location at distances approximating those encountered in nature.

METHODS AND MATERIALS

General Animal Maintenance and Experimental Design. Two groups of 5 males each were isolated from laboratory colonies, making certain only fully intact males were chosen. A 10-mm equilateral triangle coated with luminescent paint was affixed to the pronotum of each male to provide directional information, and the two groups were placed in clear plastic containers $29 \times 18.5 \times 12.5$ cm and kept for 2 weeks at 25°C on a 12:12-hr black light–white light photocycle.

The photographic design was the same as that described in Hawkins (1978). A motor-driven 35-mm single-lens-reflex camera was suspended from a wooden beam above the center of a circular arena 2.5 m in diameter. A 60-rpm synchronous motor was mounted on an adjacent beam in such a way that a slotted disk would be rotated in front of the camera lens. When photographing a cockroach, 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. During photographing, the arena was illuminated by low-intensity black light (365 nm). As a result, only the luminescent triangles on the cockroaches' pronota reproduced on film. Therefore, the light reflected from the triangle on a moving cockroach produces a series of short streaks on the film, each of which represents the distance traveled by the cockroach in 0.083 sec. While the cockroach can presumably see under UV light, preliminary experiments with a 12:12-hr white–black light photocycle revealed no significant differences in activity rhythms or mating behavior between the experimental animals and a control group maintained on a synchronized 12:12-hr light–total darkness cycle. Low-intensity UV light may well approximate crepuscular light.

Males were introduced singly into the arena. After a 30-min adjustment

period, an inverted glass jar was placed over the male to restrict its movement. Thus confined, the male was moved to the perimeter of the arena and a clean 5.5-cm disk of No. 1 filter paper was placed in the center. After 5 min, the jar was lifted and the male's movements were filmed for 7 min. Then the male was recaptured under the jar, moved to the perimeter of the arena, and a filter paper disk treated with 10 μl of sex pheromone extract was introduced. After allowing 5 min for diffusion of the pheromone, the male was released and its activity filmed for another 7 min.

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*. Sex pheromone extract was used in our experiments to ensure equal stimulus intensity for each male tested. Various concentrations of the extract were bioassayed to determine approximately one female equivalent.

Relative locomotory activity was determined by counting the number of times an individual crossed a line drawn along the long axis of the containers in 5 min in the presence and absence of sex pheromone (Block and Bell, 1974).

Analysis of Orientation Patterns. If it is assumed that an animal is receiving no directional information at a particular moment, then its movement would be expected to be random, i.e., the probability of it moving in one direction is equal to the probability of it moving in any other. Similarly, if individual animals were released from the center of a circle and their movements were completely random, the points where they left the circle would be uniformly distributed around the circumference. If one direction is arbitrarily designated as 0°, all of the angles to the various exit points measured, and a mean vector calculated, the result will be the zero vector, a vector with length 0 which is characteristic of a uniform circular (random) distribution. The radius of the circle is taken as the length of the mean vector when all the animals leave the circle at exactly the same point due to some strong directional bias. Thus, a short mean vector indicates a high degree of random movement while a longer one suggests directional orientation. This principle was applied in studying changes in the degree of randomness in the orientation patterns of male cockroaches resulting from the introduction of sex pheromone. The arena was divided into four imaginary concentric circular zones, each with a radius 30 cm longer than the one within it. Each point where a cockroach entered a particular zone was designated as the center of a unit circle in which the zero direction was toward the center of the arena. The angular deviation was measured between the zero direction and the cockroach's direction of travel within that zone (Figure 1). A mean vector was calculated for each of the three inner zones using the combined data from all males tested. Since the walls of the arena greatly reduce the opportunities for random movement within the outer zone (zone 4) and provide a bias toward the inside, that zone was not considered.

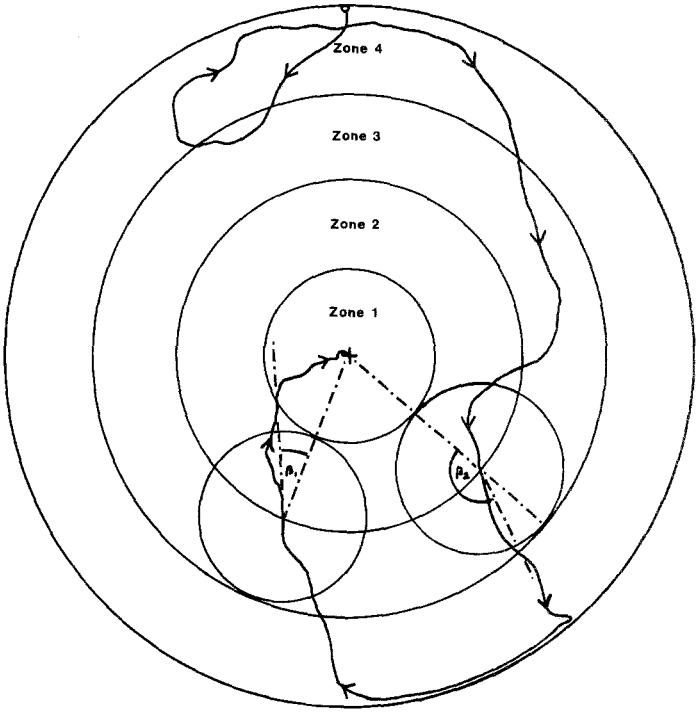

FIG. 1. Randomness values were calculated by measuring the angular deviations (β_1 and β_2) between a cockroach's directions of travel within each zone and the "correct" direction-toward the center.

RESULTS

Bioassays of the response of *P. americana* males to filter paper cages containing virgin females 15–30 days old yielded a mean of 17.63 activity counts per minute ($N = 51$ groups of 5 males). This level of activity is similar to that previously reported for this species by Hawkins and Rust (1977). Using this activity level as a standard, it was demonstrated that 10 μl of the sex pheromone extract closely approximated the average 24-hr emission of a typical virgin female. Out of 20 tests, 17 males were successful in locating the pheromone source within 7 min. The time required to reach the source varied from 20 sec to about 7 min ($\overline{X} = 3.64$ min). Interestingly, no significant difference was found between the times for males that started moving immediately and those that did not.

The distances covered before reaching the source varied considerably. While the straight-line distance was approximately 120 cm, males ran from 126.92 to 955.3 cm ($\overline{X} = 567.7$ cm). Thus, the distances traveled were from

1.03 to 7.84 times the minimum distance necessary to reach the source. It is evident that what appears to be "random wandering" is quite common (Figures 2 and 3). The male in Figure 2A took a comparatively direct route, but Figures 2B, 3A, and 3B contain numerous convolutions where the males have repeatedly crossed back over their own trails. Such random orientation generates new spatial information and is to be expected when memory and sensory information about the location of a resource is lacking. Looping patterns are more common in the presence of sex pheromone than in its absence. Loops occurred an average of 0.17 times per minute in the controls and 0.63 times per minute when pheromone was present. In the controls, the loops were restricted to the outer-most 30 cm of the arena, whle 23% occurred within 60 cm of the center when pheromone was present. In preliminary experiments involving the stimulation of males with bursts of pheromone-laden air directed into the arena from above, i.e., a nondirectional source, similar open looping patterns were elicited with no evidence of orientation toward a fixed point.

Rust et al. (1976) showed that *P. americana* utilized positive chemotropotaxis in orienting to a source of sex pheromone 20 cm away. If this mechanism is utilized as much as 1.2 m from the source, the looping patterns should not occur. Instead, the cockroach should follow a more or less straight path toward the source in the center of the arena, minimizing random orientation. It is obvious from the patterns illustrated in Figures 2 and 3 that this does not often occur, although half the animals that successfully located the pheromone source reached it without preliminary looping. These successes may have been due to coincidentally wandering close enough to the source to utilize chemotaxis to some degree. The males went straight ($\pm 3°$) to the source from up to 68 cm ($\bar{X} = 47.96$ cm) away.

When pheromone was present, the length of the mean vector was greatest for zone 1 and decreased as the distance from the center of the arena increased (Figure 4).

Since the maximum vector length in a unit circle is 1 by definition, the calculated length of the mean vector for zone 1 (0.97) indicates a very high degree of unidirectional movement. Although the lengths of the mean vectors declined to a low of 0.41 for zone 3, the values of all three zones indicated significant levels of unidirectionality ($P < 0.01$; Rayleigh test). This is in sharp contrast to controls in which the distribution did not vary significantly from a random distribution (Figure 4).

Males of *P. americana* often stay very close to the sides of the arena, maintaining contact with the walls with some portion of their bodies, usually the antennae. Occasional trips across open areas eventually result in renewed contact with the wall, which is then followed for varying distances. The presence of sex pheromone reduces the tendency of male cockroaches to

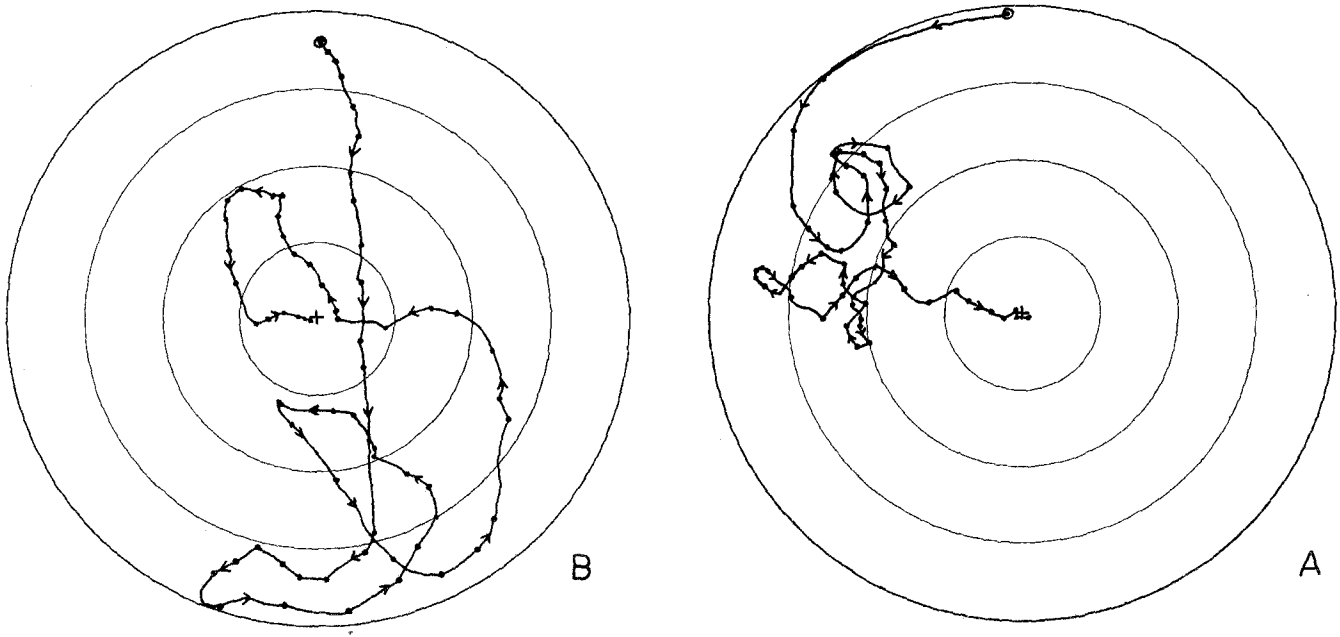

FIG. 2. Sample paths of two *P. americana* males orienting toward a source of sex pheromone located in the center of the 2.5 m. circular arena.

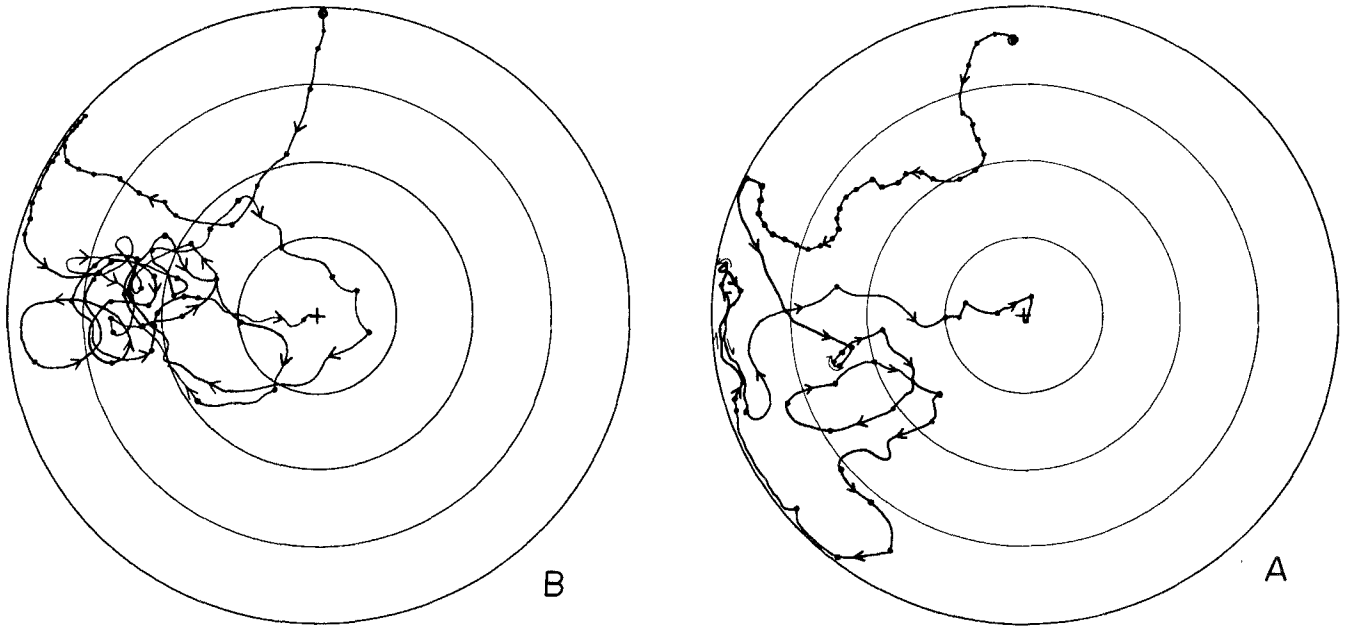

FIG. 3. Sample paths of two *P. americana* males orienting toward a source of sex pheromone located in the center of the 2.5 m. circular arena.

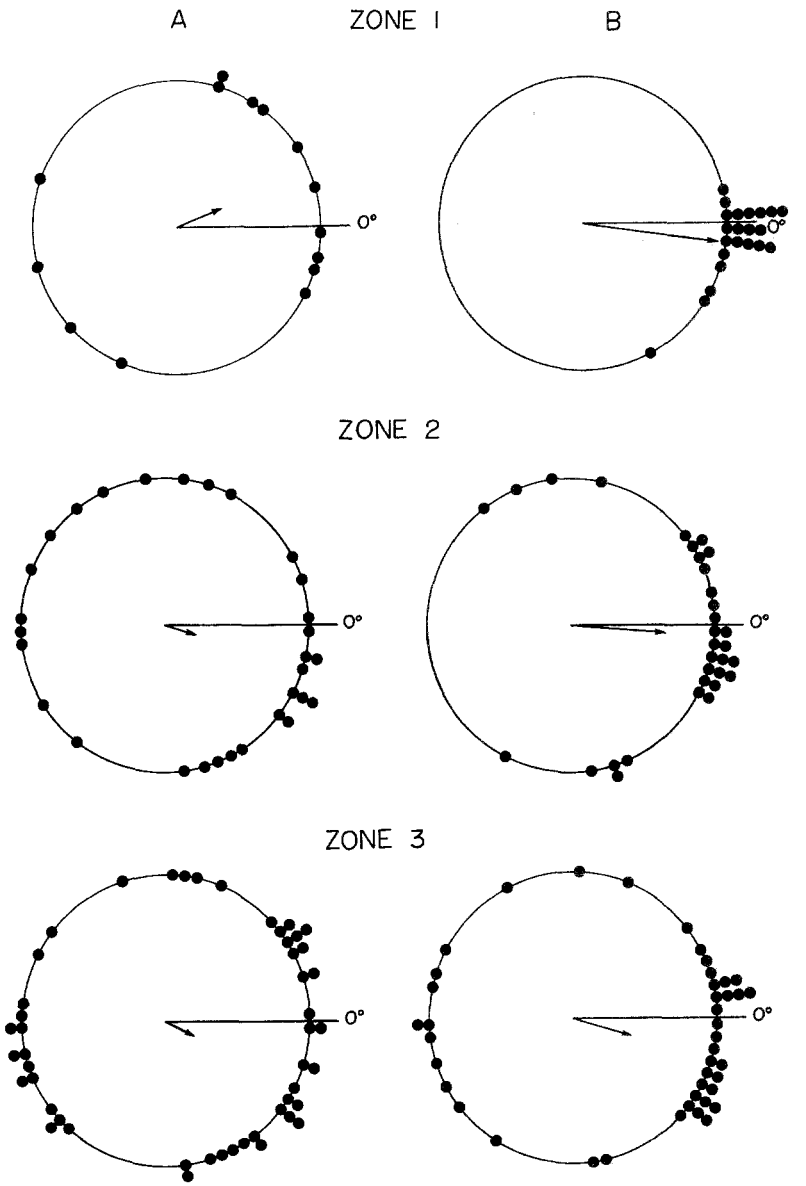

FIG. 4. Influence of sex pheromone on the directional orientation of *P. americana* males in the 2.5 m. arena as indicated by the length of the mean vectors (A = controls; B = pheromone; n = 20 males).

follow the walls. In the controls, males spent approximately 72% of the time in zone 4 and 34% of the time within 3 cm of the walls. In the presence of pheromone, the percentage of time spent in zone 4 dropped to 63% with only 10% of the total spent within 3 cm of the walls.

Even with the wall-seeking tendency reduced by the presence of sex pheromone, the number of data points found in zone 4 was greater than would be expected if the distribution were totally random. The distribution of data points (Table 1) was significantly different ($P < 0.005$; chi-square test) from what one would expect if the points were distributed among the four zones in proportion to their areas. The number of points was greater than expected in zones 1 and 4 and less than expected in zones 2 and 3. The somewhat higher number of points in zone 4 can be explained by the animals' tendency to follow walls while the value in the innermost zone is due to the attractive qualities of the sex pheromone. The distribution was also significantly different from expected in controls ($P < 0.01$) with very few points in zone 1 and a disproportionately large number in zone 4 due to the wall-seeking tendency.

TABLE 1. DISTRIBUTION OF DATA POINTS WITHIN THE ARENA[a] (PHEROMONE EXTRACT PRESENT)

	Distance from source (cm)			
Sample	0–30	31–60	61–90	91–120
1	50	77	42	87
2	24	93	132	297
3	27	15	16	123
4	11	24	82	154
5	18	10	10	4
6	48	50	88	113
7	15	21	111	37
8	22	16	58	43
9	17	12	12	34
10	34	19	34	86
11	22	124	52	83
12	49	43	64	290
13	18	11	45	140
14	14	34	49	24
Σ_X	369	549	795	1515
$\bar{X}$	26.36	39.21	56.79	108.21

[a]Each data point represents 0.167 sec that the cockroach spent in a particular area.

DISCUSSION

The behavioral responses of *P. americana* males to the females' sex pheromone have been studied both descriptively (Barth, 1970) and quantitatively (Wharton et al., 1954; Rust, 1976; Tobin et al., 1981). Rust placed the six behavioral components of the male response into three phases: (1) rapid antennal movements and erect body posture, (2) running, and (3) wing-raising, abdominal extension, and backing movements. Each of these phases is sequentially organized by increasing concentrations of sex pheromone with tactile stimulation apparently being important in eliciting backing and abdominal extension. Response thresholds for these behaviors vary greatly for individuals and within groups of males and are greatly influenced by the photocycle. Rust's third category should be subdivided into tactile and nontactile components. Wing-raising should be made a separate category because this behavior often occurs in the absence of tactile stimulation, while abdominal extension and backing require tactile stimulation and high concentrations of sex pheromones for their release.

Rust (1976) showed that the initial response of the male *P. americana* to sex pheromone is arousal. This occurs at relatively low concentrations and expresses itself as an increase in the rate of locomotion which is independent of spatial chemolocation. Similar quantitative results have been reported for another cockroach species, *Byrsotria fumigata* (Bell et al., 1974). In the present study, sex pheromone extract that was allowed to diffuse from a 5.5-mm disk of filter paper in the center of a 2.5-m circular arena produced a significant initial reduction in immobile time, i.e., more activity ($P < 0.01$) and an increase in distance travelled ($P < 0.05$).

Since it has been suggested that the most likely distribution of resources is patchey (MacArthur and Pianka, 1966), perhaps the most efficient locomotory strategy in the absence of information is to move in a straight line. Thus, a cockroach introduced into a strange environment will probably initially go straight. If it encounters an obstacle, such as a wall, it will turn to one side and go straight again. In the case of a long, curved wall, the animal will probably continue to collide with it periodically and more or less follow it for varying distances before making a more abrupt turn and getting away from it. When released in the circular arena, the cockroaches ran straight until they reached the side, turned in one direction or the other, and proceeded more or less straight ahead. Rarely did they hit the wall at right angles, generally much smaller ones. As might be expected, the bulk of the evidence suggests that they nearly always turn through the shortest turning angle.

If an organism receives a stimulus suggesting that some vital resource is nearby, and it is not immediately able to precisely locate that resource, a convoluted, looping, local search pattern is often initiated. Such a strategy is used by the blowfly (Dethier, 1957) and the three-spined stickleback

(Beukema, 1968) when searching for food. An analogous situation exists in the male American cockroach's search for a different vital resource: a mate. Response thresholds for many insect pheromones are known to be quite low. Using the data presented by Butenandt (1959), Bossert and Wilson (1963) calculated that male silkworm moths (*Bombyx mori*) respond to concentrations at least as low as 192 molecules per cubic centimeter of air. It seems likely that a male of *P. americana* will detect the presence of a sex pheromone when he is still several feet away from a virgin female, even in still air. Thus, if a male cockroach wanders into an arena where the air contains sufficient quantities of sex pheromone to exceed threshold, it will perceive that a potential mate is nearby. If no directional cues are available, the optimal strategy is to gain information as quickly as possible. From the information obtained in this study, it appears that this is chiefly accomplished in two ways: (1) by initiating a looping local search pattern, and (2) by greatly decreasing the amount of immobile time per minute. These changes, probably coupled with a slight increase in running speed, permit the animal to maximize its chances of encountering useful information regarding directional cues by covering as much territory as possible within a given time period.

A male initially responds to a sex pheromone stimulus with increased locomotion. If it is already moving toward the source of the pheromone, it may soon reach a point at which orientation guided by chemolocation can be effectively utilized. When this occurs, the source is located very quickly without initiating local search. If, on the other hand, its path does not approach the source, a convoluted local search pattern is used until the male gets close enough to the source to switch to true chemotaxis, defined here as directional orientation toward an odor source. The length of time and the distance traveled between a male's initial increase in locomotion and its arrival at the pheromone source appear to be largely fortuitous. If a male tends to restrict its search to the outer portions of the arena, it may spend several minutes and expend a considerable amount of energy before it gets close enough to the source to utilize true chemotaxis. If its search takes it to within about 45 cm of the source soon after the initial response, it will usually locate the source much more quickly.

In a concentric odor field, the locomotory response of the males was somewhat different from that reported by Rust (1976). He found that the level of locomotory activity increased with increasing concentrations of sex pheromone. In the arena, running speeds increased by more than 10% from zone 4 to zone 3 but did not increase significantly from zone 3 to zone 2. There was a significant decrease in speed from zone 2 to zone 1 in spite of an increase in the concentration of the pheromone. Lower running speeds in the immediate vicinity of the pheromone source probably facilitate chemotactic orientation.

When the mean vectors were determined from the paths of male *P.*

americana orienting to a source of sex pheromone, the values declined steadily from zone to zone as the distance from the source increased, i.e., randomness increased with increased distance. Very little randomness was found in zone 1 while the randomness in the outer zones was rather high, primarily due to the convoluted patterns of local search. The low degree of randomness and greatly reduced running speeds in the inner zone and the direct approach ($\pm 3°$) to the source from distances of up to 68 cm suggest that, in still air, males of *P. americana* are able to orient chemotactically to a fixed pheromone source from at least 45 cm away.

Bell et al. (1973) did a study of *P. americana* orientation to aggregation pheromone in which crossing the insects' antennae led to movement away from the source, indicating tropotaxis. While males may be capable of utilizing tropotactic orientation when searching for a source of sex pheromone, it is unlikely that they have the opportunity at other than close range (Bell and Tobin, 1981). Honeybees require a 2:1 ratio of odor concentrations between the two antennae to permit tropotaxis (Martin, 1964), and it is possible that cockroaches have similar requirements. Neuhaus (1965) stated that ratios of this sort rarely occur more than a few centimeters from the source, but the rather long antennae of cockroaches permit a wide separation of receptors which would aid tropotaxis. Coupled with the likelihood that the pheromone source (= female cockroach) will not be stationary, the probability that tropotactic ability will be useful at long range declines rapidly.

If virgin females commonly move about while emitting sex pheromone, the task of the male is made that much more difficult. Preliminary experiments with unmated females indicate that they do not remain stationary for prolonged periods during hours 3 and 4 of the dark cycle but move about in a stop-and-go fashion similar to that of the males. The male must therefore locate the female quickly if he is to mate. If a male is unable to get close enough to utilize chemotactic orientation before the female moves, he may not find her, or another male may find her first. With one male and one virgin female in the arena, it is not uncommon to see a male approach a female only to have her run 25–30 cm away. When this happens, the male becomes disoriented and resumes local search. On the other hand, if the male gets within 20–30 cm of the female before she moves away, he is often able to follow her and catch up the first time she stops. Selection clearly favors those males adept at quickly locating females. The need for rapid location of females may be responsible for the high running speeds that occur at intermediate distances (30–90 cm) away from the pheromone source.

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FEEDING RESPONSES OF EASTERN SPRUCE BUDWORM[1] LARVAE TO SUCROSE AND OTHER CARBOHYDRATES[2]

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Abstract—Spruce budworm larvae reared on an artificial diet were tested for preference of twelve carbohydrates over a water control. A strong preference for sucrose was seen, followed by fructose, inositol, and glucose. Male and female larvae do not differ in their responses to sucrose. The behavioral threshold for sucrose is at 10^{-4} to 10^{-3} M, with a peak response in the range of 0.01 to 0.05 M sucrose.

Key Words—*Choristoneura fumiferana*, Lepidoptera, Tortricidae, carbohydrates, sucrose, feeding preference, behavior.

INTRODUCTION

Eastern spruce budworm larvae (*Choristoneura fumiferana* Clem.) are oligophagous insects which feed on a variety of evergreen tree species, but mainly on balsam fir (*Abies balsamea*) and white spruce (*Picea glauca*). The structure and innervation of their contact chemosensilla were reported by Albert (1980), who noted the presence of a sucrose-sensitive cell in the lateral uniporous peg (sensillum styloconicum) of each galea.

The behavioral feeding response of the larvae to some host-plant chemicals was studied by Albert and Jerrett (1981). They found that a

[1]*Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae).

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sugar/glycoside fraction from balsam fir needle extracts was more stimulating than any of the other chemicals they tested, which included amino and organic acid extracts of balsam fir, as well as ethanol and petroleum ether-soluble components of various evergreens.

Sucrose is the predominant carbohydrate found in the shoots of actively growing balsam fir (Little, 1970). The importance of sucrose and other carbohydrates in feeding by other insects is well known (cf. Dethier, 1966; Dethier and Kuch, 1971; Flowers et al., 1975; Hsaio, 1969; Ishikawa, 1967; Ma, 1972; Schoonhoven, 1968; Schoonhoven and Dethier, 1966; Stadler and Hanson, 1978; Wensler and Dudzinski, 1972).

The present study was done to assess the importance of sugars in spruce budworm feeding behavior and to determine the range of their sensitivity to sucrose, which is known to be an important feeding stimulant for these larvae (Heron, 1965). This study and that of Heron (1965) will form the basis for a more detailed analysis of the insect–host-plant relationship in this species.

METHODS AND MATERIALS

The experimental animals were obtained as second-instar larvae from the Maritimes Forest Research Center, Fredericton, Canada. They were reared on an artificial diet (McMorran, 1965) Sixth-instar larvae, ≤ 1 day postmoult, were starved for 24 hr prior to testing. Those used in tests to determine the effects of starvation on the behavioral response were starved for varying lengths of time as specified in the results.

Choice tests were modified from Jermy et al. (1968) using 6.5-mm-diameter disks punched from Millipore HA 0.45-μm cellulose filters. Disks were held on color-coded pins 2 mm above the floor of a styrofoam sheet. They were arranged to form a circle, alternating experimental and control disks in a-b, etc., fashion for two-choice tests, and a-b-c, a-b-c, etc., fashion for three-choice tests. Eight disks were used in two-choice tests, nine were used in three-choice tests. They were covered with a 3.5-cm-diameter by 1-cm-high Petri dish after wetting with a 15-μl aliquot of the chemical solution to be tested. Disks treated with an equal aliquot of distilled, deionized H_2O alone served as controls. Groups of approximately 20 test arenas were placed on the same styrofoam sheet, and the whole set-up was covered with a layer of cellophane to prevent water loss from the disks. One larva was used in each test arena. Test chambers were placed in a 28° incubator with a 16-hr day. Tests averaged about 24 hr duration. At the end of the test period, the area eaten from each disk was visually estimated and recorded as disk equivalents (area of one disk = one equivalent). Data from animals which ate too little or too much were discarded (<0.5 or >3.5 disk equivalents in the two-choice tests; <0.5 or >2.5 disk equivalents in the three-choice tests) since these data are most likely biased.

The actual amounts of material eaten, whether large or small, did not affect the overall preference of animals. For each experiment, the percentage of the total amount consumed was determined for each test substance. Since the distribution of the data did not conform to a normal distribution when analyzed with the graphic Rankits test (Sokal and Rohlf, 1969), nonparametric statistics were used. Two-choice test results were analyzed with Wilcoxon's signed-ranks test; those of three-choice experiments were analyzed with the simultaneous test procedure with a significance level for rejection of the null hypothesis set a $P = 0.05$ (Sokal and Rohlf, 1969). Individual t tests on means with unequal variances were performed on the results of male versus female experiments.

RESULTS AND DISCUSSION

Since distilled, deionized H_2O was used on control disks in all our experiments, a double-control experiment was done to determine whether larvae would show a preference for a particular disk position in the experimental area. All disks were wetted with 15 μl of distilled, deionized H_2O. Half of them were held on orange-colored pins, half on blue pins. Feeding responses were the same on all disks, with no preference shown for pin color or disk position (Figure 1a).

To determine whether the duration of starvation of experimental animals had any effect on feeding preferences, two-choice tests were performed using animals starved for 0, 3, 6, 21, and 24 hr, with 0.025 M sucrose and H_2O as the test stimuli. Unstarved animals showed a slight preference for sucrose-treated disks (Figure 1b), but the preference for sucrose was stronger in animals starved for 3, 6, 21, or 24 hr. No significant differences were seen among the starved animals; that is, animals starved for 24 hr do not show different preferences than those starved for only 3 hr. Subsequently, all larvae were starved 24 hr.

Sexual differences in the feeding behavior of caterpillars were similarly investigated using randomly selected larvae in three-choice tests. Following the test, these larvae were reared to pupation and then sexed. No significant differences were found between the responses of males and females (Figure. 1c).

Preferences of budworms for 12 common carbohydrates at equivalent concentrations (0.025 M) were determined using two-choice tests. Sucrose proved to be the most stimulating while L-arabinose, D-sorbitol, and melibiose were nonstimulatory (Figure 2). Several other carbohydrates found in plants and known to stimulate other insects also were found to be stimulatory (e.g., fructose, inositol, and glucose). Different responses to α- and β-D-glucose were probably due to impurities, since an equilibrium of both forms should have been reached by rotation in the aqueous solution. The preference for

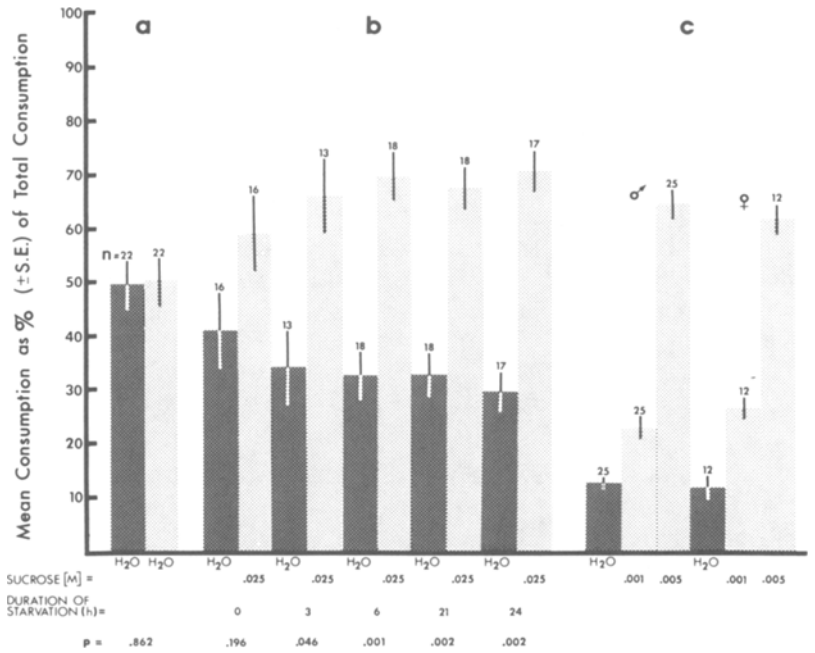

FIG. 1. (a) Control experiment using H_2O-treated disks in a two-choice situation; P = result of Wilcoxon's signed-ranks test; (b) two-choice tests using H_2O vs. 0.025 M sucrose for unstarved animals, and animals starved for 3, 6, 21, and 24 hr; P = results of Wilcoxon's signed-ranks test; (c) three-choice tests using H_2O, 1 mM and 5 mM sucrose for male and for female larvae; no significant differences were found between male and female responses to H_2O, 1 mM and 5 mM sucrose (t tests).

sucrose when compared to other carbohydrates confirms the results of Albert and Jerrett (1980), where frass weight was used as a measure of the feeding response. It supports the conclusion of Heron (1965) that sucrose is an important feeding stimulant for these larvae. He tested sucrose at concentrations from 4 mM to 0.5 M using disks of Japanese elder pith which were impregnated with the solution, then allowed to dry before testing. His results showed slightly more feeding at 0.1 M original sucrose concentration than at 4 mM, 0.02 M and 0.5 M. Only a small amount of feeding occurred in his experiments in a 48-hr test period: the mean percent area of disks eaten ranged from 0.7 to 4.2%. In contrast, our results show feeding on individual disks ranging from 0 to 100%, depending on the solution used in only a 24-hr test period. Another difference is that we found a peak preference in the range of 0.01–0.05 M (Figure 3b). Apparently, the system employed here (thin cellulose filter disks with test compounds in solution) was more stimulating for budworm larvae than dry compounds on elder pith. A system similar to

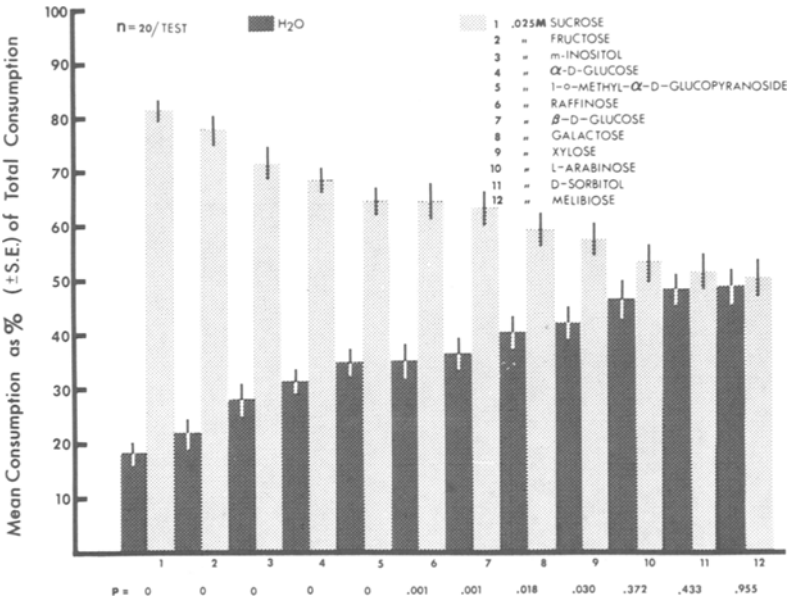

FIG. 2. Results of feeding preferences for several carbohydrates, each at a concentration of 0.025 M in two-choice tests; P = results of Wilcoxon's signed-ranks test.

ours was successfully employed by Bristow et al. (1979) for preference studies on adults of the weevil, *Sciopithes obscurus*.

In view of the pronounced effect of sucrose on feeding behavior, further analyses were done on responses to this stimulant. Data from individual three-choice tests using various concentrations of sucrose are shown in Figure 3b. With 0.01–0.05 M sucrose, larvae ate the largest percentages of the treated disks, indicating that their peak preference lies within this range of concentrations. A separate study using two-choice tests was done to establish the behavioral threshold concentration; this was found to be between 10^{-4} and 10^{-3} M sucrose (Figure 3a). The peak response between 0.01 and 0.05 M sucrose corresponds closely to the relative sucrose concentration in the diet on which the animals were reared (0.029 M). Using data on moisture content and milligrams of sucrose per gram of oven-dried needles (current growth, collected in mid-June) from Little (1970), we find a sucrose concentration equivalent to approximately 0.037 M in the host plant (balsam fir) at a time of year when larvae in their sixth instar are actively feeding on this material. This corresponds closely to the range of sucrose concentrations preferred by larvae in our tests.

The foregoing clearly demonstrate that carbohydrates, and especially sucrose, are potent feeding stimulants for spruce budworm larvae. This is in

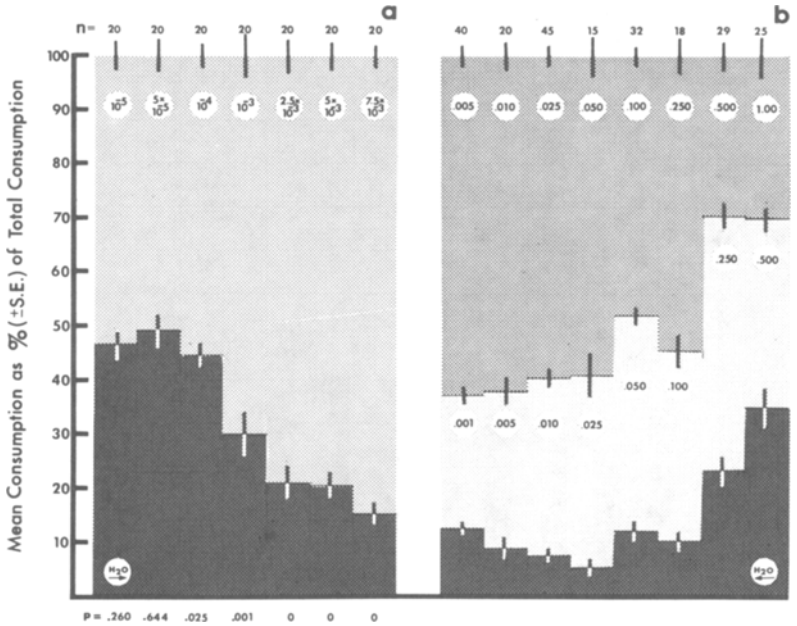

FIG. 3. (a) Two-choice tests using various concentrations of sucrose vs. H_2O to determine the threshold concentration of sucrose; P = results of Wilcoxon's signed-ranks test; numbers in white circles refer to sucrose concentrations (M); (b) three-choice tests using various sucrose concentrations (M, as indicated in white circles) and H_2O; homogeneous subsets identified using the simultaneous test procedure ($P = 0.05$) are: H_2O and 0.5 M sucrose; H_2O, 0.5 M and 1.0 M sucrose.

agreement with the data from many other insects (Dethier, 1966), which show that sucrose is a fairly universal stimulant. The present data will provide a useful data base with which to compare responses of larvae to other host plant chemicals and for correlating the results of the animal's behavior with electrophysiological experiments on the sucrose-sensitive cell of the lateral uniporous peg sensilla of the spruce budworm galea.

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TERPENES OF PONDEROSA PINE AND FEEDING PREFERENCES BY POCKET GOPHERS

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Abstract—Yield and composition of essential oils were compared in foliage, stems, and roots of ponderosa pine seedlings, and preferences for the trees by pocket gophers were determined. Test seedlings represented nine widely separated provenances in the western United States. Seed source of the trees influenced gopher feeding preferences and resulted in varied tree damage. The damage ranged from 0 to 31%, suggesting that some sources might possess sufficient natural resistance to give trees practical protection from gophers in the field. There were no morphological differences among sources to explain differential tree damage. All sources contained essential oils in all tissues examined, but oil yield varied among and within tissue types. Oils were predominantly (76–97%) composed of monoterpene hydrocarbons. Oil composition varied by source, and different tissue types varied greatly in the yield and composition of their oils. Neither yield nor constituents of foliage oils were significantly correlated with gopher damage (or preference). In contrast, some components of stem and root oils were strongly related to preference. Results of correlation and discriminant analyses showed that some oil constituents could serve as indicators of resistance (or susceptibility) to gopher damage. Such important chemical variables, when verified, could be used in selections for ponderosa pine resistant to gophers.

Key Words—Terpenes, essential oils, gopher damage, gopher feeding preference, *Pinus ponderosa*, *Thomomys* spp.

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INTRODUCTION

Ponderosa pine (*Pinus ponderosa* Laws.) is the most important conifer in interior forests of the western United States. In some areas, however, attempts to regenerate the species have repeatedly failed because of pocket gophers (Geomyidae). Gophers damage and kill trees of ponderosa pine and other coniferous species, mostly by barking and root pruning (Moore, 1940; Dingle, 1956; Tevis, 1956; Hermann and Thomas, 1963; Crouch, 1969, 1971; Barnes et al., 1970).

Gophers may be controlled by poisoning (Crouch, 1933; Barnes et al., 1970), caging individual trees (Anthony et al., 1978), or application of herbicides which reduce the animals' food supply (Crouch, 1979). Potential control methods based on natural resistance of crop trees would be more environmentally or esthetically acceptable. Development of such methods requires determination of the gophers' preferences for the trees and the factors involved.

Natural resistance of herbivores occurs among and within plant species, and essential oils and their terpenoid components have been prominent among chemical factors postulated to influence the animals' feeding preferences (Radwan, 1974). For ponderosa pine, within-species variations in resistance have been observed with many animals other than gophers (Squillace and Silen, 1962; Read, 1971). Work with gophers, however, has been limited to one unpublished study. In laboratory tests, Cummins, (1975) found that gophers consumed significantly different amounts of ponderosa pine seedlings from various sources. The gophers also showed varied preferences for the different parts of seedlings as follows: stems > roots > needles > terminal buds.

The purpose of this study was to elaborate on the differential preferences of pocket gophers for ponderosa pine and to determine relationships between the pines' terpenes and observed preferences. We used progeny of nine widely separated provenances of ponderosa pine, tested preferences for the seedlings in gopher-proof enclosures, and determined yield and composition of essential oils isolated from different parts of the trees.

METHODS AND MATERIALS

Test Seedlings. Open-pollinated seeds were obtained from natural stands in nine national forests, covering most of the range of the species in the western United States. In January 1974, seeds, stratified at 3–5° C for 3 weeks, were sown at 3-mm depth in styroblock containers filled with 1:1:1 (by volume) mixture of peat moss, vermiculite, and Tumwater sandy loam soil. Containers were placed in a greenhouse where fluorescent light was available

during a 12-hr photoperiod and temperatures were maintained at about 20° C. After germination was complete, seedlings were thinned to one per styroblock compartment. Seedlings were watered when necessary and supplied with a dilute solution of a complete fertilizer at biweekly intervals through May when they were placed outdoors. In September, seedlings were randomly selected from each source for use in the gopher preference tests at Moscow, Idaho, and chemical analysis in Olympia.

Preference Test. In November 1974, test trees were planted in each of three 0.1-hectare gopher-proof enclosures located on the University of Idaho farm at Moscow. Each source was represented by 20 trees in each enclosure and trees were planted at approximately 1.5- × 1.5-m spacing in a completely random design. Seedlings were allowed to get established in the enclosures for 10 months. In September 1975, locally trapped gophers (*Thomomys* spp.) were placed in the enclosures, and the test was run until the end of April 1976. During that time, the enclosures contained some natural forage, and the animals had free access to the trees. Inspection for gopher feeding on the seedlings and seedling mortality was made periodically. Relative preference was ranked according to the percent of trees clipped or gnawed by gophers.

Processing Plant Material. Ten seedlings of each of the nine sources were used for chemical analysis. In January 1975, seedlings of each source were randomly divided into two samples of five seedlings each. Terminal buds were removed and discarded; and the seedlings were then divided into roots, stems, and foliage by severing each tree at the root collar and just below the live needles. Roots were washed free of soil and blotted with absorbent paper to remove surface moisture. The cut seedlings were pooled by part and sample; individual parts were thoroughly mixed, weighed, and subsampled for moisture determination and distillation of essential oils.

Chemical Analysis. Isolation and analysis of the oils have been described in detail before (Radwan and Crouch, 1978). Briefly, essential oils were obtained by steam distillation and collection in *n*-heptane. Oil solutions were analyzed by gas–liquid chromatography (GLC) using flame ionization detection and open tubular columns. Compounds were identified by their retention times, infrared spectra, and peak enrichment. Compounds were quantified by electronic integrator. Average oil yields per gram of tissue and percent composition of the oils were calculated based on two samples and two injections per sample.

Statistical Analysis. Gopher damage for each source was calculated by averaging the percent damage from the three gopher enclosures. Chemical composition for each source was determined by averaging the values of the two samples for each source and tissue type.

Individual relationships between each of the chemical variables measured in the different parts of the seedlings and percent gopher damage by source

were evaluated by calculating the appropriate correlation coefficients (r) (Snedecor, 1961). Results were considered significant at $P < 0.10$.

Multivariate relationships between the chemical and damage variables were examined using stepwise discriminant analysis (Dixon, 1977). For this analysis, the populations were divided into two groups based on percent damage by gophers—low damage, 0–12%, and high damage, 16–31%. Stepwise discriminant analysis was also used to determine the chemicals which distinguished between the three different parts of seedlings. In this analysis, we assumed that the observations of each of the three tissue types were independent, when in fact they were related. This assumption, however, seemed reasonable in an analysis used for screening of variables. The F values for including variables in both discriminant functions was $P \leqslant 0.01$.

RESULTS AND DISCUSSION

Gopher Preference. The gophers fed on seedlings of all sources except those from Utah (#9) (Table 1). Like other animals (Squillace and Silen, 1962; Read, 1971), gophers discriminated among the pine sources tested, with damage ranging from 0 to 31%. In general, the gophers favored seedlings of sources from Arizona (#6), Washington (#7), Montana (#11), and Nebraska (#17) over seedlings of other sources. The least and most preferred trees were those from Utah (#9) and Arizona (#6), respectively. There were no obvious morphological differences among sources to explain the differential damage or the apparent variations in gopher preference observed.

Variations between sources of ponderosa pine, therefore, influenced the gophers' feeding preference and resulted in much varied tree damage. The 31%

TABLE 1. POCKET GOPHER DAMAGE TO DIFFERENT SOURCES OF PONDEROSA PINE SEEDLINGS IN AN ENCLOSURE FIELD TEST AT MOSCOW, IDAHO

Source identification number	National forest	Location	Gopher damage (%)
6	Coconino	central Arizona	31
7	Colville	northeastern Washington	16
9	Dixie	southcentral Utah	0
10	El Dorado	central California	10
11	Helena	western Montana	21
17	Niobrara	central Nebraska	17
18	Rogue River	southwestern Oregon	12
19	Roosevelt	northcentral Colorado	7
21	Umatilla	northeastern Oregon	12

TABLE 2. YIELD AND GROSS COMPOSITION OF ESSENTIAL OILS OF PONDEROSA PINE

Item	Oil source[a]	Source identification number								
		6	7	9	10	11	17	18	19	21
Yield (area × 10^6)[b]										
Monoterpene	F	4.30	4.95	4.31	4.07	3.62	5.33	4.15	4.69	3.01
hydrocarbons	S	1.32	2.40	2.58	1.72	4.67	2.49	1.00	2.27	1.93
	R	4.15	3.24	2.37	2.32	4.70	3.90	1.77	1.73	2.99
Oxygenated	F	1.38	0.16	0.94	0.28	0.16	0.76	0.20	0.56	0.11
monoterpenes	S	0.21	0.16	0.19	0.19	0.30	0.50	0.13	0.54	0.20
	R	1.09	0.40	0.46	0.33	0.48	0.82	0.18	0.50	0.34
Total	F	5.68	5.11	5.25	4.35	3.78	6.09	4.35	5.25	3.12
	S	1.53	2.56	2.77	1.91	4.97	2.99	1.13	2.81	2.13
	R	5.24	3.64	2.83	2.65	5.18	4.72	1.95	2.23	3.33
Composition (%)										
Monoterpene	F	75.70	96.87	82.10	93.56	95.77	87.52	95.40	89.33	96.47
hydrocarbons	S	86.27	93.75	93.14	90.05	93.96	83.28	88.50	80.78	90.61
	R	79.20	89.01	83.75	87.55	90.73	82.63	90.77	77.58	89.79
Oxygenated	F	24.30	3.13	17.90	6.44	4.23	12.48	4.60	10.67	3.52
monoterpenes	S	13.72	6.25	6.86	9.95	6.04	16.72	11.50	19.22	9.39
	R	20.80	10.99	16.25	12.45	9.27	17.37	9.23	22.42	10.21

[a] F = foliage, S = stem, R = root.
[b] Area in arbitrary units determined by electronic integrator and calculated per gram dry tissue.

TABLE 3. MONOTERPENE HYDROCARBONS OF ESSENTIAL OILS OF PONDEROSA PINE

Component composition (%)[a]	Oil source[b]	Source identification number								
		6	7	9	10	11	17	18	19	21
α-Pinene	F	30.28	12.13	19.44	10.41	12.08	14.58	9.69	12.44	10.88
	S	37.01	6.48	18.34	5.49	5.48	7.11	5.64	6.72	5.12
	R	31.09	6.78	16.58	7.41	6.19	7.85	8.03	7.04	7.06
Camphene	F	1.48	1.40	0.94	0.80	0.59	1.19	0.57	1.00	0.46
	S	0.52	0.60	1.03	0.58	0.29	1.07	0.50	0.51	0.21
	R	0.87	0.65	1.03	1.03	0.37	1.16	0.89	0.45	0.44
Unknown 6	F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	S	0.12	0.24	0.26	0.64	0.40	0.30	0.46	0.38	0.34
	R	0.72	1.37	0.75	2.64	1.67	1.07	1.76	1.13	1.24
β-Pinene + sabinene	F	20.25	38.05	27.84	33.95	39.69	33.65	42.26	34.10	36.09
	S	3.03	20.76	27.90	27.44	18.05	30.71	25.81	23.79	15.64
	R	7.30	22.81	21.81	33.61	25.97	30.38	30.57	25.22	25.29
3-Carene + myrcene	F	13.80	35.64	25.82	37.66	34.31	26.56	34.26	30.02	38.05
	S	38.25	54.66	38.31	42.33	53.61	34.27	46.74	37.91	50.92
	R	32.68	48.50	37.95	31.15	45.35	34.06	40.46	35.95	44.38
α-Terpinene	F	0.17	0.36	0.18	0.42	0.26	0.24	0.24	0.30	0.26
	S	0.22	0.39	0.30	0.26	0.45	0.33	0.27	0.24	0.33
	R	0.32	0.39	0.25	0.32	0.32	0.30	0.31	0.20	0.34

Limonene	F	4.41	2.51	2.57	3.27	2.13	3.65	1.89	4.49	2.84
	S	3.07	2.67	2.51	5.52	5.55	3.34	2.61	4.97	5.89
	R	2.32	2.40	1.96	6.02	4.70	2.74	3.23	3.28	4.27
β-Phellandrene	F	3.04	1.72	1.78	1.62	1.74	3.33	1.65	2.39	1.55
	S	0.81	1.27	1.33	1.26	1.63	1.88	1.04	1.84	1.49
	R	0.83	1.17	1.01	1.38	1.32	1.52	1.21	1.19	1.28
Ethyl caproate	F	0.18	0.02	0.10	0.00	0.00	0.40	0.00	0.54	0.00
	S	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	R	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
γ-Terpinene	F	0.36	0.71	0.46	0.68	0.67	0.49	0.62	0.52	0.67
	S	0.36	0.71	0.38	0.57	0.66	0.51	0.51	0.50	0.76
	R	0.41	0.50	0.31	0.39	0.41	0.39	0.41	0.30	0.51
p-Cymene	F	0.14	0.21	0.10	0.26	0.12	0.14	0.12	0.14	0.12
	S	0.21	0.24	0.29	0.11	0.26	0.29	0.14	0.16	0.15
	R	0.40	0.33	0.29	0.28	0.28	0.28	0.33	0.22	0.25
Terpinolene	F	1.57	4.12	2.78	4.57	4.23	3.37	4.06	3.34	5.56
	S	2.94	5.85	2.54	5.82	7.58	3.41	5.02	3.74	9.91
	R	2.32	4.32	2.32	3.41	4.19	2.64	3.48	2.11	4.69

[a]Percent of total terpenes.
[b]F = foliage, S = stem, R = root.

TABLE 4. OXYGENATED MONOTERPENES OF ESSENTIAL OILS OF PONDEROSA PINE

Component composition (%)[a]	Oil source[b]	Source identification number								
		6	7	9	10	11	17	18	19	21
Unknown 20	F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	S	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	R	0.66	0.74	0.44	0.73	0.42	0.44	0.70	0.40	0.46
Fenchyl alcohol	F	8.36	0.25	2.45	0.97	0.40	2.32	0.66	1.43	0.28
	S	9.46	2.02	4.82	4.76	1.25	11.38	3.10	11.50	2.20
	R	13.99	3.08	10.99	4.10	2.56	11.62	1.98	15.62	2.73
Terpinen-4-ol	F	0.46	0.58	0.51	0.76	0.67	0.70	0.63	0.79	0.77
	S	0.31	0.44	0.16	0.73	0.66	0.59	0.76	0.75	1.22
	R	0.58	0.47	0.41	0.45	0.43	0.44	0.49	0.37	0.44
Unknown 31	F	9.36	0.13	7.44	0.37	0.19	0.00	0.49	0.00	0.00
	S	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	R	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unknown 33	F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	S	0.51	0.26	0.39	0.33	0.22	0.43	0.64	0.65	0.25
	R	0.52	0.79	0.41	0.90	0.60	0.43	0.65	0.64	0.68
Citronellyl acetate	F	1.04	0.53	1.02	0.69	0.60	0.65	0.56	1.42	0.48
	S	0.27	0.20	0.00	0.40	0.08	0.49	0.00	0.89	0.18
	R	0.38	0.20	0.27	0.14	0.11	0.28	0.02	0.37	0.05

Estragole	F	0.00	0.19	0.00	0.07	0.14	0.02	0.11	0.11	0.10
	S	0.00	0.98	0.00	0.00	0.31	0.00	0.81	0.00	0.59
	R	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
α-Terpineol	F	0.99	0.96	1.63	2.12	1.63	1.57	1.94	1.69	1.29
	S	0.31	0.38	0.41	1.82	0.58	1.10	1.44	1.22	1.13
	R	1.06	1.63	1.23	3.02	1.10	1.86	2.49	1.83	1.17
Borneol	F	0.23	0.16	0.00	0.00	0.28	1.50	0.23	1.22	0.16
	S	0.33	0.44	0.00	0.00	1.03	1.67	1.23	1.59	1.10
	R	0.36	0.33	0.06	0.00	0.21	0.71	0.22	0.86	0.22
Unknown 41	F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	S	0.32	0.32	0.80	0.00	0.37	0.61	1.27	0.74	0.12
	R	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unknown 43	F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	S	2.00	1.14	0.28	1.98	1.48	0.09	2.07	1.46	2.39
	R	0.86	2.31	0.48	1.84	3.03	0.38	1.92	1.04	3.40
Other unknowns	F	3.89	0.39	4.99	1.42	0.30	5.72	0.04	4.10	0.46
	S	0.00	0.00	0.00	0.00	0.12	0.46	0.00	0.49	0.12
	R	2.38	1.30	1.48	1.23	0.84	1.51	0.90	1.86	1.17

[a]Percent of total terpenes.
[b]F = foliage, S = stem, R = root.

maximum difference in damage suggests that some ponderosa pine sources might possess sufficient resistance to give the trees appreciable protection from gophers in the field.

Yield and Composition of Essential Oils. Total yield of essential oils as well as yields of the monoterpene hydrocarbons and oxygenated monoterpenes are shown in Table 2. Total yield per gram dry tissue was lowest in the stems and highest in the foliage. Also, total yield of each tissue type varied by source.

As expected, the oils of the different seedling parts were predominantly (76–97%) composed of monoterpene hydrocarbons. This agrees with results obtained by others with ponderosa pine needle oil (Zavarin et al., 1971). Results also indicate that, on the basis of the monoterpenoid hydrocarbons and oxygenated compounds, ponderosa pine oil is similar to that of other conifers, such as Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Radwan and Crouch, 1978) and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) (von Rudloff, 1975).

The oils of the different parts of seedlings contained some 30 compounds each, but many were present in small or trace amounts (Table 3 and 4). Most abundant compounds present in the oils were the monoterpene "hydrocarbons" α-pinene, β-pinene + sabinene, 3-carene + myrcene, limonene, and terpinolene, and the oxygenated monoterpenes fenchyl alcohol and α-terpineol.

Composition of the oils differed by source. The sources, however, tended to fall roughly into four geographical groups as follows: northern, sources 7, 11, and 21; west-central, sources 10 and 18; eastern, sources 17 and 19; and southern, sources 6 and 9. Within each of the four groups, oils of one or more seedling parts had similar levels of some terepenes. For example, the northern sources were high in 3-carene + myrcene and γ-terpinene and low in fenchyl alcohol. In contrast, the southern sources were high in α-pinene and fenchyl alcohol and low in γ-terpinene, terpinolene, and α-terpineol.

Oil Composition of Different Tissues. Averaged over the nine sources, different tree parts varied greatly in the yield and chemical composition of their oils. Nineteen chemical variables had F values which were significant at $P \leqslant 0.05$ or $P \leqslant 0.01$ (Table 5). Differences in oils, however, were mostly quantitative, with qualitative differences noted in only seven components. These compounds, which were not always detected in oils of all tissue types, include unknowns 6, 20, 33, 41, and 43, ethyl caproate, and estragole.

The six most important variables which distinguished between the three tissue types were p-cymene, citronellyl acetate, and unknowns 6, 20, 33, and 41. The first variable in the discriminant function was unknown 20. This component, however, did not distinguish between stems and foliage because it was not detected in either tissue. The discriminant function with unknowns 20 and 33 correctly distinguished between the three tissues.

TABLE 5. CHEMICAL VARIABLES SHOWING SIGNIFICANT DIFFERENCES BETWEEN DIFFERENT PARTS OF PONDEROSA PINE SEEDLINGS

Item[a]	Mean value[b]			F value	Statistical significance[c]
	Foliage	Stem	Root		
Unknown 6	0.00	0.35	1.37	36.6	**
β-Pinene + sabinene	33.99	21.46	24.77	6.6	**
3-Carene + myrcene	30.68	44.11	38.94	8.0	**
β-Phellandrene	2.09	1.39	1.21	9.4	**
Ethyl caproate	0.14	0.00	0.00	4.2	*
γ-Terpinene	0.58	0.55	0.40	6.0	**
p-Cymene	0.15	0.21	0.30	14.6	**
Terpinolene	3.73	5.20	3.28	3.4	*
Unknown 20	0.00	0.00	0.55	126.8	**
Fenchyl alcohol	1.90	5.61	7.41	3.9	*
Unknown 33	0.00	0.41	0.62	52.4	**
Citronellyl acetate	0.78	0.28	0.20	13.3	**
Estragole	0.08	0.30	0.00	4.0	*
α-Terpineol	1.54	0.93	1.71	5.0	*
Unknown 41	0.00	0.51	0.00	15.0	**
Unknown 43	0.00	1.43	1.70	12.3	**
Other unknowns	2.37	0.13	1.41	6.2	**
MTH yield	4.27	2.26	3.02	10.2	**
Total terpene yield	4.78	2.53	3.53	9.2	**

[a]MTH = monoterpene hydrocarbons.
[b]Means of all nine sources. Yield = area ($\times 10^6$) in arbitrary units determined by electronic integrator and calculated per gram dry tissue. All other values are percents.
[c]*, $P \leq 0.05$; **, $P \leq 0.01$.

Some of the discriminating properties which separated the tissues indicated that important characteristics of the oils were: high concentrations of *p*-cymene, and unknowns 6, 20, and 33 in the roots; medium levels of *p*-cymene, citronellyl acetate, and unknowns 6 and 33 in the stem; and absence of unknowns 6, 20, 33, and 41 from the foliage.

Relationships of Oil Yield and Composition to Gopher Preference. Correlation analysis between yield and chemical components of the oils and gopher preference, as measured by observed gopher damage to seedlings, resulted in six significant correlation coefficients. Neither yield nor constituents of foliage oils were significantly correlated with feeding preference. In contrast, the β-pinene + sabinene component of stem oils was negatively related to preference ($r = -0.74$, $P \leq 0.05$). The other five significant correlations involved root oils. All were positive and included *p*-cymene ($r = 0.65$, $P \leq 0.10$), terpinen-4-ol ($r = 0.76$, $P \leq 0.05$), yield of monoterpene hydrocarbons ($r = 0.77$, $P \leq 0.05$), yield of oxygenated monoterpenes ($r = 0.66$, $P \leq 0.10$), and total terpene yield ($r = 0.80$, $P \leq 0.01$).

Discriminant analysis of the high- and low-gopher-damage seedlings showed that total terpene yield of root oils was the variable most closely associated with preference; the average yield was almost twice as high in the high-damage sources as that of the low-damage trees. The discriminant function with total terpene yield of root oils and monoterpene hydrocarbon yield in foliage oils correctly classified all sources in their correct damage group. Association of the monoterpene hydrocarbon and total terpene yields with feeding preference is in agreement with results of the correlation analyses.

Results of the correlation and discriminant analyses suggest important possible practical applications. For example, the β-pinene + sabinene component of stem oil, which was negatively correlated with preference may be useful as a measure of pine resistance to damage by gophers. On the other hand, high levels of p-cymene, terpinen-4-ol, monoterpene hydrocarbon yield, oxygenated monoterpene yield, and total terpene yield in root oils might serve as indicators of potential high susceptibility to damage.

In general, the components which best distinguished between the different tree parts (Table 5) were not the same as those associated with gopher damage in the previous analysis. Some of these components, however, could be influential in determining feeding preference. Of particular importance is the component β-pinene + sabinene. This component, which was negatively correlated with gopher damage, was also found in highest levels in the foliage, the least preferred seedling tissue (Cummins, 1975).

CONCLUSIONS

This study helped generate hypotheses concerning the relationships between yield and composition of oil and damage by gophers in ponderosa pine seedlings. Additional research in now needed to confirm these relationships. Chemical variables associated with resistance (or low preference), when verified, could be used for indirect selection of planting or breeding stock suitable for areas where pocket gophers are a serious obstacle to reforestation of ponderosa pine. Alternatively, some of the same components could be tested for biological activity and possible development as repellents.

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SEX PHEROMONE OF THE AVOCADO PEST, *Amorbia cuneana* (WALSINGHAM) (LEPIDOPTERA: TORTRICIDAE): Structure and Synthesis[1]

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Abstract—The major volatile components in the extract of the female sex pheromone gland of *Amorbia cuneana* consisted of (*E,E*)- and (*E,Z*)-10,12-tetradecadien-1-ol acetates. The identification was based on electroantennogram bioassay of gas chromatographic effluent from sex pheromone gland extract, relative retention times on polar and nonpolar gas chromatographic columns, chemical degradation (ozonolysis, saponification), mass spectrometry, chemical synthetic methods, and field tests. Based on mass spectrometry and retention times by capillary gas chromatography, traces of (*E*)-10-tetradecen-1-ol acetate and 1-tetradecanol acetate were also present in the extract. Traps baited with a combination of synthetic (*E,E*)- and (*E,Z*)-10,12-tetradecadien-1-ol acetates caught more males than did traps baited with females.

Key Words—Western avocado leafroller, *Amorbia cuneana*, sex pheromone, Tortricidae, (*E,E*)- and (*E,Z*)-10,12-tetradecadien-1-ol acetate, Lepidoptera.

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

INTRODUCTION

In California, an estimated 1.3×10^8 kg of avocados are produced annually on approximately 2.2×10^4 hectares (Rock and Platt, 1978). In the autumn of 1976, one of us (J.B.B.) initiated the development of an integrated pest management program for the four-pest complex of this crop consisting of *Amorbia cuneana* (Walsingham) (western avocado leafroller), *Sabulodes aegrotata* (Guenée) (omnivorous looper), *Heliothrips haemorrhoidalis* (Bouché) (greenhouse thrips), and *Oligonychus punicae* (Hirst) (avocado brown mite). Subsequently, as part of this program a study of the structures of the sex pheromones of *A. cuneana* and *S. aegrotata* were undertaken in order to provide attractant-baited traps for population monitoring of these insects. Here we report the isolation, determination of structure, and synthesis of the sex pheromone of *A. cuneana*.

METHODS AND MATERIALS

Insects

Pupae or larvae were collected from avocado groves and the larvae were reared in a greenhouse on a diet developed for *Trichoplusia ni* (Hübner) (Shorey 1963) at the University of California's South Coast Field Station, Santa Ana. The pupae were separated according to sex and sent to Yakima, Washington, where they were placed in a bed of moist vermiculite in a room at $21 \pm 1°C$, at a relative humidity of $68 \pm 2\%$, and a photoperiod of 14 hr light and 10 hr dark. Emerging moths were caged daily. Adults were maintained on a diet of beer, sucrose, and ascorbic acid (Calkins and Sutter, 1976).

Collection of Pheromone

Female moths (2–4 days old) were collected 7–10 hr after the beginning of scotophase and placed in a refrigerator for 10–60 min prior to dissection. Pheromone was obtained by extracting abdominal tips (McDonough and Kamm, 1979) or excised epithelial tissue containing the pheromone gland (McDonough et al., 1980). These methods were of equal efficacy and produced 30–110 ng of pheromone per female.

Chromatography

The following gas chromatography analytical columns were used: (1) 1.8 m × 2.3 mm OD silanized glass column containing 3% polydimethyl siloxane (SE-30®) on 80/100 mesh Gas Chrom Q® operated at 135° or 160° C; (2) same as 1 except the liquid phase was 5% polyethylene oxide (Carbowax 20M®) operated at 175° C; (3) 30 m × 0.25 mm ID glass capillary column coated with

Carbowax 20M operated in the splitless mode with the following program: 40 sec delay on inlet purge; 2 min at 80°; 32°/min to 180°; samples in heptane solvent; and (4) same as 3 except the column was 60 m and was operated at 200°C.

Retention times were determined on packed columns relative to standard saturated homologs. Retention indexes (Kovats, 1965) were determined for products of the ozonolysis experiment. In the ozonolysis experiment, appropriate analytical standards were not available, and the retention index allowed the ozonolysis product to be identified. Because of the temperature program used with the capillary column, relative retention times did not have the same meaning as they did on packed columns. Thus, absolute retention times of test compounds and appropriate standards are listed separately for the capillary columns.

Liquid chromatography was conducted on silica gel (2% added water) in 28 × 3.3 cm OD column with 9:1 hexane–ether as the elution solvent.

Purification of Sex Pheromone Gland Components

Purification of sex pheromone gland components (substance X or Y) from extracts was effected by collection from a gas chromatograph (Hewlett-Packard, model 5711, Avondale, Pennsylvania) equipped with a flame ionization detector and a column effluent splitter (one part of effluent to detector and 10 part to collection trap). Column (1) was used. Eluting materials were collected with an efficiency of 40–70% in glass tubing cooled in dry ice-acetone.

Electroantennogram Analysis (EAG)

Natural, synthetic, and isomers of synthetic pheromone samples were bioassayed by the EAG procedure as described previously (Kamm and McDonough, 1980).

Ozonolysis

Ozonolysis of purfied substance X was conducted in predistilled dichloromethane at −70°C with an ultraviolet ozonizer (Orec Co. model 03VI, Phoenix Arizona) and the ozonide was reduced with triply recrystallized triphenylphoshine. The ozonized sample was injected on column (1) at 80° for 2 min, programed at 4°/min to 190° and held at that temperature. It was also injected on column (2) at 155°C.

Saponification

Purified substance X from extract of 7 female glands was dissolved in 200 μl of 1 M KOH in 9:1 methanol–water. After 2 hr, 200 μl of dichloromethane

and 200 μl of water were added. Then the dichloromethane was removed with a syringe and the methanol–water solution was extracted twice more with 200 μl of dichloromethane. The combined extracts were concentrated to 100 μl and used directly for the packed GC column analysis. For capillary GC analysis, 50 μl of solution was added to 40μl of heptone, and the solution was allowed to concentrate to 40 μl to remove most of the dichloromethane.

Gas Chromatography–Mass Spectrometry (GC-MS)

A quadrupole mass spectrometer (Finnegan Corp., model 4000 Sunnyvale, CA) with computerized data collection and a GC inlet was used. The 30-m × 0.25-mm ID polydimethyl siloxane (SP-2100®) capillary column was held at 50° C for 2 min and then programmed at 20° / min to 220° C and held at this temperature. The volatiltiy range over which mass spectra were obtained encompassed the retention times of decyl through octadecyl acetate. All spectra were taken in the electron impact mode.

(Z,Z)-9,11-Tetradecadien-1-ol Acetate

A sample of this compound was kindly furnished to us by Dr. David Warthen, Beltsville, Maryland.

Synthesis

Isomers of 10,12-tetradecadien-1-ol acetate were prepared by two procedures. Procedure A was designed to produce all four isomers and procedure B to produce (*E,E*)- and (*E,Z*)-10,12 isomers.

Procedure A

10-[(Tetrahydro-2H-pyran-2-yl)oxy]-1-decanol (I). 1,10-Decanediol (20.0 g) (115 mmol) and 4.83 g of dihydropyran (57.5 mmol) in 1400 ml of dichloromethane containing 5 drops of concentrated HCl were stirred for 2 hr (see Figure 1). Then 10 g of $NaHCO_3$ was stirred in and the solution was filtered. Dichloromethane was removed with a rotary evaporator, and the resulting residue was triturated with pentane; the undissolved solid was 1,10-decanediol. The solid was filtered from the pentane solution and the pentane was removed with a rotary evaporator to give 14.8 g (99.8%) of crude product oil.

10-[(Tetrahydro-2H-pyran-2-yl)oxy]decanal (II). I (14.8 g) 57.4 mmol) in 75 ml of dichloromethane, that had been dried by distillation from P_2O_5, was added to a stirred solution of 18.6 of pyridinium chlorochromate and 1.41 g of sodium acetate in dried dichloromethane (Corey and Suggs, 1975). After 2 hr, 400 ml of anhydrous ether was added; the solution was filtered, and the solvent was removed with a rotary evaporator. The residue was extracted with

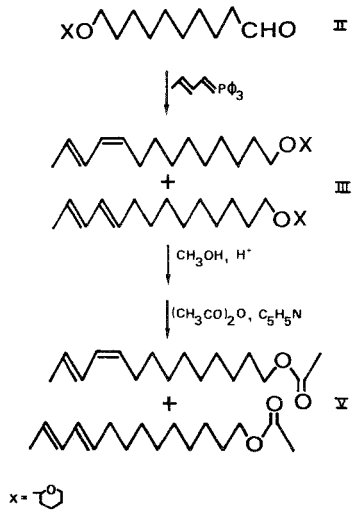

FIG. 1. Synthesis of all four isomers of 10,12–14:Ac which produced mainly *Z*10,*E*12- and *E*10,*E*12- as shown.

hexane, and the hexane was removed to give the crude product which was then purified by liquid chromatography. The yield was 9.0 g (61%). IR: 2700 cm^{-1} (w) and 1725 cm^{-1}(s), aldehyde; no 3330 cm^{-1} absorption for alcohols; typical tetrahydropyranyl ether pattern from 1470 to 1250 cm^{-1}.

Tetrahydro-2-[(10,12-tetradecadienyl)oxy]-2H-pyran (III). Crotyl bromide (13.0 g, Aldrich Chemical Co., Milwaukee, Wisconsin) (96 mmol) and 25.3 g of triphenyl phosphine (96 mmol) in 200 ml of benzene were stirred for 48 hr. The benzene was decanted from the precipitate, and 300 ml of dry tetrahydrofuran was added. The mixture was stirred during the slow addition of 96 mmol (60 ml of 1.6 M) of *n*-butyllithium in hexane. After the addition, the solution was deep red, and all of the Wittig salt had dissolved. II (4.06 g) (16 mmol) in 50 ml of tetrahydrofuran was added dropwise to the red phosphorane solution, and the solution was stirred for 2 hr after the addition of II. Acetone was added to destroy excess phosphorane. The solution was filtered to remove lithium bromide, and solvent was removed with a rotary evaporator. The resultant residue was extracted with hexane and water, and the hexane layer was separated and dried with sodium sulfate. The hexane was removed, the residue was extracted with pentane, and the solution was filtered to remove a white precipitate. After evaporation of the pentane, 3.9 g (83%) of crude product was obtained that was subsequently purified by liquid chromatography.

10,12-Tetradecadien-1-ol(IV). III in 100 ml methanol containing 3 drops of concentrated HCl was refluxed overnight. Water (200 ml) was added and the product was extracted with ether. Gas chromatography (column 3)

showed four peaks at the following retention times (min) and relative peak intensities: 36.9 (0.92), 38.2 (1.00), 38.9 (0.11), 39.4 (0.085); hexadecanol had a retention time of 36.0. The yield was 0.99 g.

10,12-Tetradecadien-1-ol acetate (*V*). IV (0.99 g) in 30 ml of pyridine and 2 ml of acetic anhydride was refluxed for 2 hr and cooled. Pentane (ca. 150 ml) was added and the solution was extracted once with water, twice with 6 N HCl (to remove pyridine), and once more with water. After drying, the product was purified by liquid chromatography. Gas chromatography (column 3) showed peaks corresponding to the four geometrical isomers at the following retention times (min) and relative peak intensities: 22.8 (0.88), 23.4 (1.00), 23.8 (0.09), 24.1 (0.08). The natural pheromone peaks had retention times of 23.4 and 23.8 min. The infrared and mass spectra were consistent with the expected structures.

Procedure B

(E)-*12-[(Tetrahydro-2H-pyran-2-yl)oxy]-2-dodecenal* (*VI*). II (5.1 g) (20 mmol) and 6.1 g (20 mmol) of formylmethylenetriphenylphosphorane (Alfa Products, Danvers, Maryland) were refluxed in 300 ml of benzene for 72 hr (see Figure 2). Benzene was removed with a rotary evaporator and the residue was extracted with pentane. After the solution was filtered, the pentane was removed with a rotary evaporator to give crude product (VI). IR: 2700 cm^{-1} (w) and 1690 cm^{-1}(s), aldehyde; 3000 cm^{-1} (w) and 1637 cm^{-1}(m), olefin. A weak to medium absorption at 1725 cm^{-1} indicated the presence of unreacted

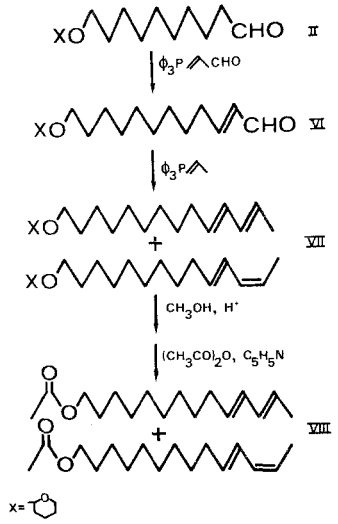

FIG. 2. Synthesis of *E*10,*E*12- and *E*10,*Z*12-14:Ac in a ratio of 1:1.6.

II. Purification of VI was effected by liquid chromatography. II was only partially removed (weaker IR absorption at 1725 cm^{-1}); yield was 2.8 g (50%).

1-[(Tetrahydro-2H-pyran-2-yl)oxy]-10,12-tetradecadiene (VII). Ethyltriphenylphosphonium bromide (11.1 g) (30 mmol) (Alfa Chemicals, Danvers, Maryland) in 200 ml of dry tetrahydrofuran in a 3-neck flask was stirred for 0.5 hr under nitrogen. The salt did not completely dissolve. *n*-Butyllithium (20 ml of 1.6 M in hexane) (32 mmol) was added by syringe through a rubber septum on one neck of the flask. The solution became deep red and the rest of the salt dissolved. Then, VI (2.8 g) (10 mmol) in 30 ml of tetrahydrofuran was added dropwise, and the solution was stirred for 1 hr after the addition was complete. Excess phosphorane was discharged with acetone and the solvent was removed with a rotary evaporator. The residue was extracted with water and pentane. The pentane extract was dried with sodium sulfate, and the pentane was removed with a rotary evaporator to give crude product.

10,12-Tetradecadien-1-ol Acetate (VIII). Crude VII was converted to the alcohol and then the acetate by the same procedures used for the preparation of IV and V. VIII was cleaned up by liquid chromatography. The yield of VIII was 0.8 g (32% from VI). Gas chromatography (column 4) showed two peaks at the following retention times (min) and relative peak intensities: 32.0 (1.0) and 32.5 (1.6). The natural pheromone peaks and the second and third peaks of V were also at these retention times. Mass spectral analyses confirmed these structures.

Separation of Isomers of 10,12-Tetradecadien-1-ol Acetate

The synthesized 10,12-tetradecadien-1-ol acetate isomers of V and VIII were separated by silver resin liquid chromatography (Houx et al., 1974; Warthen, 1976), and methanol was used for elution. The eluate was monitored with a refractive index detector (Varian Aerograph, high-pressure liquid chromatograph, model 281, Walnut Creek, California).

Infrared Spectra

A grating infrared spectrometer (Perkin-Elmer, model 337, Norwalk, Connecticut) was used. Liquid synthetic intermediates were determined between sodium chloride plates, and the purified isomers of V and VIII were determined as dichloromethane solutions.

Field Tests

Field tests were conducted in avocado groves at the University of California's South Coast Field Station, Santa Ana, California. Synthetic pheromone (compound VIII from procedure B) was tested after impregnation of dichloromethane solutions into rubber septa (West Co., Phoenixville,

Pennsylvania) which were placed in Pherocon® 1 traps (Zoecon Corp., Palo Alto, California). In the avocado groves, the traps were placed 25 m apart. When traps were baited with females, 2 females (2–4) days old) in a screen cage were placed in the traps and changed every 3 days. Tests were conducted with five replicates.

RESULTS

Structure Determination. A gas chromatographic trace of the extract of the sex pheromone gland (SPG) on column 1 (SE-30) showed one prominent peak in the volatility range encompassing decyl (10:Ac) through octadecyl acetate (18:Ac). The retention time of this peak was 21.8 min (1.22 × 14:Ac). The only other significant peak was broad and unsymmetrical (suggesting that it was composed of more than one substance) and occurred at 17.8 min (peak height was 4% of the major component and occurred at the retention times of the tetradecenyl acetates). When the major substance X was collected from the gas chromatograph, a 200-ng sample evoked an EAG response of 0.85 mV—the same as a sample of unpurified SPG extract containing 200 ng of this material (air response 0.2 mV). We concluded that X was either the sex pheromone or the major component of it. X had a retention time of 9.0 min (1.10 × 16:Ac) on column 2 (Carbowax 20M).

Next, purified X was analyzed by capillary gas chromatography (column 3). Then two peaks were obtained in a peak height ratio of 1:2.1 (earlier peak–latter peak) with retention times of 35.5 and 36.1 min (16:Ac at 33.6 min).

If the components of X were acetates, the GC behavior on columns 1, 2, and 3 would be consistent with an isomeric pair of conjugated tetradecadien-1-ol acetates. Consequently, a sample of purified X was saponified and analyzed on GC columns 1 (SE-30) and 3 (capillary Carbowax 20M). The resultant substance had a retention time of 1.15 × 14:OH on SE-30 and gave two peaks on the capillary Carbowax column at 43.9 and 44.7 min (16:OH at 41.7 min) with a peak height ratio of 1:2.3 (earlier–later peak). This experiment indicated that the components of X were acetates.

The mass spectra of the two components of X were identical to each other and to that of reference *Z*9, *Z*11–14:Ac. The spectra, of course, do not discriminate between geometrical or positional isomers. The principal diagnostic peaks were M^+ at *m/e* 252 and M^+-60 at *m/e* 192. A peak at *m/e* 61 further confirmed that these components were acetates.

After X was ozonized, the product had a retention index (Kovats, 1965), I_x, of 1600 on column 1 (SE-30), and the ozonolysis product of *Z*9–14:Ac had $I_x = 1500$ on column 1. On column 2 (packed Carbowax 20M) the ozonolysis product of X had $I_x = 2246$, and the ozonolysis product of *Z*9–14:Ac had $I_x = 2148$. These data and the preceding information established that the

pheromone components were two geometrical isomers of 10,12- tetradecadien-1-ol acetate.

The small peak (substance Y) of the GC trace on column 1 of the orginal extract of the SPG, which occurred in the retention time range of the tetradecenyl acetates, was also investigated. About 50 ng of Y was purified and injected on column 3 (capillary Carbowax). A peak eluted in the retention time region of the tetradecen-1-ol acetates; it was not coincident with either *Z*9-, *E*9-, *Z*11-, or *E*11–14: Ac, but was bracketed by *E*9- and *E*11–14: Ac. A GC-MS analysis of the rest of the 50-ng sample showed the presence of a tetradecen-1-ol acetate (M^+-60 at *m/e* 194) and tetradecan-1-ol acetate (M^+-60 at *m/e* 196). We concluded that the tetradecen-1-ol acetate was *E*10–14: Ac. 14: Ac and *E*10–14: Ac were each about 1–2% of the peak height of 10,12–14: Ac.

Synthesis. Because the double-bond configurations of the pheromone components were not known, a synthetic procedure (Figure 1) was devised which we expected would generate all four possible isomers. The procedure did generate all four geometrical isomers as shown by GC and GC-MS, but the first two eluting isomers predominated. Based on studies of 8,10–12: OH (Roelofs et al., 1972), we expected the order of elution to be (1) *Z*10,*E*12-, (2) *E*10,*E*12-, (3) *E*10,*Z*12-, and (4) *Z*10,*Z*12-. The peak height ratios we obtained indicated that the product was mainly *Z,E* and *E,E* and therefore the crotyl bromide used to make the phosphorane had mainly the *E* configuration. Since GC peaks 2 and 3 were coincident with the pheromone peaks, the pheromone components have *E,E,* and *E,Z* configurations.

Procedure B (Figure 2) (based on the procedure of Bestmann, 1977) was devised to generate the two desired isomers only. We expected VI to have only the *E* configuration and the Wittig step to produce both *Z* and *E* with the *Z* configuration predominating. These expectations were realized. Procedure B produced *E,E* and *E,Z* in a 1:1.6 peak height ratio, respectively, and the retention times coincided with the aforementioned peaks (2 and 3). To further confirm our assignment of configuration, the principal isomers V (*Z,E* and *E,E*) and VIII (*E,E* and *E,Z*) were isolated by silver resin chromatography, and their infrared spectra were taken. The ratios of the intensity of the 980 cm^{-1} band (*trans*-olefin) to the 1725 cm^{-1} band (acetate carbonyl) were: *Z,E* (0.15); *E,E* (0.37); and *E,Z* (0.13). Therefore, the configurational assignments were confirmed.

The EAG of the three purified synthetic isomers were determined at concentrations of 10 and 0.1 μg, and the responses, in mV were: *Z,E* (0.65, 0.35); *E,E* (0.88, 0.53) *E,Z* (0.78, 0.48). Thus, the *E,Z* and *E,E* isomers were equal and *Z,E* appeared to be slightly weaker, but the difference was comparable to the experimental error of the measurement.

Field Tests. Tests were conducted with 3 mg/septum of the synthetic pheromone, VIII from procedure B. During an 8-day period in June 1980,

traps baited with synthetic pheromone captured 4.7 males/trap-day, and traps baited with females captured 0.7 males/trap-day. Unbaited traps caught no males.

DISCUSSION

A large number of sex attractant pheromones have been identified for the subfamily Tortricinae. Roelofs and Cardé (1974) have characterized these pheromones as having 14-carbon straight chains, terminated with an alcohol, aldehyde, or acetate group. Most of these have Δ11 double bonds, but Δ9 have also been reported (Tamaki et al., 1971; Meijer et al., 1972). The pheromone of *A. cuneana* is therefore of the type expected, but this is the first time multiple double bonds or the 10,12 positions of unsaturation have been found for a species of Tortricinae.

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SEX PHEROMONE OF FALL ARMYWORM: Laboratory Evaluation of Male Response and Inhibition of Mating by Pheromone Components

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Abstract—Two components of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), sex pheromone, (*Z*)-9-dodecen-1-ol acetate (DDA) and (*Z*)-9-tetradecen-1-ol (TDA), were tested alone and in combination to determine their effects on male sexual response and inhibition of mating in the laboratory. The threshold response for FAW males was lower for TDA than for DDA, and males responded to TDA over a wider range of dosages. Although TDA is not attractive to FAW males in the field, this compound was highly effective in reducing mating under laboratory conditions.

Key Words—Fall armyworm, *Spodoptera frugiperda,* Lepidoptera, Noctuidae, (*Z*)-9-dodecen-1-ol acetate, (*Z*)-9-tetradecen-1-ol acetate, pheromone, mating behavior.

INTRODUCTION

Two components of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), sex pheromone have been identified, (*Z*)-9-tetradecen-1-ol acetate (Sekul and Sparks, 1967) and (*Z*)-9-dodecen-1-ol acetate (Sekul and Sparks, 1976). Of these, only (*Z*)-9-dodecen-1-ol acetate (DDA) consistently attracted male fall armyworms (FAW) to traps in the field (Mitchell and Doolittle, 1976). (*Z*)-9-Tetradecen-1-ol acetate (TDA) apparently acts as a secondary sex pheromone which, when blended (2–10%) with DDA, gives a slight increase in attractancy to FAW over DDA alone (Jones and Sparks, 1979).

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Mitchell et al. (1974) showed that DDA disrupts pheromone communication of the FAW when evaporated into the atmosphere surrounding traps baited with virgin females. They also suggested that TDA might be considered a candidate material for a regional suppression program against this species. In subsequent field trials TDA did indeed prove to be an effective disruptant of pheromone communication and mating (McLaughlin et al., 1981, Mitchell and McLaughlin, 1981). The present study was conducted to determine the influence of pheromone components (DDA and TDA) alone and in combination upon male sexual response and inhibition of mating by the FAW in the laboratory.

METHODS AND MATERIALS

Spodoptera frugiperda adults used in these experiments were reared from larvae that were maintained on artificial diet (Burton, 1970). Insects were sexed as pupae and held in separate cabinets that were maintained at ca. 20° C (12 hr L–12 hr D) until eclosion. Newly emerged moths were transferred to holding cages (33 × 23 × 21 cm) that were constructed of screenwire (males) or to 3.8-liter paper cartons (females) equipped with screenwire tops. Prior to testing they were held in the same cabinet in which they eclosed. A 10% sucrose solution on cotton was provided to ensure a continuous supply of food and water.

Test compounds were obtained from Chemical Samples Company, Willoughby, Ohio, and each sample was at least 97+% pure when analyzed by gas chromatography.

Male Response to Sex Pheromone Components. Approximately 1–2 hr before the onset of the scotophase, male moths (3 days old) were placed into screenwire cages (11 × 9 cm) fitted with ½ of a polystyrene Petri dish at the top and the bottom. Five moths were used per replicate with six replicates per chemical dosage. Bioassays were conducted 3–4 hr after lights-off under diffuse light (1.1 lux). Timing for bioassays was based upon similar behavioral experiments by Sekul and Cox (1967). Serial dilutions of the test chemicals were made in reagent-grade hexane. Five μl of the test sample was introduced into a 2.5-ml transfer pipet that was equipped with a rubber bulb, and the solvent was allowed to evaporate. The tip of the pipet was then directed toward individual males in the flask or cage as the bulb was squeezed (10×/male). A positive response was recorded if the "puffed" male responded with a full copulatory response, i.e., the abdomen was curved toward the pipet opening and the claspers were extended. Other reactions such as wing vibration, vibration of antennae, and erratic flight were not considered because preliminary observations indicated that such movements frequently

were induced by handling procedures, light, crowding, and movement of other males in the bioassay chamber.

Mating Suppression by Sex Pheromone Component(s). The test insects (2 females and 3 males, each 3 days old) were placed into a screenwire cage (11 × 9 cm) 3 hr after lights-off. Each cage was wrapped with aluminum foil and closed at the ends with ½ of a polystyrene Petri dish. The test chemicals, DDA, TDA, and a 1 : 1 blend thereof in 5 μl hexane, were introduced into each cage on a stainless steel planchet (2.5 cm diam). The hexane was allowed to evaporate before the planchet was attached to the top of the cage with a small piece of tape. The cage was then sealed at both ends with vinyl tape and placed into a cardboard box for 7 hr, after which the insects were removed and separated by sex. Females were dissected to determine if they had mated, as indicated by the presence of a spermatophore in the bursa copulatrix. Control insects were handled in the same manner. Each treatment (chemical dosage) and corresponding controls were replicated 14–15 times. The percentage of mating suppression (MS) was calculated as follows:

$$\text{MS}(\%) = \frac{\%\ \text{♀ mated in control} - \%\ \text{♀ mated in treatment}}{\%\ \text{♀ mated in control}}$$

RESULTS AND DISCUSSION

The sexual responses of male FAWs to TDA over a range of dosages from 1×10^{-8} to 1×10^{1} μg were examined using the screenwire cage described herein and a 250-ml glass flask. The cage gave the most consistent results, and the mean percentage of males responding at dosages of 1×10^{-4}, 1×10^{-3}, 1×10^{-2} and 1×10^{-1} μg was significantly higher (5% level, Student's *t* test) for the cage than the flask. Therefore, screenwire cages were used in all subsequent bioassays.

Figure 1 shows the response of FAW males to DDA and blends of DDA–TDA (100 : 1) ranging in dosage (μg) from 1×10^{-6} to 1×10^{1} DDA to 1×10^{-8} to 1×10^{-1} TDA. The 100 : 1 ratio of the two components was selected because of the reported increase in catches of FAW males in traps baited with this combination of attractants over DDA alone (Jones and Sparks, 1979). Males responded clearly to DDA at a dosage of 1×10^{-4} μg and maximum responsiveness was recorded at 1×10^{-2} μg. Maximum responsiveness to TDA also occurred at 1×10^{-2} μg (Figure 2). However, the percentage of FAW males responding to TDA was significantly greater than those recorded for DDA at 1×10^{-4}, 1×10^{-3}, and 1×10^{-1} μg, respectively.

Figure 3 illustrates the effects of DDA and TDA and a 1 : 1 blend on

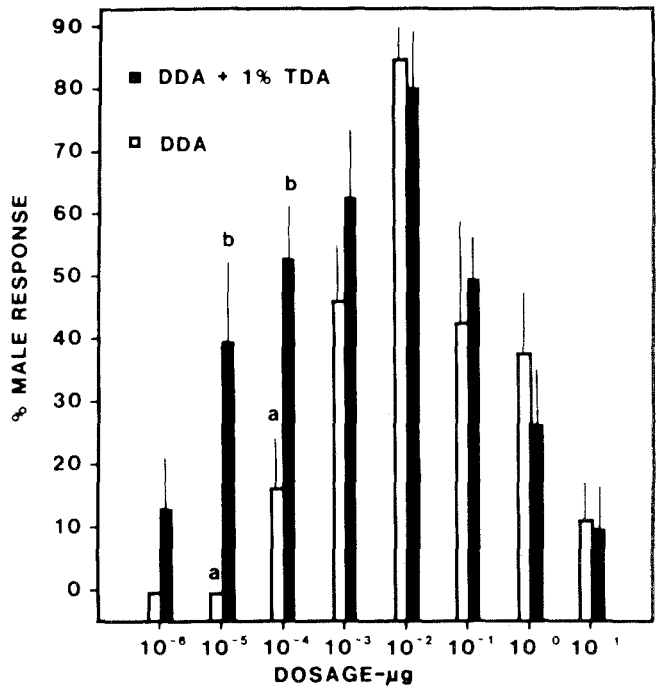

FIG. 1. Response of *Spodoptera frugiperda* males to (*Z*)-9-dodecen-1-ol acetate alone and in combination with 1% (*Z*)-9-tetradecen-1-ol acetate. Thirty males tested per dosage. Thin vertical lines indicate SE. Means in the same grouping with different letters indicate significant differences (5% level, Student's *t* test); the absence of letters indicates no significant difference between means.

mating by the FAW. The blend used was selected because the previous tests (Figures 1 and 2) had shown that TDA was a powerful sexual stimulant, although it is not a long-range attractant for FAW males (Mitchell and Doolittle, 1976). Hence we wanted to ascertain if an uncommonly high proportion of TDA to DDA would result in an overall reduction in the quantity of materials required for significant mating suppression over either chemical alone. In each case, the percentage of females mating was reduced with increasing dosages of the test chemicals. Maximum reductions in mating (ca. 80%) for each chemical and the blend occurred at the highest dosage tested (1×10^1 µg). The impact of the 1 : 1 blend of DDA and TDA on mating by the FAW was about the same as either chemical alone, suggesting an additive effect. Therefore, such a pheromone blend would provide no particular cost advantage in a mating suppression program for the FAW because the price differential between DDA and TDA would be negligible.

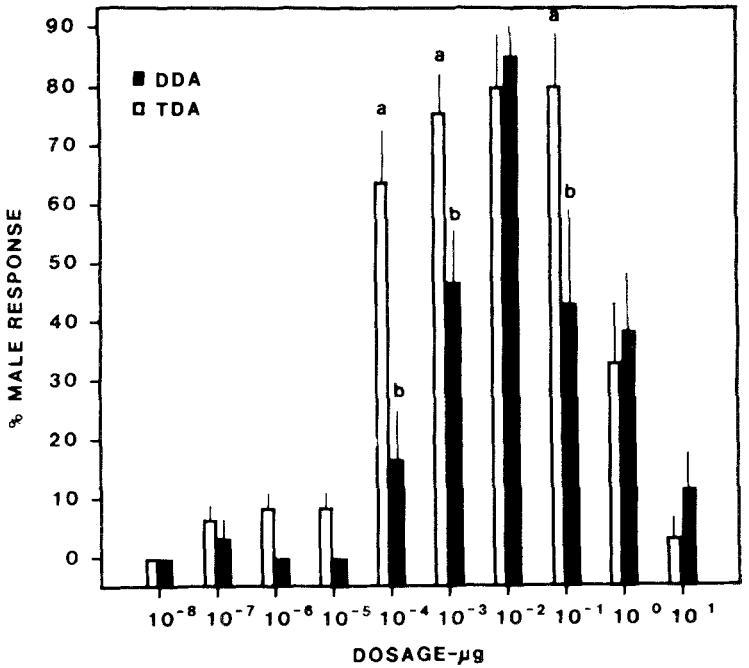

FIG. 2. Response of *Spodoptera frugiperda* males to (*Z*)-9-tetradecen-1-ol acetate and (*Z*)-9-dodecen-1-ol acetate. Thirty males tested per dosage. Thin vertical lines indicate SE. Means in the same grouping with different letters indicating significant differences (5% level, Student's *t* test); the absence of letters indicates no significant difference between means.

The results obtained so far suggest different roles for DDA and TDA in the sexual behavior of the FAW, although their function is unclear at this time. When males were exposed to the highest dosage (1×10^1 μg) of either compound, they flew about without exhibiting the characteristic copulatory behavior observed at the lower dosages, suggesting an avoidance reaction. In the stimulation bioassay, there appeared to be synergism at the lower dosages when TDA (1%) was admixed with DDA (Figure 1). Jones and Sparks (1979) reported "low-level synergism" in field trapping experiments with the FAW when 2 and 10% quantities of TDA were added to 100 μg of DDA and dispensed from dental wicks placed in Stickem®-coated pie-plate traps.

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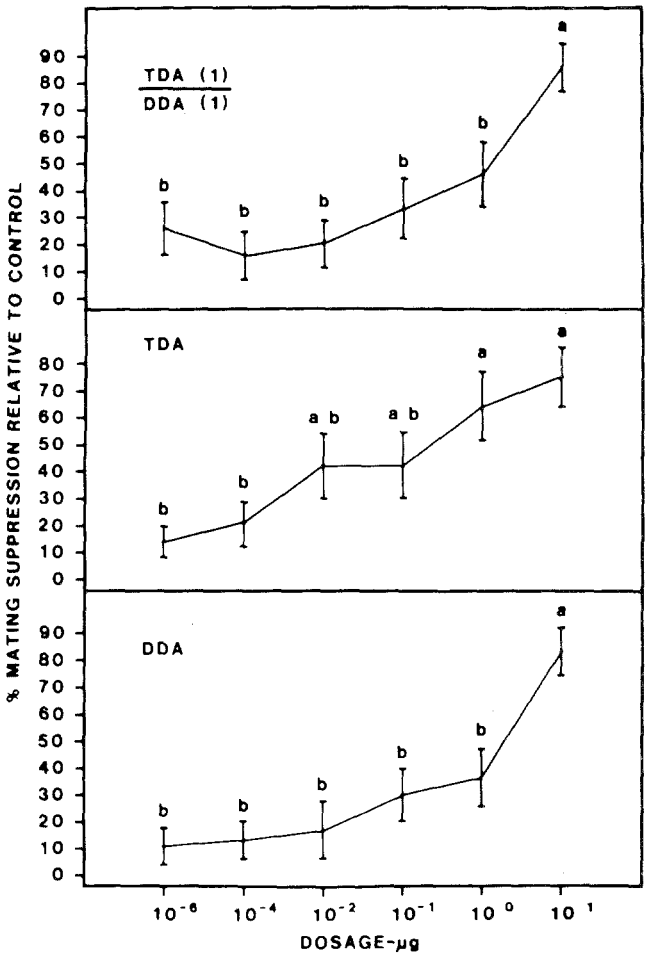

FIG. 3. Mating suppression in *Spodoptera frugiperda* with (*Z*)-9-dodecen-1-ol acetate, (*Z*)-9-tetradecen-1-ol acetate, and a 1:1 blend of these compounds. Each dosage for the blend represents the sum of the two chemicals combined. Thirty females and 45 males tested per dosage; the females were dissected to check mating status. Means in the same grouping with different letters indicate significant differences between treatment dosages (5% level, Duncan's multiple-range test).

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EFFECTS OF PINE-PRODUCED CHEMICALS ON SELECTED UNDERSTORY SPECIES IN A *Pinus ponderosa* COMMUNITY

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Abstract—*Pinus ponderosa* accounted for more than 98% of all tree and shrub stratum stems in a climax community with low herb coverage and aboveground biomass, 35% and 60 g/m^2, respectively. Because of our previous report that nitrification and nitrifying bacteria in the same community were allelopathically inhibited, we speculated that the pine-produced allelochemics might also directly influence the development and growth of the herb stratum. In most cases decaying needles, needle leachate, and field soils significantly reduced germination and radicle growth of *Andropogon gerardii* and *A. scoparius*, pine-associated herbaceous species. Additionally, growth of *Andropogon scoparius* seedling radicles was reduced 28–56% by pine needle extracts, 33% by pine bark extracts, and 67% by soil hydrolysate extracts. *Andropogon* seed germination was reduced 20–25% by pine needles and soil. Phytotoxins identified in various plant parts and associated soils were caffeic acid, chlorogenic acid, quercetin, and condensed tannins. Pine needle water and soil hydrolysate extracts were most inhibitory to the radicle growth of the test species. Thus it appears that the limited growth of the herbaceous stratum in the pine community may be accounted for, in part, by allelopathy. Such allelopathic interactions may have an adaptive ecological significance in various forest and other plant communities.

Key Words—Allelopathy, *Pinus ponderosa*, herb stratum, physioecology, phytotoxins, *Andropogon gerardii, Andropogon scoparius.*

INTRODUCTION

Characteristics generally associated with coniferous forest ecosystems such as low nitrification rates, slow litter mineralization rates, and low herb stratum productivity and biomass have often been attributed to the low light

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intensities and the highly acidic soil-litter complex under most conifer canopies. Recently, Lodhi and Killingbeck (1980) reported that a community dominated by ponderosa pine (*Pinus ponderosa* Dougl.) in western North Dakota influenced nitrification and mineralization by reducing nitrifier populations even though the soils under the pine stands were slightly alkaline (pH 7.25–7.75). The reduction in nitrifiers was caused by ponderosa pine-produced allelochemics rather than by low soil pH.

The relatively low herb stratum coverage (35%) (Potter and Green, 1964) and biomass (<60 g/m^2) (Lodhi and Killingbeck, 1980) in the above-mentioned North Dakota pine stands, coupled with the nonacidic soils and open canopy, indicated that factors other than soil pH and light were restricting herbaceous growth. The allelochemics identified from ponderosa pine and associated soils were caffeic acid, chlorogenic acid, quercetin, and condensed tannins (Lodhi and Killingbeck, 1980). These compounds are widely known to inhibit the growth and seed germination of herbaceous understory species in various plant communities (Rice, 1974; Lodhi, 1976). Because production of allelochemics by the ponderosa pine in these stands had already been demonstrated, it seemed plausible that pine-produced biochemicals might also directly influence the herb stratum development and growth.

Appropriate experiments were initiated in 1980 to determine the effects of allelochemics released from ponderosa pine and associated soils on selected understory herbaceous species. Herbaceous species selected for experimentation were *Andropogon scoparius* Michx. (little bluestem) and *A. gerardii* Vitman (big bluestem).

METHODS AND MATERIALS

Field Methods. Ponderosa pine needles, bark, and soils were collected from stands located in southwestern North Dakota (103° 30′W, 46° 35′N). More than 98% of the tree stratum stems and 67% of the shrub stratum stems were ponderosa pine (Wali et al., 1980). Ten samples from 0–15 cm of the mineral soil profile and ten samples of live pine needles and outer bark were collected from a representative ponderosa pine stand for further experimentation.

Effects of Decaying Pine Needles. The effects of pulverized, dried, green pine needles were determined by a method modified from that used by Del Moral and Muller (1970). Petri plates were supplied with 0.2 g of pine needle powder layered under a cellulose sponge and a filter paper on top. After the moistened plates were incubated at 25°C for 48 hr to initiate decomposition, 50 seeds each of *A. scaparius* and *A. gerardii* were placed in separate plates on top of filter paper and returned to the incubator. Control plates were prepared

in the same way except that needle powder was replaced with 0.2 g of air-dried peat moss powder to keep the organic matter content the same as in the test plates. Replicate plates were used for all treatments. Percent germination was recorded after 96 hr of incubation. Only 15 seedlings with the longest radicles were retained in each plate. Radicle length was measured after an additional 48 hr of incubation.

Effects of Pine Needle Leachate. Fifty seeds each of *A. scoparius* and *A. gerardii* were placed in Petri plates containing a cellulose sponge below filter paper. Treatment plates were watered with 10 ml of distilled water that had been passed over living pine needles several times in the form of a mist. Control plates were watered with untreated distilled water. Percent germination was recorded after 96 hr at which time all seedlings except the 15 with longest radicles were removed from each plate. Radicle length was measured after an additional 48 hr of growth.

Effects of Pine Soils. Replicate germination plates were prepared as in the pine needle experiment except that 1.0 g of soil collected from the 0- to 15-cm depth under the ponderosa pine stands was used instead of needle powder in the test plates, and 1.0 g of greenhouse potting soil was used in place of peat moss in the control plates. Percent germination and radicle growth were determined as above.

Extraction and Effects of Various Allelochemic Fractions. Ten percent aqueous extracts of air-dried pine needles were acidified to pH 2.5 with HCl and extracted with three half-volumes of diethyl ether. Ether and water fractions were evaporated to dryness and taken up in 5 ml of 95% ethanol and 10 ml of distilled water, respectively. Ten grams of tree bark were refluxed with acetone for 24 hr. The acetone fraction was evaporated to dryness and taken up in 5 ml of 75% ethanol. The hydrolysis method used to isolate and identify allelochemics from the pine soils was the same as described previously by Lodhi (1975) with the modification suggested by Rice and Pancholy (1973). They found that 150 g of soil with 150 ml of 1 N NaOH, when autoclaved for 10 min, gave the best yields of condensed tannins and other allelochemics. Fifty grams of soil were also extracted with acetone for 24 hr, evaporated to dryness, and taken up in 5 ml of 75% ethanol. No attempts were made to identify the allelochemics since Lodhi and Killingbeck (1980) previously isolated and identified various allelochemics in the same fractions. However, various spots of allelochemics were eluted from chromatography paper and verified by determining and comparing their maximum absorption spectra with known compounds.

To determine the allelochemic activity of extracts, all fractions of extracts were evaporated to dryness before preparing final dilutions. Final dilutions based on 10% aqueous extracts were prepared as follows (1) ether and water fractions were in 20 ml and 40 ml of water, respectively; (2) acetone

extracts of the tree bark were in 20 ml of water; (3) soil hydrolysate extracts were taken up in 40 ml of water; and (4) soil acetone extracts were taken up in 20 ml of water. Test and control plates were prepared as given above for the needle leachate experiment except that test plates were watered with needle-water, needle-ether, bark-acetone, soil-acetone, or soil-hydrolysate extracts. Percent germination and radicle growth of *A. scoparius* seeds and seedlings, respectively, were determined as above.

RESULTS

Germination of *Andropogon* seeds was suppressed by pine needles and by soils taken from under the pine stands (Figure 1). Leachate from water-misted needles had no appreciable effect on germination. The pine needle powder treatment reduced germination by almost 50%.

Seedling growth, as measured by radicle length, was reduced below expected levels by treatment with pine needles and soil (Table 1). The needle

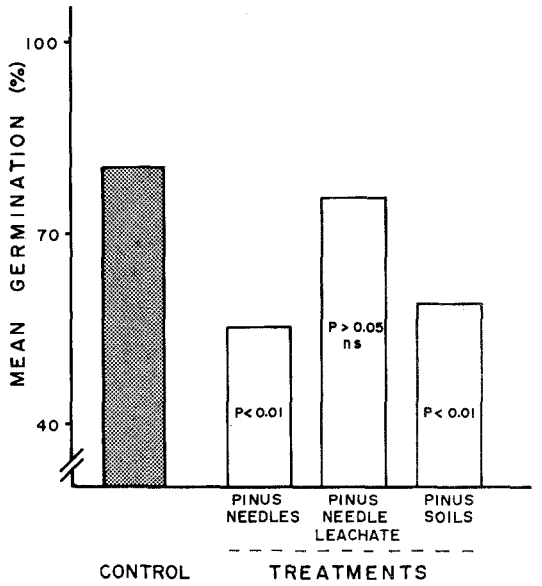

FIG. 1. Effects of *Pinus ponderosa* needles, needle leachate, and soil on the germination (%) of *Andropogon* seeds ($N = 100$ *A. scoparius* plus 100 *A. gerardii* seeds for each treatment and control group). Probabilities (P) are from pairwise Student's t tests performed on percent germination data transformed (arcsin $\sqrt{\%}$) to meet normality assumptions. Probabilities indicate differences between the control and each treatment (ns = control and treatment not significantly different).

TABLE 1. EFFECTS OF *Pinus ponderosa* NEEDLES, NEEDLE LEACHATE, AND SOIL ON GROWTH (LENGTH IN mm) OF *Andropogon scoparius* AND *A. gerardii* SEEDLING RADICLES[a]

Treatment	Species	Mean growth of radicles (mm ± SE)		Probability of difference
		Control	Treatment	
Pinus needles	*A. gerardii*	26.6 ± 1.0	18.7 ± 0.8	*
		23.7 ± 0.9	14.5 ± 0.9	*
	A. scoparius	19.5 ± 1.1	11.7 ± 1.0	*
		28.3 ± 2.0	17.4 ± 1.6	*
Pinus needle leachate	*A. gerardii*	26.6 ± 1.0	21.0 ± 0.8	*
		23.7 ± 0.9	16.8 ± 1.2	*
	A. scoparius	19.5 ± 1.1	20.8 ± 1.8	ns
		28.3 ± 2.0	18.6 ± 0.8	*
Pinus soils	*A. gerardii*	26.6 ± 1.0	20.3 ± 1.6	*
		23.7 ± 0.9	17.0 ± 0.7	*
	A. scoparius	19.5 ± 1.1	13.6 ± 0.3	*
		28.3 ± 2.0	16.5 ± 1.2	*

[a]Probabilities were derived from Student's t tests. * = $P \leq 0.05$, control and treatment growth were significantly different, ns = $P \geq 0.05$, control and treatment growth were not significantly different; SE = standard error or the mean; $N = 15$ in all cases.

TABLE 2. ALLELOCHEMICS IDENTIFIED IN EXTRACTS FROM *Pinus ponderosa* NEEDLES, BARK, AND SOILS AND THEIR MAXIMUM ABSORPTION SPECTRA IN 95% ETHANOL WITH OR WITHOUT NaOH.

Allelochemics	Maximum absorption spectra	
	−NaOH	+NaOH
Caffeic acid	282	265
Suspected caffeic acid	283	265
Chlorogenic acid	301	370
Suspected chlorogenic acid	301	368
Quercetin	375, 257	257
Suspected quercetin	370, 257	257
Cyanidin (condensed tannin)	460, 558[a]	–

[a]Maximum absorption in *n*-butanol and compared with the maximum absorption published by Feeny and Bostock (1968).

TABLE 3. EFFECTS OF FIVE EXTRACTS FROM *Pinus ponderosa* NEEDLES, BARK, AND SOILS ON GROWTH (LENGTH IN mm) OF *Andropogon scoparius* SEEDLING RADICLES[a]

Treatment (extract)	Mean growth of radicles (mm ± SE)		% difference	Probability of difference
	Control	Treatment		
Pinus needles, water	33.9 ± 2.3	14.8 ± 0.8	−56	*
Pinus needles, ether	33.9 ± 2.3	24.3 ± 1.1	−28	*
Pinus bark, acetone	33.9 ± 2.3	22.7 ± 2.0	−33	*
Pinus soils, acetone	33.9 ± 2.3	36.0 ± 2.4	+6	ns
Pinus soils, hydrolysate	33.9 ± 2.3	11.3 ± 0.7	−67	*

[a]Probabilities were derived from Student's t tests (* = $P \leq 0.05$, control and treatment growth were significantly different; ns = $P \geq 0.05$, control and treatment growth were not significantly different; SE = standard error of the mean; $N = 30$ in all cases).

powder treatment suppressed radicle growth more than either of the other two treatments, yet significant reductions occurred with all treatments. The highest growth reductions (up to 44% reduction compared to control) occurred in the *A. scoparius* seeds treated with pine needle powder. With the exception of one group, *A. scoparius* seedlings were more sensitive to the treatments than were *A. gerardii* seedlings.

Allelochemics identified from different sources in the ponderosa pine community were the same as reported previously by Lodhi and Killingbeck (1980). Caffeic acid, chlorogenic acid, quercetin, and condensed tannins (cyanidin) identified previously were verified by their absorption spectra using paper chromatography (Table 2).

Four of five specific extracts from ponderosa pine needles, bark, and soils significantly reduced *A. scoparius* radicle growth (Table 3). The acetone extract from pine soils did not reduce radicle growth, yet the hydrolysate extract from the same soils reduced radicle growth by 67%. Of the two pine needle extracts, the water extract was almost twice as inhibitory as the ether extract.

DISCUSSION

Andropogon gerardii and *A. scoparius* are two of ten nonwoody species found in more than 5% of the herb stratum quadrats sampled in the North Dakota ponderosa pine stands by Wali et al. (1980). As previously mentioned,

the overall herb stratum coverage and biomass in these stands was low. The combined coverage of *A. gerardii* and *A. scoparius* was less than 2% and patches of forest floor devoid of all vegetation were common.

The sporadic distribution pattern of the herb stratum could not be totally attributed to soil pH and light. The open nature of the stands (referred to as having "the appearance of a pine savannah in matrix of mixed grassland" by Potter and Green, 1964) allowed for much light penetration to the forest floor (Figure 2). Another factor that has been reported to restrict or inhibit herbaceous growth in forest ecosystems is allelopathy. Many allelochemics were found in the pine community (Table 2), and these have also been found to be responsible for inhibiting the growth of nitrifying bacteria in the same community (Lodhi and Killingbeck, 1980).

Germination success and seedling radicle growth were inhibited by pine allelochemics that were released by leaching and decomposition of ponderosa pine tissues (Figure 1, Tables 1 and 3). Because germination and radicle growth affect plant density and productivity, reduction in both accounted for at least some of the low biomass/coverage found in the pine understories.

The most drastic reductions in radicle growth were induced by extract fractions containing tannins. Lodhi and Killingbeck (1980) found that ponderosa pine needles and associated soils contained more tannins than any other allelochemics. Tannins were identified in the water fraction of the aqueous extracts and in soil hydrolysate. This is important because water-soluble tannins can be leached from pine needles and litter more easily than water-insoluble compounds. The low rainfall and gradual leaching of the litter

FIG. 2. Ponderosa pine field site showing the open nature of the stand and light penetration of the forest floor.

by snowmelt in this region may intensify the accumulation of tannins and other phytotoxins in the rooting zone of the herbaceous plants. Some of the inhibitors from the soil were extracted only after hydrolysis; there is no doubt that most bound compounds would be released readily by decomposers in the soil. Lodhi (1978) reported that the bound phenolics in plant litter were high in the associated soil in January, whereas free phenolics (released from bound form) were high in samples collected in April. This supports the idea that bound compounds are probably more detrimental because they are released in soils at a lower rate and are in the rooting zone in large amounts during the active growth period.

Inhibitory actions caused by the allelochemics identified in this study (Table 2) are well documented. Green and Corcoran (1975) found that tannins inhibited gibberellin (GA) induced growth and were particularly inhibitory to GA_4 and GA_{14}. Chlorogenic acid has been reported to decrease potassium and calcium uptake by *Amaranthus retroflexus* (Olmsted and Rice, 1970), reduce phosphorylase activity in potato plants by 50% (Schwimmer, 1958), and cause stomatal closure in *Nicotiana tabacum* L. and *Helianthus annuus* (Einhellig and Kuan, 1971). The reduction of stomatal opening in *Nicotiana* plants was correlated with reduced photosynthesis (Einhellig et al. 1970). Lodhi (1979a,b) reported that 10^{-3} to 10^{-4} M concentrations of caffeic acid, chlorogenic acid, and quercetin, in addition to other phytotoxins, significantly reduced the germination, radicle growth, and seedling growth of radish (*Raphanus sativus*, Crimson Giant), *Kochia scoparia*, and *Salsola kali.*

Germination and seedling growth are complex phenomena which are often affected by a wide range of abiotic and biotic factors. For *A. scoparius* and *A. gerardii* inhabiting the understory of *Pinus ponderosa* stands in western North Dakota, one mechanism that influences the above phenomena is pine-induced allelopathy. Ponderosa pine, by way of reducing the growth of the herbaceous stratum, possibly maximizes the availability of nutrients (if limiting) to its advantage. Additionally, many polyphenols are also known to serve as defense mechanisms against grazing, viral, fungal and bacterial pathogens (Feeny, 1970), and to perform other protective functions in plants (Johnson and Schaal, 1952). Speculatively, production of such chemicals has highly beneficial adaptive value for ponderosa pine communities, because forest and agricultural ecosystems that approach a monoculture are highly susceptible to large-scale losses from insect and pathogen infestations (Watt, 1974).

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VENOM CHEMISTRY OF ANTS IN THE GENUS *Monomorium*

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Abstract—A comparative analysis of the venomous alkaloids produced by ant species in the subgenus *Monomorium* of the genus *Monomorium* has been undertaken. All species produce mixtures of unsymmetrical *trans*-2,5-dialkylpyrrolidines, but the proportions of the constituents may vary considerably between species. All alkaloids contain both C_6 and C_9 side chains which are present as C_9-saturated. C_6- monounsaturated, and both C_6-and C_9-monounsaturated dialkylpyrrolidines. The structure of 2-(1-hex-5-enyl)-5-(1-non-8-enyl)pyrrolidine, a previously undescribed alkaloid, was proved by unambiguous synthesis after the location of the double bonds was established by the methoxymercuration–demercuration followed by mass spectrometry. The possible chemotaxonomic significance of the mixtures of venomous alkaloids produced by these species of *Monomorium* is discussed.

Key Words—*Monomorium* spp., Hymenoptera, Formicidae 2,5-dialkyl-pyrrolidines, ant venom alkaloids, methoxymercuration–demercuration, chemotaxonomy.

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INTRODUCTION

A large variety of novel alkaloids has been identified in the venoms of ant species in the myrmicine genus *Solenopsis*, a taxon particularly well represented in tropical areas. Species in the subgenus *Solenopsis* produce characteristic 2,6-dialkylpiperidines (MacConnell et al., 1971, 1976; Brand et al., 1972), whereas some species in the subgenus *Diplorhoptrum* synthesize 2,5-dialkylpyrrolidines in their poison glands (Pedder et al., 1976; Jones et al., 1979, 1980). However, that alkaloidal venoms are not restricted to the genus *Solenopsis* is demonstrated by the identification of a series of 2,5-dialkylpyrrolidines in the venom of *Monomorium pharaonis* (Ritter and Persoons, 1975; Ritter et al., 1973, 1975; Talman et al., 1974), a "tramp" species that is quite abundant in the Old World tropics. The venom of *M. pharaonis* is particularly distinctive in also containing at least two 3,5-dialkylindolizidines, a class of compounds that has never been encountered as venomous products of *Solenopsis* species.

In order to determine whether the venomous alkaloids produced by *M. pharaonis* are typical of those produced by other species in this genus, we have analyzed the poison gland products of additional species, all of which are in the same subgenus (*Monomorium*) as the former. The results indicate that considerable alkaloidal diversity that may be chemotaxonomically useful characterizes the chemistry of *Monomorium* (*Monomorium*) venoms. These results further demonstrate that the venoms of *Monomorium* species may provide useful characters whenever this rich but taxonomically difficult genus, which has not been analyzed in nearly 60 years (Emery, 1922), is subjected to revisionary studies.

METHODS AND MATERIALS

Ants

Ants were collected in the areas indicated in Table 1 and immediately placed in glass vials containing 1–2 ml of CH_2Cl_2. Since it was demonstrated that the alkaloids produced by *Monomorium* species were poison gland products, analyses were subsequently conducted on extracts of whole ants. For analyses, these solutions were reduced in volume to 0.2 ml with a slow stream of nitrogen.

Chemical Analyses

Gas chromatography was performed on a Tracor model MT-160 chromatograph using a 2.5-m × 2-mm ID column packed with 10% SP-1000 on Gaschrom Q, on a Varian model 90-P chromatograph using a 2-m × 4-mm ID column packed with 10% SP-1000 on Gaschrom Q, or on a Varian model

TABLE 1. ALKALOIDS IDENTIFIED AS VENOM CONSTITUENTS OF *Monomorium* (*Monomorium*) SPECIES

Monomorium species[a] (collection site)	Alkaloids[b]						
	1	2	3	4	5	6	7
M. near *metoecus* (Athens, Georgia)	+	*	○	–	○	○	○
M. viridum (Gainesville, Florida)	*	*	○	○	○	○	○
M. floricola (Vero Beach, Florida)	–	–	–	–	–	–	–
M. floricola[c] (Bahia Honda Key, Florida)	–	–	○	–	–	–	–
M. floricola (San Lorenzo, Puerto Rico)	–	–	–	–	–	–	–
M. minimum (Douglas Co., Kansas)	*	–	–	○	–	–	–
M. minimum (Harvey Co., Kansas)	*	–	–	○	–	–	–
M. minimum (Otero Co., Colorado)	*	–	–	○	–	–	–
M. new sp. near *minimum* (Gainesville, Florida)	*	*	–	–	–	–	–
M. near *emersoni* (Taos Co., New Mexico)	○	*	–	–	○	–	–
M. near *emersoni* (Bernadillo Co., New Mexico)	○	*	–	–	○	–	–
M. near *emersoni* (Lubbock, Texas)	○	*	–	–	○	–	–
M. cyaneum (Hualapai Mt. Park, Arizona)	–	*	–	–	+	–	–
M. ebeninum (San Lorenzo, Puerto Rico)	*	○	○	–	–	*	*

[a] Taxonomy as determined by M.B.D. who is revising the North American species in this genus.

[b] * = major component; + = minor component (10–12%); ○ = trace; – = not detected.

[c] Traces of related unidentified alkaloids are also present.

1400 chromatograph equipped with a 2-m × 2-mm ID column packed with 4% OV-1 on Gaschrom Q. Infrared spectra were obtained from neat liquid films with a Perkin-Elmer 297 grating infrared spectrophotometer. NMR spectra were taken on a Varian T-60 instrument. Combustion analyses were performed by Atlantic Microlabs, Atlanta, Georgia.

The initial gas chromatographic–mass spectroscopic (GC/MS) analyses were performed on a LKB-2091 mass spectrometer equipped with a 2-m × 2-mm glass column packed with 10% SP-1000 on Supelcoport. Subsequent mass

spectral analyses of the natural and synthetic alkaloids and their derivatives were performed on a Hewlett-Packard 5710A GC-5983A mass spectrometer interfaced to a Hewlett-Packard 5933 data system. This instrument was equipped with either a 1.2-m × 2-mm ID glass column packed with 2% OV-101 on Chromosorb W-AWS or a 3-m × 2-mm ID nickel column packed with 2% OV-101 on Chromosorb W-AWS. Both mass spectrometers were operated at 70 eV.

Preparation of Heptafluorobutyramodes

Approximately 50 μg of crude pyrrolidine dissolved in CH_2Cl_2 was placed in a 15-ml Teflon-lined screw-cap centrifuge tube, the solvent was removed with nitrogen, and the residue was dissolved in 0.2 ml of neat heptafluorobutryic anhydride. The mixture was warmed for 10 min at 50°C and treated with 2 ml of hexane and 2 ml of saturated $NaHCO_3$. After shaking, the aqueous phase was removed, and the hexane phase was extracted twice with saturated $NaHCO_3$, once with water, and dried over anhydrous sodium sulfate.

Preparation of Methoxyethers

A gentle stream of nitrogen was used to remove the solvent from the crude hexane solutions of the heptafluorobutyramides. The residue was taken up in 2 ml of hexane-methanol (60:40), treated with approximately 50% excess of mercuric acetate, and stirred in the dark for 24 hr. The mixture was then treated with a slight excess of sodium borohydride and after 5 min, with two drops of glacial acetic acid and 2 ml of water. The hexane phase was separated and extracted twice more with water. The combined aqueous phases were extracted with hexane, and then the combined hexane phases were dried over anhydrous sodium sulfate. Following concentration, the hexane solutions were analyzed directly by GC-MS.

Synthesis of 2-(1-Hex-5-enyl)-5-(1-non-8-enyl)pyrrolidine.

9-Decenal(8). A solution containing 10.0 g (64mmol) of 9-decenol-1 in 64 ml of CH_2Cl_2 was added all at once to a rapidly stirred suspension of 21 g of pyridinium chlorochromate (Corey and Suggs, 1975) in 130 ml of Ch_2Cl_2. After 2 hr, 900 ml of ether was added and the mixture was filtered through Florisil, Distillation gave 6.7 g of pure aldehyde (68% yield), at 61–65° C (1.0 mm Hg); IR 3075, 2845, 2710, 1730, 1645, 995, and 905 cm^{-1}; NMR, δ9.7(1H, t, J = 2Hz, C<u>H</u>O), 5.9(1H, d of d of t, J = 18, 10, and 6 Hz, CH_2=C<u>H</u>—), 5.0(1H, br d, J = 18 Hz, CH_2=CH—), 4.9(1 H, br d, J = 10 Hz, CH_2=CH—), 2.38 (2H, br t, C<u>H</u>$_2$CHO), 2.0 (2H, m, C<u>H</u>$_2$CH=CH_2), 1.35[10H, br s, —$(CH_2)_5$—].

Analysis: Calculated for $C_{10}H_{18}O$: C, 77.87; H, 11.76; found: C, 77.09; H, 11.53.

1,11-Dodecadien-3-ol (9). A solution containing 6.7 h (43.5 mmol) of aldehyde **8** in 10 ml of tetrahydrofuran was added dropwise to a well-stirred solution of 1.5 equivalents of vinyl magnesium bromide in tetrahydrofuran under a nitrogen atmosphere. The mixture was stirred at room temperature for 45 min, heated to reflux for 2 hr, cooled, and quenched with saturated NH_4Cl solution. The mixture was extracted with ether (3 × 50 ml), and the ether extracts were dried over anhydrous magnesium sulfate. After filtration, distillation gave 5.8 g of pure diene alcohol **9** (73% yield), bp 72–76° C (0.2 mm Hg): IR 3330 (br), 3070, 1640, 990, and 905 cm^{-1}; NMR, δ5.71(2H, complex m, CH_2=C<u>H</u>—), 5.0(4H, complex m, C<u>H</u>$_2$=CH—), 4.0(1H, br m, C<u>H</u>—OH), 4.03(1H, s, —OH), 1.95(4H, m, CH_2=CH—C<u>H</u>$_2$— and C<u>H</u>$_2$CH—OH), 1.3 [10H, br s, —$(CH_2)_5$—].

Analysis: Calculated for $C_{12}H_{22}O$: C, 79.06; H, 12.16; found: C, 79.11; H, 12.17.

1,18-Nonadecadien-7,10-dione (10). A solution containing 5.8 g (32 mmol) of dienol **9** in 10 ml of CH_2Cl_2, was added to a suspension of pyridinium dichromate (Corey and Schmidt, 1979), in 50 ml of CH_2Cl_2, and the mixture was stirred 10 hr. After the usual work-up, distillation gave 3.0 g of the unstable 1,11-docecadien-3-one (53% yield, bp 68–73° C (0.3 mm Hg): IR 3070, 1695, 1685, 1645, 1615, 990, 960, and 904 cm^{-1}. This vinyl ketone was immediately mixed with 1.87 g (16.6 mmol) of 6-heptenal (Jones et al., 1980) and 0.5 g of 5-(2′-hydroxyethyl)-4-methyl-3-benzylthiazolium chloride (Stetter and Kuhlmann, 1974). After purging with nitrogen, 2.3 ml of triethylamine were added, and the mixture was refluxed for 20 hr. After cooling, the mixture was taken up in ether and filtered through a short Florisil column. The solvent was removed, and kugelrohr distillation at 0.2 mm Hg gave 3.3 g of diketone **10** as a waxy solid (68% yield), mp 41–44° C. Sublimation of the pot residue produced another 0.65 g of **10** (13% yield), mp 42–43° C; NMR, δ5.8(2H, d of d of t, J = 18, 10, and 6 Hz, CH_2=C<u>H</u>—), 5.0 (2H, br d, J = 18 Hz, trans C<u>H</u>$_2$=CH—), 4.9(2H, br d, J = 10 Hz, cis C<u>H</u>$_2$=CH—), 2.55(4H s, CO<u>CH</u>$_2$<u>CH</u>$_2$CO), 2.41(4H, m, C<u>H</u>$_2$CO), 2.0(4H, m C<u>H</u>$_2$C=C), 1.3(14H, br s, alkyl —CH_2—); MS m/z(rel. intensity) 292 (0.5, M+), 237(2), 224(2), 209(2), 195(2), 182(2), 181(4), 173(1), 167(2), 154(10), 153(4), 149(5), 138(8), 136(15), 135(5), 127(4), 125(3), 122(2), 121(5), 114(12), 112(1), 111(20), 110(8), 109(5), 107(13), 99(10), 98(8), 95(11), 93(9), 83(28), 81(10), 79(9), 71(20), 69(31), 68(5), 67(25), 57(10), 56(11), 55(100), 53(9).

Analysis: Calculated for $C_{19}H_{32}O_2$: C, 78.03; H, 11.03; found: C, 77.78; H, 11.06.

trans-*2-(1-Hex-5-enyl)-5-(1-non-8-enyl) pyrrolidine (1) and its cis Isomer.* A solution containing 2.0 g of diketone **10** (6.85 mmol), 0.1 g of KOH, 0.54 g of ammonium acetate, and 0.5 g of sodium cyanoborohydride in 15 ml of methanol was stirred for 15 hr. An excess of sodium borohydride was added, and the mixture was stirred an additional hour. The usual work up gave 1.8 g of pyrrolidine **1** as a 50:50 mixture of *cis* and *trans* isomers that was greater

than 95% pure by GLC analysis (OV-1, SP—1000) (approx. 90% yield): IR 3260 (w), 3070, 1640, 990, and 905 cm^{-1}; NMR δ of the mixture 5.8(2H, d of d of t, J = 18, 10, and 6 Hz, CH_2=C<u>H</u>—), 5.0(2H, br d, J = 18 Hz, trans C<u>H</u>$_2$=CH—), 4.9 (2H, br d, J = 10 Hz, cis C<u>H</u>$_2$=CH—), 3.0 (3H, br m, C<u>H</u>—N and N—H), 2.1(4H, m, CH_2—CH=CH_2), 1.3(22H, br s, alkyl —CH_2—); MS m/z (rel. intensity), 277(3, M+), 276(2), 234(12), 220(14), 195(15), 194(100), 178(10), 153(9), 152(83), 150(4), 136(3), 124(2), 122(2), 110(2), 109(1), 108(2), 96(5), 95(3), 94(2), 83(7), 82(30), 81(5), 70(2), 69(4), 68(10), 67(18), 57(1), 56(4), 55(20), 54(3), 53(2).

Analysis: Calculated for $C_{19}H_{35}N$: C, 82.24; H, 12.71; N, 5.05; found: C, 82.10; H, 12.72; N, 5.02.

Treatment of a small sample of the mixture of pyrrolidine **1** and its *cis* isomer with heptafluorobutyric anhydride as described above produced the *N*-heptafluorobutyramide derivative, MS m/z (rel. intensity) 473(1, M+), 416(3), 391(10), 390(45), 350(1), 349(18), 348(100), 305(2), 304(21), 280(2), 267(3), 266(40), 240(5), 169(3), 135(3), 135(12), 121(1), 109(8), 107(4), 95(12), 93(8), 81(18), 79(10), 69(15), 67(33), 55(22). Methoxymercuration–demercuration of this derivative as described above gave 2-(5-methoxyhex-1-yl)-5-(8-methoxynon-1-yl)-*N*-heptafluorobutyrylpyrrolidine as a major product whose mass spectrum is identical with that shown in Figure 1, which was obtained from the natural compound.

2-(1-Hex-5-enyl)-5-nonylpyrrolidine (2)

The preparation of the monounsaturated pyrrolidine **2** has already been described (Jones et al., 1980). A small sample of **2** was treated with heptafluorobutyric anhydride to give the *N*-heptafluorobutyryl derivative, MS m/z (rel. intensity) 475(0.5 M+), 446(1), 393(18), 392(100), 369(3), 368(8), 351(7), 350(62), 349(5), 348(50), 306(10), 280(3), 267(11), 266(60), 264(6), 240(15), 197(2), 180(1), 169(7), 145(2), 135(18), 123(12), 111(10), 110(7), 109(28), 107(10), 97(35), 95(47), 93(18), 91(5), 85(10), 83(44), 81(57), 79(20), 71(20), 70(15), 69(84), 68(22), 67(82), 57(33), 55(98). Methoxymercuration-demercuration of this derivative as described above gave 2-(5-methoxyhex-10-yl)-5-nonyl-*N*-heptafluorobutyryl-pyrrolidine as the major product. MS m/z (rel. intensity) 507(0.5, M+), 492(3), 407(18), 393(10), 392(44), 380(1), 368(12), 353(2), 349(8), 348(45), 306(3), 294(3), 281(2), 280(4), 266(28), 264(40), 197(2), 182(8), 169(5), 152(3), 147(2), 145(2), 135(18), 123(9), 121(4), 119(2), 111(7), 109(21), 107(10), 105(6), 97(22), 95(30), 93(17), 91(8), 85(13), 83(23), 81(30), 79(20), 71(16), 69(40), 67(38), 59(100), 57(20), 55(48).

Mass Spectral Analysis of M. ebeninum *Alkaloids*

The GC/MS from the extracts of *M. ebeninum* showed one broad peak whose mass spectrum had ions at m/z = 277 (M+), 194, and 152, indicative of pyrrolidine **1**. In addition there were ions at m/z = 275, 274, 207, 206, 192,

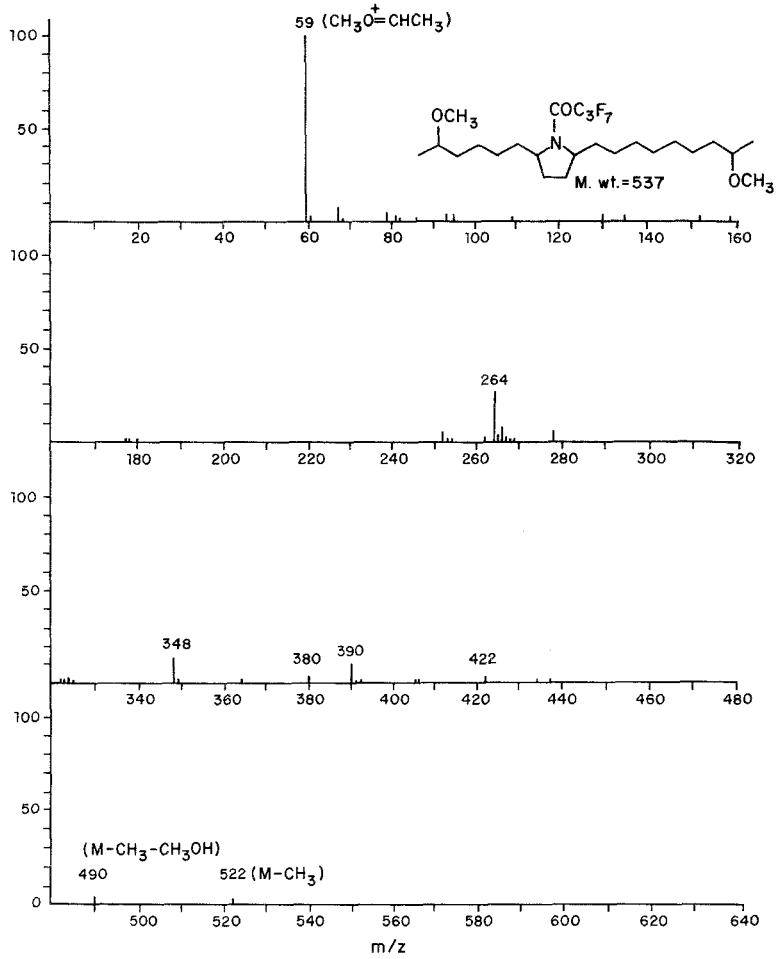


FIG. 1. MS of the dimethoxyheptafluorobutyryl derivative of **1**.

165, 164, 150, and 82, indicative of the pyrrolines **6** and **7**. Treatment of a portion of the extract with sodium borohydride converted the mixture to a single component with a mass spectrum identical to that of **1**. Treatment of another portion of the extract with sodium borodeuteride gave an inseparable mixture of isotopomers with a mass spectrum containing fragment ions at $m/z = 278$, 195, and 153 as well as those at $m/z = 277$, 194, and 152. The intensities of these ions indicated that there were essentially equivalent amounts of deuterated and undeuterated pyrrolidines. Traces of pyrrolidines **2** and **3** were also present in these extracts as well separated GC peaks. Compound **2** mass spectrum identical with that previously reported (Jones et al., 1980), while **3** showed the following important ions; $m/z = 281$(1, M+), 196(85), and 154(100), indicative of its saturated side chains.

Quantitation of Alkaloids in M. *near* metoecus

Five or ten ant workers were taken from a group of ants freshly killed by freezing and macerated in 10 μl of either CH_2Cl_2 or ethanol. A 1.0-μl sample of the supernatant liquid was immediately analyzed by gas chromatography. This was repeated seven times using a total of 60 ants. The results were calibrated with the gas chromatogram from 1.0 μl of a solution containing 2.3 mg/ml of pyrrolidine **2** in CH_2Cl_2, and they indicate that each ant worker contains between 0.5 μg and 2.0 μg of alkaloid.

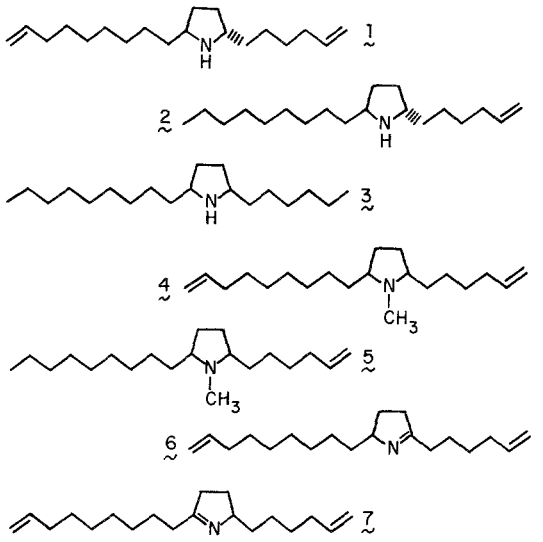
Methylation of Alkaloids in M. *near* metoecus

A CH_2Cl_2 extract of a sample of *M.* near *metoecus* containing pyrrolidines **1**, **2**, and **3** was taken to dryness with a gentle stream of nitrogen and treated with one drop of 28% formaldehyde and one drop of formic acid. The resulting mixture was heated in a closed vial overnight. The solution was made basic with 10% KOH and extracted with chloroform to provide a mixture containing only *N*-methyl-pyrrolidines. GC-MS (2-m × 2-mm column packed with 10% SP-1000 on Supelcoport) showed **4** and **5** eluting at about the same temperature and characterized by their mass spectral fragmentation patterns: **4**, m/z = 291(M+), 208(M—C_6H_{11}), and 166(M—C_9H_{17}); **5**, m/z = 293(M+), 210(M—C_6H_{11}), and 166(M—C_6H_{19}). The methylated derivative of **3** was well separated under these conditions and its mass spectrum had important ions at m/z = 295(M+), 210, 168. The mass spectrum of the nearly inseparable mixture of **4** and **5** prepared this way was almost identical to that of these two alkaloids and served to identify them when they were found in fresh venom extracts of the various species listed in Table 1.

RESULTS

The *trans*-2,5-dialkylpyrrolidines **1–5** are characteristic venomous components of all species of *Monomorium* analyzed. While quantitative and qualitative variations are evident, the venoms of the five native species, and those of *M. ebeninum* and one population of *M. floricola* as well, are all characterized by the presence of 2,5-dialkyl-pyrrolidines with the same C_{19} carbon skeleton (Scheme 1).

The novel 2-(1-hex-5-enyl)-5-(1-non-8-enyl) pyrrolidine (**1**) and the previously reported 2-(1-hex-5-enyl)-5-nonylpyrrolidine (**2**) (Jones et al., 1980), like the major alkaloidal components in every species studied, are easily recognizable from their mass spectra (Pedder et al., 1976). The mass spectra of these compounds show a pair of intense peaks resulting from α-cleavage of side chains and a weak molecular ion of odd mass: M+ 277(3%), 194(100), 152(83), and M+ 279(4%), 196(83), 152(100). These data indicate a 6-carbon monounsaturated side chain and either a 9-carbon monounsaturated or a



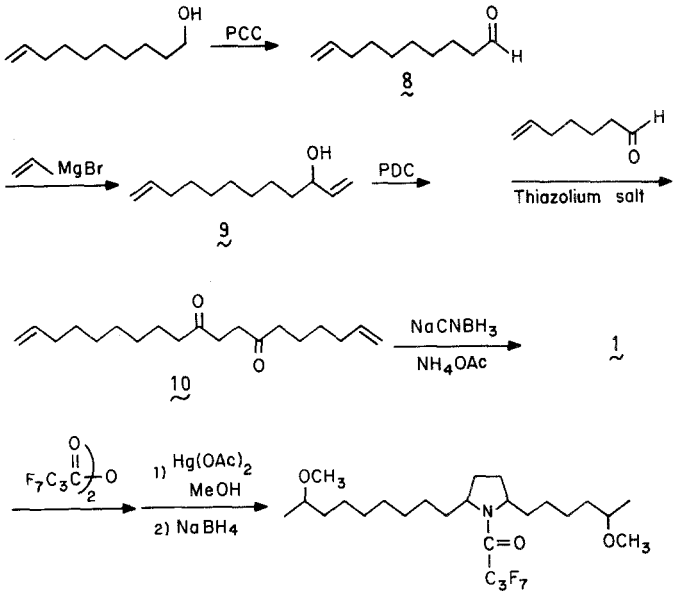
SCHEME 1

saturated side chain, but do not reveal the positions of the double bonds.

The technique of methoxymercuration–demercuration was utilized to locate the double bonds in the side chains (Howard et al., 1978; Blomquist et al., 1980). Since this technique was not successful with the free amines, the pyrrolidines were converted to the corresponding *N*-heptafluorobutyramides. These derivatives, upon treatment with mercuric acetate and methanol, followed by reduction with sodium borohydride, yielded the methoxylated compounds, albeit in rather modest yields. When the starting alkaloid was diunsaturated, both the di- and monomethoxylated derivatives were formed. The mass spectra of these compounds, via the exclusive formation of ions at $m/z = 59$, showed the incorporation of methoxy ether groups on the penultimate carbon of the side chains, indicating terminal double bonds in every case (Figure 1).

Pyrrolidine **1**, prepared by previously described methodology (Scheme 2) (Jones et al., 1980), was identical by GC/MS to the natural alkaloid; the derivatives of the natural alkaloid and synthetic compound were also identical. The monomethoxy-*N*-heptafluorobutyramide derivative of pyrrolidine **2** was also prepared, and again its mass spectrum as well as the mass spectrum of the free base matched the corresponding spectra from the ant extracts.

The synthetic alkaloids **1** and **2** are a 1:1 mixture of *cis* and *trans*-2,5-dialkylpyrrolidine isomers separable by gas chromatography (Jones et al., 1980). As shown previously, the *cis* configuration can be assigned to the isomer that elutes first. Comparison by retention time and coinjection showed that



SCHEME 2

the naturally occurring pyrrolidines **1** and **2** were of the *trans* configuration in every sample of *Monomorium* extract available. In *M. viridum* the ratio of pyrrolidine **1** to pyrrolidine **2** was found to be approximately 3:2, while in *M.* new sp. near *minimum* this ratio was about 1:2.

The 1-pyrrolines **6** and **7**, major components in the alkaloidal mixture from *M. ebeninum*, were characterized by their parent ion at $m/z = 275$ as well as the M-1 ion at 274 and the McLafferty rearrangement ions at 207 and 165 in **6** and **7**, respectively. They also showed characteristic allylic cleavage ions at $m/z = 206$ and 150 from **6** and $m/z = 192$ and from **7**. An ion at $m/z = 82$ arises from both pyrrolines. The intensities of these ions indicated that there was slightly more of **6** and **7**.

These Schiff bases were further characterized by their conversion to pyrrolidine **1** upon treatment with sodium borohydride. In *M. ebeninum*, their incorporation of deuterium upon treatment with sodium borodeuteride was evident because of the appearance of ions at m/z =278, 195, and 153; the intensity of these ions relative to the undeuterated ions showed that the total amount of **6** and **7** was roughly equal to the amount of **1** in the alkaloid mixture from this ant.

Three minor alkaloidal components were also found in the *Monomorium* species included in this investigation. In extracts of *M.* near *metoecus*, *M. viridum*, and *M. ebeninum*, a minor component (approximately 1–2% of the mixture) had a characteristic mass spectrum [281(1%), 196(85), 154(100)] that

could be attributed to 2-hexyl-5-nonylpyrrolidine (**3**). In addition, small amounts of the *N*-methyl and analogs of pyrrolidines **1**, and **2** (**4** and **5**) were detected. Their structures were suggested by their gas chromatographic behavior and their mass spectra. Confirmation of the structures of **4** and **5** was obtained by *N*-methylation of the extracts of a sample of *M.* near *metoecus* with formaldehyde and formic acid, which converted all of the N-H alkaloids present into their *N*-methyl analogs.

With the exception of *M. floricola*, each species appeared to produce a characteristic venom fingerprint with no significant differences in alkaloidal composition being associated with different populations of the same species (e.g., *M. minimum*) (Table 1). No alkaloids were detected in extracts of two populations of *M. floricola* whereas that from Bahia Honda Key, Florida (Table 1) contained a very small quantity of pyrrolidine **3**, along with traces of several unidentified alkaloids. The venom of *M. viridum* was found to be especially distinctive in being the only one of the venoms studied that contained all of the dialkylpyrrolidines detected in these secretions. In addition, it and *M.* new sp. near *minimum* produce the only venom in which both pyrrolidines **1** and **2** are major constituents. Among the *Monomorium* species, the venom of *M. ebeninum* was especially characteristic in containing two pyrrolines as major constituents; that of *M. cyaneum* was exceptional in producing the *N*-methylpyrrolidine **5** as a major venomous alkaloid.

Since *M.* near *metoecus* was readily available, samples of these ants were analyzed in order to determine the approximate amount of alkaloid produced by each ant. The extracts from five or ten individuals macerated in a known volume of solvent were compared to a standard solution of pyrrolidine **2** by gas chromatography. Seven replicates of this comparison indicated that each *M.* near *metoecus* worker contained between 0.5 μg and 2.0 μg of alkaloids.

DISCUSSION

M. ebeninum and the native North American species of *Monomorium* appear to have limited biosynthetic capacities in that they produce only *trans* isomers of 2,5-dialkylpyrrolidines possessing C_6 and C_9 side chains. The most characteristic compound produced by these ants, pyrrolidine **1**, which has not been previously detected as a natural product, appears to be the only known example of a diunsaturated pyrrolidine synthesized by animals. While terminally unsaturated side chains on 2,5-dialkylpyrrolidines have been previously reported as being produced by *Monomorium* species (Jones et al., 1980; Ritter and Persoons, 1975; Ritter et al., 1975), and the synthesis of one of these compounds has been described (Ritter and Stein, 1978), this is the first report of experimental evidence for this assignment. The technique of methoxymercuration-demercuration produced the dimethoxy-*N*-heptafluoro-

butyramide derivative of natural and synthetic alkaloid **1** on a microscale. Figure 1 depicts the mass spectrum of this compound from the major venom component of *M. minimum* (Douglas Co., Kansas), and Figure 2 shows the molecular fragments that can be assigned to this spectrum. The base peak at $m/z = 59 (CH_3CHOCH_3)^+$ indicates incorporation of the methoxyl groups at the penultimate carbon atoms of the side chains. There is no evidence for the presence of methoxyl groups at any other site on the molecule. An analogous fragmentation pattern was also observed for the monomethoxy-*N*-heptafluorobutyramide derivative of pyrrolidine **2**, which again indicated a terminal double bond.

Since oxymercuration reactions proceed to give mostly Markovnikov addition of the hydroxyl species (Brown and Geoghegan, 1970), it is not surprising that no observable terminal methoxylation occurred. The *Monomorium* unsaturated pyrrolidines thus contain only terminal double bonds, similar to the unsaturated pyrrolidines reported in *M. pharaonis* (Ritter et al., 1975, 1980).

Synthesis by an unambiguous route confirms the above structural assignments for pyrrolidines **1** and **2** (Scheme 1). This synthetic methodology also permits assignment of stereochemistry of the alkyl groups about the

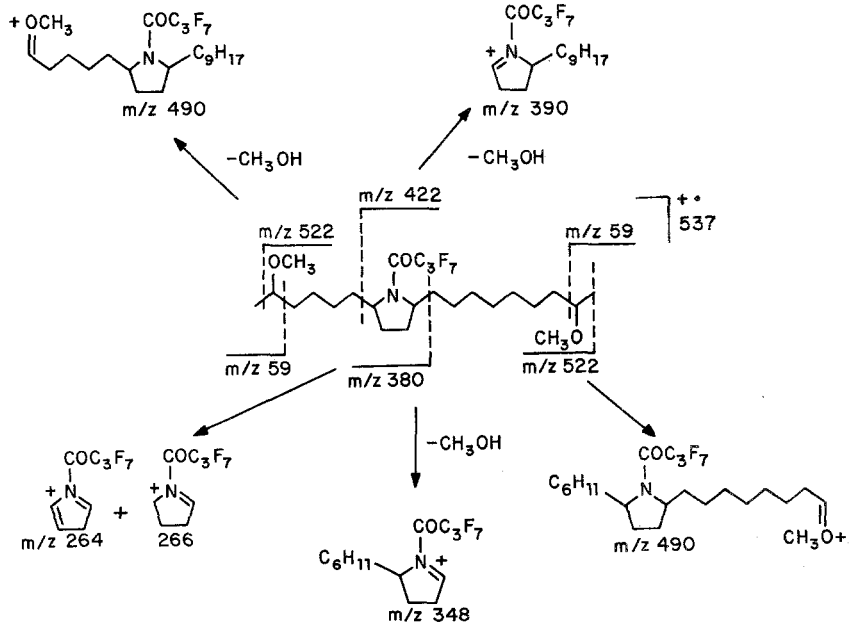


FIG. 2. Suggested interpretation of the MS of the dimethoxyheptafluorobutyryl derivative of **1**.

pyrolidine ring since both *cis* and *trans* isomers are formed (Jones et al., 1980) having different gas chromatographic retention times but identical mass spectra. Comparison of the natural and synthetic alkaloids shows that in every case, the later-eluting *trans* isomer is produced by the *Monomorium* species listed in Table 1.

Since it has been shown that 1-pyrrolines such as **6** and **7** can be formed as artifacts in GC-MS instruments (Fales et al., 1980), their appearance in trace amounts is always somewhat suspect. On the other hand, reduction of the extract of *M. ebeninum* with sodium borodeuteride shows that the 1-pyrrolines **6** and **7** make up at least half of the alkaloid mixture in this species. The allylic cleavage ions and McLafferty rearrangement ions that characterize these compounds arise from previously described fragmentation pathways (Pedder et al., 1976; Fales et al., 1980).

The minor constituents of these alkaloidal mixtures, pyrrolidines **3**, **4**, and **5**, serve to further characterize the venom mixtures from each *Monomorium* species. 2-Hexyl-5-nonylpyrrolidine **3** is immediately identifiable from its mass spectral fragmentation pattern which is characteristic of other 2,5-dialkylpyrrolidines (Jones et al., 1980). The *N*-methylpyrrolidines, on the other hand, have mass spectral fragmentation patterns identical with their parent pyrrolidines **1** and **2**, with the α-cleavage and parent ions increased by 14 mass units (Figure 3). Computer-constructed, selected ion chromatographs of these compounds show symmetrical peaks, whereas the parent pyrrolidines

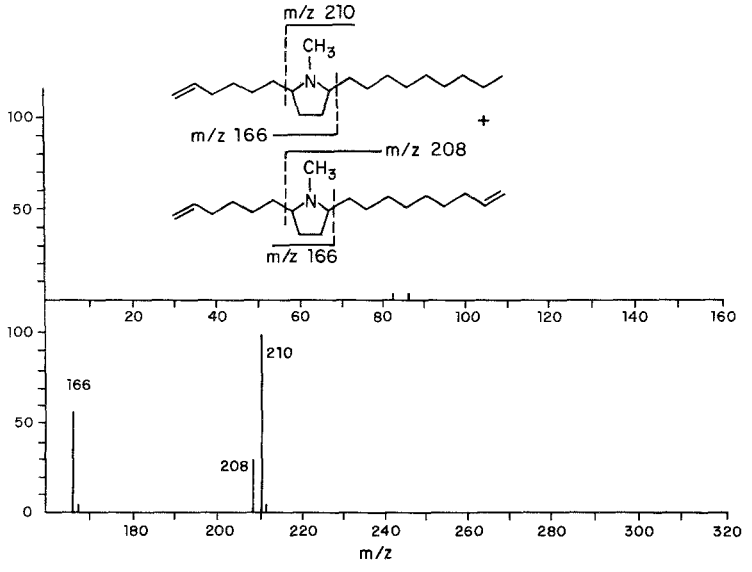


FIG. 3. *N*-Methylpyrrolidines **4** and **5** from *M. viridum*.

show distinctly unsymmetrical peaks (OV-101), presumably because of interactions of the active hydrogen on the nitrogen with the column substrate. The symmetrical shape of the peaks for these compounds tends to exclude the possibility that the compounds are dialkyl or dialkenyl piperidines. In addition, their mass spectra are essentially identical to those of the *N-methylpyrrolidines prepared from a sample of natural Monomorium* pyrrolidines by *N*-methylation. Since there is so little of **4** and **5** present, there is no direct evidence that the position of their double bonds is terminal. However, the fact that several species of *Monomorium* that were examined contain these *N*-methyl analogs in exactly the same relative proportions as pyrrolidines **1** and **2** in their venoms, suggests that perhaps *N*-methylation is a terminal biosynthetic process, so it would be unlikely that the compounds differ in the location of their double bonds.

From a chemotaxonomic standpoint, these *Monomorium* venoms would appear to possess at least a modicum of value. All apparent species that were analyzed produce distinctive venoms in terms of their qualitative and quantitative compositions. For example, *M. viridum* is the only species that produces all the dialkylpyrrolidines detected in these venoms, being further distinguished by synthesizing both the diunsaturated and monounsaturated compounds, **1** and **2**, as major constituents. The venom of the closely related species *M.* near *metoecus* is almost as qualitatively rich as that of *M. viridum*, differing only in the absence of the *N*-methyl compound **4**. The only pyrrolidine-producing species lacking the diunsaturated pyrrolidine **1**, *M. cyaneum*, is especially distinctive in producing significant amounts of the *N*-methyl pyrrolidine **5** (Table 1).

Even though relatively few *Monomorium* (*Monomorium*) species have been analyzed, there appears to be a considerable amount of variation in the chemistry of the venoms synthesized by different species. Although the venoms of native North American species in this subgenus contain only variations on 2-hexyl-5-nonylpyrrolidine, that of *M. pharaonis* contains a diversity of other nitrogen heterocycles which include pyrrolidine **2**, a major component in the venoms of the *Monomorium* species analyzed in this investigation. In addition, the venom of *M. pharaonis* contains 2,5-dialkylpyrrolidines with different side-chain lengths, as concomitants of dialkylindolizidines (Ritter et al., 1975).

The dialkylpyrrolidines are probably utilized as repellents for ants in interspecific contexts. Hölldobler (1973) demonstrated that *M. pharaonis* can utilize its venom as a powerful repellent while plundering brood from the nests of other species. This plundering *modus vivendi* is characteristic of thief ants in the genus *Solenopsis*, many of which produce dialkylpyrrolidines in their venoms (Jones et al., 1979, 1980). It has been recently demonstrated that the thief ant *S. fugax* produces 2-butyl-5-heptylpyrrolidine in its poison gland,

utilizing this compound as a venom mace while stealing brood from the nests of foreign ant species (Blum et al., 1980). It appears that the dialkylpyrrolidines synthesized by *Monomorium* species are also utilized to repel other species of ants in competitive situations involving the acquisition of food. One of us (J.T.) has observed workers of *M. ebeninum* and *M. floricola* effectively repelling other species of ants by utilizing their venomous secretions. The biochemical evolution of alkaloidal repellents may have enabled these diminutive species to exploit sociality in a way that would not have been otherwise possible.

Note Added in Proof—As this manuscript was going to press, we had the opportunity to examine two other *Monomorium* species. *M. minutum* (San Sebastian, Spain) was found to contain only pyrrolidines **1** and **2** in approximately a 20:1 ratio. *M. carbonarium* (Key Largo, Florida) was found to contain only pyrrolidine **1**. None of the other alkaloids listed in Table 1 were detected in these species.

Acknowledgments—We thank J. Moody for collecting *M.* near *emersoni* (Lubbock, Texas) and James Trager for providing *M. floricola* (Vero Beach, Florida) and *M.* new sp. near *minimum* (Gainesville, Florida), Dennis Howard and Mike Tomalski for collecting *M. floricola* (Bahia Honda Key, Florida) and *M. carbonarium*, and C. Collingwood for collecting and identifying *M. minutum*.

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FIELD ATTRACTIVENESS OF CHIRALLY DEFINED SYNTHETIC ATTRACTANTS TO MALES OF *Diprion similis* AND *Gilpinia frutetorum*

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Abstract—Field attractiveness of synthetic attractants toward males of two introduced species of sawflies was examined. It was first established that the esters of 2*S*,3*S*,7*S*-3,7-dimethylpentadecan-2-ol (diprionol), which have been active toward males of many *Neodiprion* species, were inactive toward males of *Diprion similis* and *Gilpinia frutetorum*. To determine the chiral combination of the alcohol moiety, four different isomers, each containing specific chirally defined carbons, were synthesized. As a result it was concluded that the most active chiral arrangement of diprionol for these species is 2*S*, 3*R*, 7*R*.

Key Words—Synthetic attractant, introduced pine sawfly, chiral specificity, *Diprion similis*, *Gilpinia frutetorum*, esters, 2*S*,3*S*,7*S*-3,7-dimethylpentadecan-2-ol acetate and propionate, Hymenoptera, Diprionidae.

INTRODUCTION

Jewett et al. (1976) reported the identification of the chemical structure of the sex attractant of diprionid sawflies as either the acetate or propionate of 3,7-dimethylpentadecan-2-ol. Subsequently Matsumura et al. (1979) and Kraemer et al. (1979) established that the chiral configuration of the major sex-pheromone of *Neodiprion lecontei* (Fitch) and *Neodiprion pinetum* (Norton) is 2*S*, 3*S*, 7*S* with respect to the three optically active carbon centers of the alcohol moiety. This confirms the earlier diagnosis from the nuclear

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magnetic resonance spectra of the natural pheromone that the pheromone of *N. lecontei* has the erythro configuration (Jewett et al., 1976). The term erythro configuration is applied to the optical arrangement of diastereomeric 2- and 3-carbons, indicating the arrangement is either 2*S*,3*S* or 2*R*,3*R*. The threo arrangement requires that the chiral property of these positions are either 2*S*,3*R* or 2*R*,3*S*. These studies were facilitated by the work of Tai et al. (1976), who synthesized two erythro isomers [2*R*,3*R*,7(*R*/*S*) and 2*S*,3*S*,7-(*R*/*S*)], a 1:1 mixture of 2*S*,3*R*,7(*R*/*S*) and 2*R*,3*S*,7(*R*/*S*), and a 1:1 mixture of threo isomers, and by the work of Mori et al. (1978), who synthesized four erythro isomers.

As to the variation of the sex-pheromone among diprionid species, it has been hypothesized by Jewett et al. (1976) that the difference in pheromones between *Neodiprion* species and *Diprion* species resides in the optical configuration of the alcohol (diprionol) moiety. To prove the above point, we tested two threo isomers with the racemic configuration at the 7 position 2*S*,3*R*,7(*R*/*S*) and 2*R*,3*S*,7(*R*/*S*), and four isomers with the racemic configuration at the 2 position 2(*S*/*R*),3*R*,7*R*; 2(*S*/*R*),3*R*,7*S*; 2(*S*/*R*),3*S*,7*R*; and 2(*S*/*R*),3*S*,7*S* by modifying the optically pure erythro isomers from Mori et al. (1978) for the determination of the 7 position. We now report the results of the field attractiveness of these preparations to the males of *Diprion similis* (Hartig) and *Gilpinia frutetorum* (Fabricius).

METHODS AND MATERIALS

Method of Bioassay. Trapping studies were conducted from the end of May to June 20 for first-generation adults and from August 2 to August 28 for second-generation adults, in 1978 and 1979 in white pine stands at Crossmore, North Carolina; Siren, Wisconsin; Hartwick Pines Park and Rose Lake, Michigan.

The traps used were white Pherocon II traps (Zoecon Corporation, Palo Alto, California). Synthetic pheromones were dissolved in *n*-hexane and 1.0-ml aliquots were stored in sealed glass ampules. Each ampule was broken in the field and its contents poured onto a ca 2.5-cm cotton wick held with a pair of forceps. The wick was then attached to the inside roof in the center of a trap. The forceps used were washed each time with alcohol to avoid contamination. The traps were hung from pine branches at approximately 1.5 m above the ground and spaced at 5- to 10-m intervals (Jewett et al., 1978; Matsumura et al., 1979).

Synthesis of 2-Position Mixture Threo Isomers from Erythro Isomer Synthesized by Mori et al. (1978). For the identification of the optical configuration of the second carbon position of diprionol, the four-erythro enantiomers described by Mori et al. (1978) were racemized at the 2 position

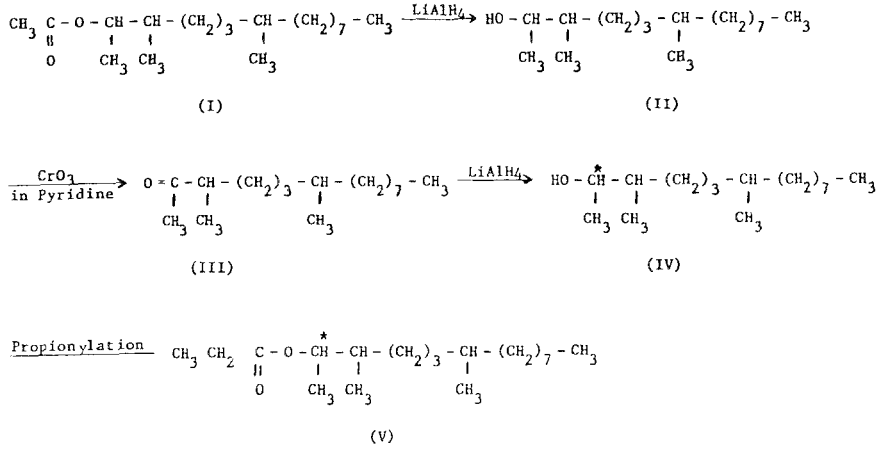

FIG. 1. The route of modification of pure erythro enantiomer from K. Mori to the 1 : 1 mixture of threo and erythro. Asterisk indicates racemic on that position.

via oxidation followed by reduction (Figure 1). Only the example for conversion of one isomer is shown, but the processes employed for other isomers were identical.

Hydrolysis of Acetate of 2 R,*3* R,*7* S-*Diprionol (I).* To the diprionol ester (5.3 mg in 1 ml anhydrous ether) 2 equimolar equivalents of lithium aluminum hydride in 1 ml ether were gradually added with stirring by using a magnetic stirrer in an acetone–ice bath. The reaction mixture was brought to room temperature and was kept for 2 hr. Moistened ether was added dropwise to the reaction product. The aluminum hydroxide formed was separated by using a centrifuge. The organic phase was separated and extracted with ether for three times.

Oxidation of (2 R,*3* R,*7* S)-*Diprionol (II) by CrO_3–Pyridine Complex.* To a solution of CrO_3 (10 mg in 2 ml pyridine), the whole amount of (2*R*,3*R*,7*S*)-diprionol, as obtained above in 1 ml pyridine, was added at approximately −5° C. The system was maintained at the same temperature for 1 hr with stirring. It was then allowed to warm to room temperature and kept for 1 hr. The mixture was poured into 10 ml of water and extracted by *n*-hexane three times, dried over anhydrous magnesium sulfate, and the solvent evaporated using a stream of dry nitrogen gas.

Reduction of (3 R,*7* S)-*3,7-dimethypentadeca-2-one (III) to 2(*R/S*),3* R, *7* S-*Diprionol (IV).* To $LiAlH_4$ (approximately 10 mg) in 1 ml ether, the above ketone III in 1 ml of ether was added dropwise with stirring at room temperature. The $LiAlH_4$ complex obtained was hydrolyzed by adding moistened ether. The subsequent steps to obtain the reduction product were identical to those described previously. The alcohol thus obtained was a 1 : 1

mixture of erythro and threo enantiomers at the 2 position as judged by proton magnetic resonance (PMR) analysis using a 180 04 MH_z model of Bruker Co. The PMR data for the other preparations were obtained similarly. The alcohol was converted to acetate or propionate by treating with acetic anhydride in pyridine or with propionyl chloride with magnesium. The stereochemical and optical purities of these products were examined by GLC and PMR. The methods of synthesis for the 2*S*,3*R*,7(*R*/*S*) and 2*R*,3*S*,7(*R*/*S*) enantiomers will be published elsewhere.

Preparation of Modified Natural Extract Attractive to D. similis. Virgin females of *D. similis* were obtained from cocoons from mature larvae collected in the field at Hartwick Pines Park, Michigan. Two hundred females were immersed in 30 ml methanol in a 100-ml round-bottomed flask and refluxed overnight. This extraction process was repeated by using the same volume of fresh methanol. The combined methanol phase was filtered and evaporated to ca. 50 ml in a rotary evaporator and 0.5 g KOH was added. The mixture was refluxed for 2 hr. Most of the methanol was removed in a rotary evaporator. Diethyl ether (100 ml) and water (20 ml) were added, and the mixture was transferred to a separatory funnel, shaken, and the aqueous layer was removed. The solvent layer was passed through anhydrous Na_2SO_4 and evaporated. The pale yellow oil remaining after evaporation was treated with propionyl chloride and Mg (metal) at room temperature overnight. The reaction mixture was dissolved in *n*-hexane and extracted with water, dilute HCl, and saturated $NaHCO_3$ solution. The hexane phase was dried over anhydrous $MgSO_4$ and evaporated.

The residue was spotted on thin-layer chromatographic (TLC) plates (HF 254 + 366 silica gel, thickness 0.25 mm, 20 × 20 cm, and activated at 120° for 2 hr). The plates were developed until 15 cm from the origin with a mixture of hexane and ether (4:1) and four zones (Figure 2) were collected and eluted with ether. After removal of the ether, the residue was first adjusted to 2 ml with *n*-hexane (i.e., 100 female equivalents per ml) and then further diluted with *n*-hexane to give 4 females equivalents per ml. Each 1-ml aliquot was transferred to an ampule in preparation for the field tests. After the field test, the active fraction was further spotted on another silica gel plate and developed first with *n*-hexane and then with benzene. Thereafter the plate was divided to four fractions as shown in Figure 2 (R_f of propionate of diprionol was 0.45 by the same method). Each fraction was adjusted to 4 female equivalents per ml for field assay as previously mentioned. The total scheme of purification is described in Figure 2 with the biological activity for each fraction.

For GLC analysis the most active fraction, (3)-III in Figure 2, was purified once more by TLC (silica gel HF 254 + 366), hydrolyzed, and reesterified to the acetate. The plate was divided to five fractions corresponding to R_f 0–0.36, 0.36–0.54, 0.54–0.68, 0.68–0.82, and 0.82–1.0, and the region

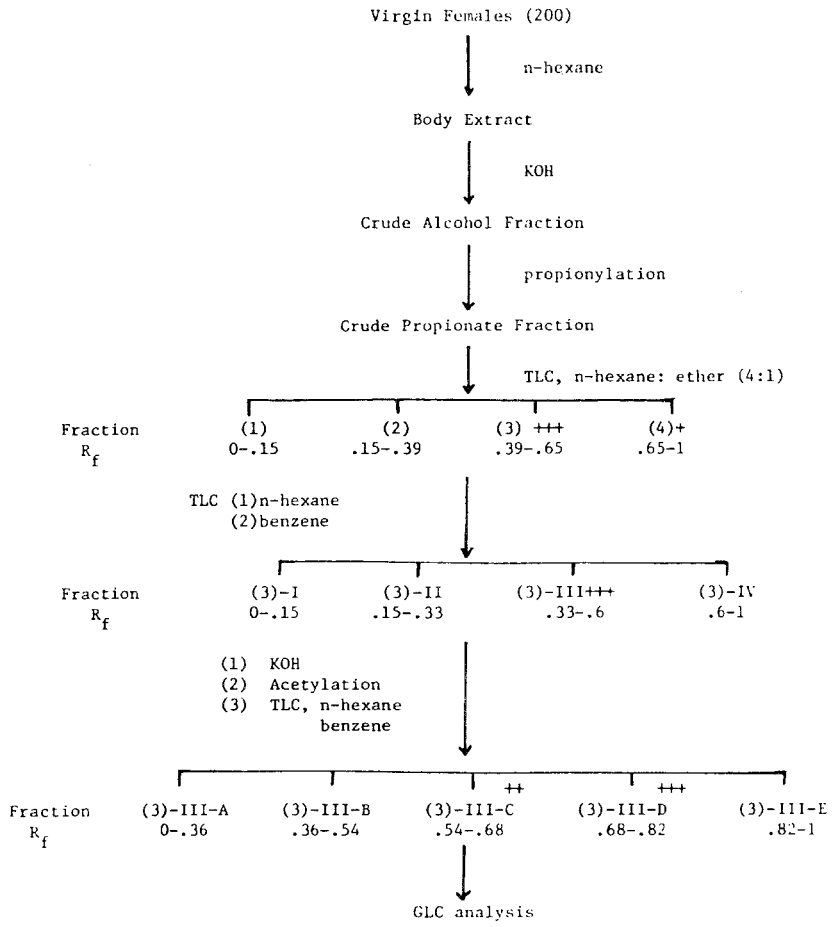

FIG. 2. Schematic representation of the purification method employed for the biological activities of fractions isolated from females of *D. similis*. The test was conducted July 25 to August 6, 1979, Hartwick Pines Forest, Grayling, Michigan, with two replicates. The biological activities are shown by +, ++, +++ (i.e., weak, medium, and strong response).

corresponding to R_f 0.68–0.82 was used for further GLC analysis. This process was necessary because of some unknown material which masked the pheromone peak when tested in the form of propionate on our GLC system.

RESULTS

The results of PMR analysis on 3,7-dimethylpentadecan-2-ol show that $2S,3R,7(R/S)/2R,3S,7(R/S)$ preparations by Tai et al., (1978) contained ca.

5% of the erythro isomer. This was probably due to epimerization at the Grignard reaction step of the synthetic process. As mentioned previously the racemized products from the erythro isomers of Dr. Mori consisted of a 1 : 1 ratio of erythro and threo isomers.

To clarify the source of the preparations, bioassay samples were coded as shown in Tables 1 and 2. All preparations originating from Dr. Mori's group bear T as the first letter of the code.

The results of field bioassay tests are summarized in Tables 1 and 2. Traps baited with the propionate of 2*S*,3*R*,7(*R*/*S*) or 2(*R*/*S*),3*R*,7*R* were most active toward *D. similis*. It is important to note here that neither 2*S*,3*S*,7*S*-*P* nor 2*R*,3*R*,7*R*-*P* were active toward the males of this species as reported by Longhurst et al. (1980). On the other hand, as noted by Jewett et al. (1976), the propionate form is very important to the males of this species as they showed no activity toward the acetate (i.e., 2*S*,3*R*,7(*R*/*S*)-A). To our knowledge, this is the first demonstration that some species of pine sawflies respond exclusively to the threo configuration (i.e., 2*S*,3*R* configuration) as a sex attractant. In a side-by-side comparison test involving three locations, 2(*R*/*S*),3*R*,7*R*-P (TMP-1) performed best among the three active threo preparations (Table 3). Since 2(*R*/*S*),3*R*,7*R*-P was more active than 2(*R*/*S*),3*R*,7*S*-P (TMP-2), the active form at the 7-carbon position was reasoned to be *R*. At the same time 2*S*,3*R*,7(*R*/*S*)-P was active while 2*R*,3*S*,7(*R*/*S*)-P was not, indicating the active forms at the second and third positions must be 2*S*,3*R*. It has been suggested that an attractant with a

TABLE 1. TRAP CATCHES OF *D. similis* MALES BY ACETATE OR PROPIONATE OF FOUR ERYTHRO AND TWO THREO ISOMERS OF 3,7-DIMETHYL PENTADECAN-2-OL PREPARED BY TAI ET AL. (1978)[a]

Chiral configuration	Code name	Amount (μg)	Number of males captured per trap		
			90 min	7 hr	Total
2*R*,3*R*,7(*R*/*S*)-A	A-1[c]	300	0	0	0
2*S*,3*S*,7(*R*/*S*)-A	A-2[c]	300	0	0	0
2*S*,3*R*,7(*R*/*S*)/ 2*R*,3*S*,7(*R*/*S*)-A[b]	A-3[c]	300	0	0	0
2*R*,3*R*,7(*R*/*S*)-P	P-1[d]	300	0	0	0
2*S*,3*S*,7(*R*/*S*)-P	P-2[d]	300	0	1	1
2*S*,3*R*,7(*R*/*S*)/ 2*R*,3*S*,7(*R*/*S*)-P[b]	P-3[d]	300	5	10	15

[a]The test was conducted on August 11, 1977, in Amery, Wisconsin with one replicate.
[b]1 : 1 mixture.
[c]A for acetate.
[d]P for propionate.

TABLE 2. FIELD RESPONSE INDEX OF *D. similis* MALES TO VARIOUS SYNTHETIC SEX ATTRACTANTS[a]

Chiral configuration	Code name	Amount (μg)	Replicates A	Replicates B	Average catch
2*R*,3*R*,7*R*-P[b,d]	TP-1	25	0	0	0
2*R*,3*R*,7*S*-P[b]	TP-2	25	0	0	0
2*S*,3*S*,7*R*-P[b]	TP-3	25	0	0	0
2*S*,3*S*,7*S*-P[b]	TP-4	25	0	0	0
2*S*,3*R*,7(*R*/*S*)-P[c]	CP-1	50	3	1	2
2*R*,3*S*,7(*R*/*S*)-P[c]	CP-2	50	0	0	0
2(*S*/*R*),3*R*,7*R*-P	TMP-1	50	14	0	7
2(*S*/*R*),3*R*,7*S*-P	TMP-2	50	0	0	0
2*R*,3*R*,7*R*-A[b,e]	TA-1	25	0	0	0
2*R*,3*R*,7*S*-A[b]	TA-2	25	0	0	0
2*S*,3*S*,7*R*-A[b]	TA-3	25	0	0	0
2*S*,3*S*,7*S*-A[b]	TA-4	25	0	0	0
2*S*,3*R*,7(*R*/*S*)[c]	CA-1	50	0	0	0
2*R*,3*S*,7(*R*/*S*)-A[c]	CA-2	50	0	0	0

[a]The test was conducted June 2 to June 18, 1979 in Rose Lake, Lansing, Michigan, with two replicates.
[b]Supplied by Dr. K. Mori (Mori, et al., 1978).
[c]Supplied by Dr. A. Tai (Tai et al., 1978).
[d]P for propionate.
[e]A for acetate.

correct optical configuration shows a dose-related response (Carde et al., 1977). The amount of 2*S*,3*R*,7(*R*/*S*)/2*R*,3*S*,7(*R*/*S*)-P (P-3) was varied for this purpose. The result (Table 4) indicates that the response of *D. similis* males is dose-related.

To test whether some combinations of enantiomers give high biological activity, several proprionate preparations were added to 2*S*,3*R*,7(*R*/*S*)-P. The results (Table 5) show that in all cases trap catch decreased with the presence of another enantiomer in the trap.

To confirm the identity of these active isomers, PMR spectra were obtained and chemical shifts of protons were assigned (Table 6). It is clear from the spectra that the signals due to threo protons at the 2- and 3-carbon positions are clearly differentiated from those due to the erythro protons. The ratio of the 2*S*,3*R* component of the 2(*R*/*S*),3*R*,7*R*-A and 2(*R*/*S*),3*R*,7*S*-A preparations as a result of the racemization reaction at the 2 position on 2*R*,3*R*,7*R* and 2*R*,3*R*,7*S* was thereby identified as 1:1.

An effort was then made to compare the potency of these threo isomers to that of the natural pheromone. The method of purification of the pheromone and the biological activities of isolated fractions are shown in Figure 2. The biological activity was always aligned in one region of the thin-layer

TABLE 3. FIELD RESPONSE OF *D. similis* MALES TO SYNTHETIC PROPIONATE OF THREO ISOMERS, 2*S*, 3*R*, 7 (*R*/*S*), 3*R*, 7*R*, AND 2*S*, 3*R*, 7 (*R*/*S*)/2*R*, 3*S*, 7 (*R*/*S*) IN THREE AREAS

		Average per trap catch by two replicates								
		NC[a] (5/9-6/7 1979)			WI[b] (6/6-9/1 1978)			MI[c] (6/2-6/18 1979)		
Code name	Amount (μg)	Replicates A	B	Average	Replicates A	B	Average	Replicates A	B	Average
2*S*,3*R*,7(*R*/*S*)-P	50	1	2	1.5	1	1	1	3	1	2
2(*R*/*S*),3*R*,7*R*-P	50	2	16	9	5	15	10	14	0	7
2(*R*/*S*),3*R*,7*S*-P	50	0	0	0	0	0	0	0	0	0
2*S*,3*R*,7(*R*/*S*)/ 2*R*,3*S*,7(*R*/*S*)[d]	100	0	1	0.5	2	9	5.5	3	3	3

[a]Crossnore, North Carolina.
[b]Siren, Wiconsin.
[c]Rose Lake, Lansing, Michigan.
[d]1 : 1 mixture of 2*S*, 3*R*, 7 (*R*/*S*) and 2*R*, 3*S*, 7 (*R*/*S*). From Tai et al. (1978).

chromatogram. The final GLC fractions, (3)-III-C and (3)-III-D were examined for the presence of a peak corresponding to the acetate of diprionol. As a result of a capillary GLC analysis (SE-30 and OV-101), the presence of the pheromone was ascertained and its quantity was assessed (Figure 3). The biological activity of a known quantity of the propionate form of the natural pheromone was then compared to the synthetic counterpart under the same

TABLE 4. EFFECT OF CHANGES IN AMOUNT OF SYNTHETIC ATTRACTANT PER TRAP ON NUMBER OF TRAP CATCHES OF *D. similis* MALES AT SIREN, WISCONSIN, JULY 19 THROUGH AUGUST 18, 1979.

Chiral configurations (code name)	Amount (μg)	Number of catches
2*S*,3*R*,7(*R*/*S*)/2*R*,3*S*,7(*R*/*S*)-P (P-3)	1	0
	3	0
	10	1
	30	9
	100	24
	300	57
	600	84

TABLE 5. FIELD RESPONSE OF *D. similis* MALES TO VARIOUS COMBINATIONS OF SYNTHETIC SEX ATTRACTANTS[a]

Chiral configuration	Amount (μg)	Replicates A	B	C	Total
2*S*,3*R*,7(*R*/*S*)	50	5	6	9	20
2*R*,3*R*,7*R*-P/2*S*,3*R*,7(*R*/*S*)-P[b]	50/50	1	5	–	6
2*R*,3*R*,7*S*-P/2*S*,3*R*,7(*R*/*S*)-P	50/50	1	0	5	6
2*S*,3*S*,7*R*-P/2*S*,3*R*,7(*R*/*S*)-P	50/50	3	6	1	10
2*S*,3*S*,7*S*-P/2*S*,3*R*,7(*R*/*S*)-P	50/50	7	0	1	8
2*S*,3*S*,7*S*-A/2*S*,3*R*,7(*R*/*S*)-P	50/50	3	1	11	15
2*R*,3*S*,7(*R*/*S*)-P/2*S*,3*R*,7(*R*/*S*)-P	50/50	1	2	1	4
2*S*,3*R*,7(*R*/*S*)-A/2*S*,3*R*,7(*R*/*S*)-P	50/50	1	0	2	3
2*S*,3*S*,7*S*-P	50	0	0	0	0
2*S*,3*S*,7*S*-A	50	0	0	0	0

[a]The tests were conducted June 2 to June 18, 1979, in Rose Lake, Lansing, Michigan (replicates A and B) and at the Hartwick Pines State Forest, Michigan (replicate C).
[b]2*R*,3*R*,7*R*-P/2*S*,3*R*,7(*R*/*S*)-P means a mixture of propionate of 2*R*,3*R*,7*R* and that of 2*S*,3*R*,7(*R*/*S*) at the quantities indicated in the second column (i.e., 50 μg each).

experimental conditions. It was determined that the threshold value for 2*S*,3*R*,7(*R*/*S*)-P was in the order of 0.1 μg per trap, while the trap baited by the propionate of the natural diprionol at 10 ng attracted males at a level comparable to traps baited with 25 μg of 2*R*,3*R*,7(*R*/*S*)-P (data obtained in Rose Lake, Michigan, in August 1980).

There are two possibilities to explain the above discrepancy. First there might be a synergistic component(s) which facilitates the action of the major

TABLE 6. ASSIGNMENT OF METHYL GROUPS OF 3,7-DIMETHYL PENTADECAN-2-YL ACETATE[a]

Compounds	Shift value of methyl group from benzene as standard ppm (*J* value, Hz) in C_6D_6 I	II	III	IV[b]	V[b]
2*R*,3*R*,7*S*-A	1.343	0.712 (erythro, *J* = 6.3)	0.543	0.515	0.500
2(*R*/*S*),3*R*,7*S*-A	1.345	0.718 (erythro, *J* = 6.3)	–	0.52	0.507
		0.691 (threo)	–	0.52	0.466
2*S*,3*R*,7(*R*/*S*)-A	1.346	0.687 (threo, *J* = 6.48)	0.546	0.52	0.465

[a]$O{=}C(-O-CH(CH_3)-CH(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_7)$
I II III IV V
[b]Estimated values.

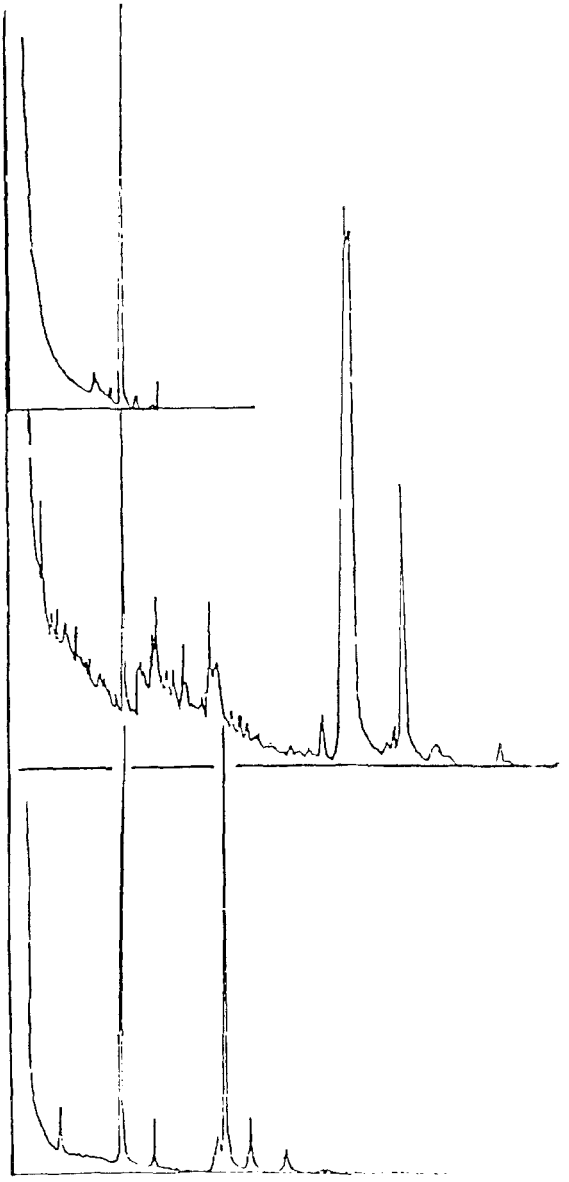

FIG. 3. GLC comparison by capillary column of synthetic acetate of 3,7-dimethyl pentadecan-2-ol and acetate of natural pheromone. In the upper figure, the synthetic acetate of 3-7-dimethyl pentadecan-2-ol (using 2*R*,3*R*,7(*R*/*S*)-A as standard sample), the major peak at 7.5 min in retention time corresponds to the synthetic pheromone; in the middle figure, 2.5 female equivalents of fraction (3)-III-D in Figure 2; and in the bottom figure 2.5 female equivalents of fraction (3)-III-C in Figure 2, respectively. Note that these two active fractions contain the peak identical in retention time to the synthetic attractant. The SE-30 capillary column was used at 180° at 30 ml/min of N_2. The detector was supplied with 30 ml/min of H_2 and 300 m/min of air. Recorder speed was 8 in./hr. The R_t of the pheromone peak was 7.5 min.

TABLE 7. TRAP CATCHES BY MIXTURE OF CP-1 WITH FOUR TLC FRACTIONS FROM *D. similis* FEMALES, ALCOHOL, ALDEHYDE, ESTER, OR PARAFFIN FRACTION (FIGURE 1)[a]

TLC fractions tested[b]	Replicate A	Replicate B	Total trap catch
2*S*,3*R*,7(*R*/*S*)-P, 50 μg	28	13	41
Alcohol fraction + 50 μg 2*S*,3*R*,7(*R*/*S*)-P	23	14	37
Aldehyde fraction + 50 μg 2*S*,3*R*,7(*R*/*S*)-P	4	4	8
Ester fraction + 50 μg 2*S*,3*R*,7(*R*/*S*)-P	13	21	34
Paraffin fraction + 50 μg 2*S*,3*R*,7(*R*/*S*)-P	28	13	41

[a]The test was conducted August 2 to August 11, 1979, at Siren, Wisconsin, with two replicates.

[b]R_f values for each fraction in this TLC system were: alcohol fraction 0–0.15 [corresponding to 10(*E*)-dodecen-1-ol], aldehyde fraction 0.15–0.39 (corresponding to cinnamaldehyde), ester fraction 0.39–0.65 [corresponding to 10(*E*)-dodecen-1-yl acetate], and paraffin fraction all materials above the ester fraction.

pheromone in the extract of the virgin females. Second it is possible that the synthetic attractants are not pure enough and the contaminants reduce the field effectiveness.

To test the first possibility, we mixed various chromatographically separated fractions from the extract of virgin females with 2*S*,3*R*,7(*R*/*S*)-P. In the first experiment (Table 7) various TLC fractions were mixed with 50 μg of 2*S*,3*R*,7(*R*/*S*)-P. It is clear that none of the fractions has synergistic action. In the second experiment the volatile portion of the TLC-purified fraction containing the pheromone in the propionate form was mixed with 2*S*,3*R*,7(*R*/*S*)-P (Table 8). The results indicate that there is no apparent synergistic component in the volatile fraction to drastically increase the field affectiveness of CP-1.

Finally some of the enantiomers were tested toward males of *G. frutetorum*, an Old World species. The results (Table 9) clearly indicate that the most active enantiomer for this species is 2*S*,3*R*,7(*R*/*S*)-A followed by 2*S*,3*R*,7(*R*/*S*)-P.

DISCUSSION

Concerning the effectiveness of the propionate of 2*S*,3*R*,7*R*-diprionol the most puzzling aspect is that our data do not fully agree with those of Longhurst et al. (1980), who have found the effectiveness of the proprionate of 2*R*,3*R*,7*R* and to a lesser extent 2*S*,3*S*,7*S*-diprionol towards the males of *D. similis*. Since in our current work these two identical preparations from Mori et al. (1978) were inactive under our conditions, the difference cannot be

TABLE 8. EFFECT OF COMBINATION OF GLC-PURIFIED PHEROMONE FRACTION AND A SYNTHETIC ATTRACTANT, CP-1 (2*S*, 3*R*, 7 (*R*/*S*)

Fractions	Rose Lake, MI			Siren, WI		Average catch per trap
	A	B	C	D	E	
GLC[a] pheromone fraction	16	3	13	0	3	7
2*S*,3*R*,7(*R*/*S*)-P (50 μg)	1	3	4	1	5	2.8
2*S*,3*R*,7(*R*/*S*)-P (50 μg) plus GLC pheromone fraction	1	–[b]	–[b]	5	16	7.3

[a]GLC effluent collected by using a 10% SE-30 column (6 mm × 1.8 m) at 250°C and 30 ml/min of N_2 flow. Five female equivalents of the TLC fraction (3)-III (see Figure 3) from the body extract from virgin females was injected and the effluent portion corresponding to 0–16 min was collected.
[b]Traps were vandalized and lost.

ascribed to the source of the attractants. Three possibilities are: (1) the population of *D. similis* in Wales belongs to a different biotype, (2) their requirement for chiral specificity is not rigid and therefore they respond to many isomers, and/or (3) they responded to a small amount of a contaminant in the 2*R*,3*R*,7*R*-P preparation. As far as the populations of *D. similis* in this region of the United States, however, the superiority of the preparations containing 2*S*,3*R*,7*R* component over others is apparent.

TABLE 9. FIELD RESPONSE OF *G. frutetorum* MALES TO VARIOUS SYNTHETIC SEX ATTRACTANTS[a]

Chiral configuration	Amount (μg)	Trap catch		Average trap catch
		8/7–8/19/1979	6/10–9/4/1980	
2*R*,3*R*,7*R*-A	25	–	0	0
2*R*,3*R*,7*S*-A	25	–	0	0
2*S*,3*S*,7*R*-A	25	–	0	0
2*S*,3*S*,7*S*-A	25	–	0	0
2*S*,3*R*,7(*R*/*S*)-A	50	21	6	13.5
2*R*,3*S*,7(*R*/*S*)-A	50	0	1	0.5
2*R*,3*R*,7*R*-P	25	–	0	0
2*R*,3*R*,7*S*-P	25	–	0	0
2*S*,3*S*,7*R*-P	25	–	0	0
2*S*,3*S*,7*S*-P	25	0	1	0.5
2*S*,3*R*,7(*R*/*S*)-P	50	1	2	1.5
2*R*,3*S*,7(*R*/*S*)-P	50	0	0	0

[a]The test was conducted August 7 to August 19, 1979, at Mondovi, Wisconsin, and from June 10 to September 4, 1980, at Trempeleau, Wisconsin.

As to the actual potency of the synthetic attractants in comparison with the natural pheromone, our conclusion is that the natural pheromone is still far superior, the difference being in the neighborhood of 2500-fold in the case of 2*S*,3*R*,7(*R*/*S*)-P.

The most likely explanation of this discrepancy is that even the most active synthetic attractant does not have the totally satisfactory chiral configuration. There is evidence to support this view. First, the addition of 2(*R*/*S*),3*R*,7*R*-P (TMP-1) to 2*S*,3*R*,7(*R*/*S*)-P decreased the field effectiveness of the latter (Table 5), indicating that the activity of 2(*R*/*S*),3*R*,7*R*-P may be improved, if one could eliminate or reduce the coexisting 2*R*,3*R*,7*R*-P enantiomer. Second, the difference in activity between 2(*R*/*S*),3*R*,7*R*-P and 2(*R*/*S*),3*R*,7*S*-P is significant and therefore the racemic nature of the 7 position of the 2*S*,3*R*,7(*R*/*S*)-P preparation could be detrimental to its field effectiveness. Third, there appear to be no significant synergistic components available to the extract from the virgin females to increase the effectiveness of the attractant. The biological activity in this species is always associated with one region of chromatography, and the final pheromone fraction that consisted of a single peak on the GLC system was quite active.

In conclusion, the most active enantiomer toward *D. similis* and *G. frutetorum* has been identified to be the proprionate of 2*S*,3*R*,7*R*-diprionol. This chiral configuration the alcohol moiety is very different from the 2*S*,3*S*,7*S* form reported to be attractive to two species of *Neodiprion* (Matsumura et al., 1979; Kraemer et al., 1979).

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Book Review

Olfaction and Taste VII; Proceedings of the Seventh International Symposium on Olfaction and Taste and of the Fourth Congress of the European Chemoreception Research Organization. 1980. Edited by H. van der Starre. IRL Press Ltd., London. 500 pp. £22/$50.

In this volume we have further evidence of growing international scientific interest in the chemical senses. At the joint ECRO IV/ISOT VII congress held in the Netherlands in the summer of 1980 the focus of interest was, of course, chemoreception rather than chemical ecology, although the two fields clearly share much in common. As we turn the pages of the proceedings, we can appreciate that this was indeed a good congress. Contributions are classified under the headings of molecular aspects of chemoreception, chemosensory coding at the peripheral level, information processing in the central nervous system and behavioral aspects of chemoreception. There are also papers from workshops on problems of sensory analysis and olfactometry.

However, good congresses do not automatically generate memorable books. Congresses are about personal encounter and this, their most valuable dimension, evaporates in the cold light of the printed page, leaving behind a factual residue which does not always adequately recompense the reader for his absence from the event. Whatever the participants took from this congress, it was probably considerably more than the reader takes from this volume, unless of course the reader was there.

So, what does the reader receive? The most satisfactory parts of the book are the six fairly short review lectures which are interesting, if not entirely unexpected in their content. Of the chemically oriented lectures, Ohloff (Firmenich, Geneva) talks again about his triaxial rule of odor sensation, and van der Wel (Unilever Research, Vlaardingen) presents a short but very readable account of the organic chemistry of taste sensation, introducing us to some of his work on sweet-tasting proteins, while Cagan and Rhein (Monell/University of Pennsylvania) discuss their biochemical studies on the adsorption of odor and taste stimulus molecules on receptor cell surfaces. Of the other lectures, Boeckh (University of Regensburg) presents a useful brief

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summary of some ideas of how, since individual chemoreceptor cells are not tuned to single compounds, the discrimination of sensory quality can arise, while Kauer (Yale) discusses spatial characteristics of central information processing in the vertebrate olfactory pathway and Cain (Yale) explores the role of cognitive factors in odor classification.

The vast bulk of the 500 pages of this book, however, are less satisfactory, comprising some 70 brief "oral presentation" papers and 140 abstracts of poster presentations. Certainly a large quantity of stimulating work is mentioned, but the accounts are usually of necessity far too brief and the overall impression is disjointed and insubstantial. There is seldom sufficient space available for the authors to satisfy adequately the interest they generate, so that most of what we find is either a cursory summary of work which will no doubt be published more satisfactorily subsequently, or else a brief reiteration of earlier work.

Embedded in the text I found a number of items of some chemical ecological interest. Some of these are represented in the briefest poster presentation abstracts. Burger, le Roux, and Bigalke list 24 components they have identified in the dorsal gland secretion of the springbok (*Antidorcas marsupialis*) and 34 compounds in the preorbital secretion of the grysbok (*Raphicerus melanotis*). Goodrich writes tantalizingly briefly of his chemical and behavioral studies on the volatile components of rabbit anal gland secretion; Vernet-Maury reports the presence of a component of fox feces which elicits strong fear reactions in the rat and names this as *cis*- and *trans*-trimethyl-Δ^3-thiazoline; Verberne and Ruardij mention some work on the flehmen response in cats, as does Müller-Schwarze in relation to deer. A number of poster abstracts deal with 5α-androst-16-en-3-one. One (Pershaud, Pelosi, and Dodd) concerns binding studies in relation to the sheep olfactory mucosa. Another (Hancock, Gower, and Bannister) deals with its reduction to the corresponding 3α- and 3β-alcohols in the pig nasal epithelium. There is also a paper by Kirk-Smith and Booth on the effect of the application of this odorous steroid to a seat on sex-related seating patterns in a dentist's waiting-room. There is talk of affinity labeling of rat olfactory receptors (Persaud, Wood, and Dodd) and of the difference in gas chromatograms deriving from frustrated and rewarded rats (Eslinger, Ludvigson, and Reinecke). There are references to unconscious odor conditioning in human subjects (Kirk-Smith, van Toller, and Dodd), to responses to human axillary odor (Schleidt), to the ways in which tamarins use their scent glands (French and Snowdon), and to much else in the 200 other papers.

But, in summary, I feel that a very big question must hang over volumes of this type, for it is just not sufficient to aggregate so many brief reports and abstracts and call them a book. The frustration of this publication is that in its 500 pages it says so very little about so very many topics. It is not a text which I

would recommend, for it seems probable that the interested reader will need constantly to be reaching for an ample supply of postage stamps and writing to the contributors to expand on their brief summaries, to say what they would have written if they had had more space.

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MANDIBULAR GLANDS OF MALE *Centris adani*, (HYMENOPTERA: ANTHOPHORIDAE) Their Morphology, Chemical Constituents, and Function in Scent Marking and Territorial Behavior[1]

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Abstract—Males of the solitary Central American bee *Centris adani* Cockerell possess enlarged mandibular glands filled with a fragrant fluid that was shown by gas chromatographic and mass spectral analysis to consist of nerol, geraniol, neral, ethyl laurate, and geranyl acetate. Male bees set up territories by marking a semicircular array of grass stems with these compounds. Grass stems marked by the bees showed the presence of the same chemicals.

Key Words—*Centris adani*, mandibular gland, geraniol, nerol, neral, geranyl acetate, ethyl laurate, territoriality, pheromone, solitary bee.

INTRODUCTION

Males of some Hymenoptera species are known to establish and maintain territories against conspecific males for the suspected purpose of attracting and mating with conspecific females. In the case of territorial bees, most

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defended areas cover a few square meters and are often associated with either nesting sites (Cruden, 1966; Alcock et al., 1978) or with food plants (Haas, 1960; Crazier and Linsley, 1963; Jaycox, 1967).

Males of several *Centris* species have been reported to defend territories (Frankie and Baker, 1974; Alcock, 1976), and in some cases the presence of an odor emanating from the males has been noted (Raw, 1975; Frankie et al., 1980). In this regard, males of some *Centris* species resemble males of *Bombus* and *Psithyrus* bee species and some *Philanthine* and *Nyssonine* wasp species that mark their territorial sites with cephalic secretions (Kullenberg, 1956; Free and Butler, 1959; Calam, 1969; Borg-Karlson and Tengö, 1980).

Frankie et al. (1980) reported that male *Centris adani* Cockerell, observed in Costa Rican dry forests, were territorial in certain grassy habitats not necessarily associated with nest sites or food plants. Territories were also observed in the crowns of some flowering host trees. These territories appeared to be maintained by compounds that the bees deposited on surrounding vegetation. In this paper we identify the mandibular glands as the source of these chemicals, describe the morphology of these glands, and analyze the compounds extracted from them. We also describe how these chemicals are deposited on vegetation by the bees.

METHODS AND MATERIALS

The study site at which collections were made was located in the Pacific lowland dry forest (<200 m elevation) of Guanacaste Province, approximately 8 km WSW of the town of Liberia. Vegetation consisted of pasture and rice fields mixed with strips and patches of second-growth forest and open grassy areas. Vinson and Frankie (1977) provide additional details on the site.

For morphological studies *C. adani* males were collected from grassy habitats with a net immediately after or 2 hr after territorial initiation (Frankie et al., 1980). Males for chemical studies were also collected from sleeping aggregations (Frankie et al., 1980) or while foraging on flowering host trees. Bees were placed in vials on ice and taken to the field laboratory where the heads were removed or the mandibular glands were extracted.

Severed heads and glands were placed in redistilled hexane. Samples were collected in 1976 and 1980. The 1976 samples consisted of individual bee heads, while the 1980 samples consisted of about 7 individual bee heads from males collected from a sleeping aggregation or at various times after initiating their territories, a pooled sample of mandibular glands dissected from 20 males, and a sample of 30 heads of foraging males collected from flowering trees. The 1976 samples were held for 6 months often under warmer than ideal conditions before analysis, while the 1980 samples were kept below −2° C

except during 2 days of shipment to the laboratory in College Station, Texas, where they were held at −20°C and were analyzed within 4 weeks.

In addition to the bees, grass stems were collected just after they had been marked by a bee, and the marked area was cut from the grass stem with a pair of twig clippers and placed in redistilled hexane. To ascertain whether marked grass retained the bee compounds for 24 hr, the area of grass stem marked by bees was indicated with a marking pen and complete grass stems were removed to the field laboratory and held outside in the shade. The stem area that was scent marked by the bees was removed 24 hr later and placed in hexane. Control samples consisted of grass stems collected several meters from known territories; that is, in areas where territories had not previously been observed over a several day period.

The extirpated mandibular gland contents exhibited a pronounced geraniol-like odor. Gas chromatographic studies were performed using a Tracor MT-550 GC with flame ionization detection. A 1.83-m × 4-mm ID silanized column, packed with 3% OV-101 on Chromosorb G 80–100 mesh, AW, HMDS, with a nitrogen flow rate of 60 ml/min, programed from 80 to 250° at 10°/min, was used to analyze volatile components. Since further separation of several components eluting at 150°C was necessary, a second 1.83-m × 4-mm ID silanized glass column packed with 5% Carbowax 20 M on Chromosorb G, 80–100 mesh, AW, HMDS was used, temperature programed from 140 to 200°C at 10°/min.

Mass spectral data were obtained on a Varian MAT CH-7 90° sector magnetic scan GC-MS unit equipped with a Varian 620L data system. Columns used were 1.83-m × 2-mm, 3% OV-101, and 3% Carbowax 20 M on Chromosorb G, 80–100 mesh, helium flow rate 30 ml/min, temperature programed from 80 to 250°C at 10°/min at the OV-101 column and from 80 to

TABLE 1. COMPOUNDS ISOLATED FROM MANDIBULAR GLAND OF *Centris adani*, THEIR RETENTION TIMES AND PERCENT OF TOTAL VOLATILES EXTRACTED

Compound	Retention time (min)[a]	% of total
Heptadecane standard	5.12	
Neral	5.31	0.4
Geranyl acetate	6.50	17.2
Nerol	7.48	0.8
Geraniol	8.76	80.0
Ethyl laurate	9.45	0.6

[a]5% Carbowax 20M on 80–100 mesh Chromosorb G-HP, N_2 flow 60 ml/min, 1.82-m × 4-mm ID glass column, 150°.

225°C at 10°/min on the Carbowax 20 M column. ^{1}H Nuclear magnetic resonance spectra were obtained on a JEOL FX90Q FT-NMR using deuterated benzene as solvent.

Molecular weights of the unknown compounds were determined from mass spectral data and possible molecular formulas were computed. Data comparison with that found by Stenhagen et al. (1969) allowed the identification of compounds in Table 1. Gas chromatographic analysis by coinjection of commercial samples of these compounds and comparison of mass and NMR spectra of commercial samples with those of the unknown materials confirmed the identification (see Tables 1 and 2).

RESULTS

Marking Behavior. Males initiated territories between 0830 and 0900 local time in grassy habitats (Frankie et al., 1980). A male would hover or perch on a small stick or stem often in the center of an open area surrounded by grass. Every few minutes the male flew to and alighted on a grass stem or twig several centimeters upwind or to either side of a perch. The male then moved a short distance along the stem before returning to a perch or hovering. We refer to such activity as marking. More specifically, marking was exhibited by the male running up the stem while moving slightly from side to side with wings open and apparently assisting in the movement. Mandibles were closed and pressed closely to the substrate during this process. Grass stems or twigs thus marked possess a faint terpene-like odor similar to geraniol. This behavior resulted in a well-defined territory that the male maintained and defended against intruders until about 1100–1200 local time (Frankie et al., 1980).

Male Mandibular Glands. Figure 1A shows location and relative size of the mandibular glands in the head of a *C. adani* male. Figure 1B shows gland size of males collected immediately after territorial initiation; Figure 1C shows gland size just before territorial abandonment. These glands consist of a two-branched reservoir, the lumina of which are swollen (Figure 1B) with an oily material having a strong geraniol-like odor. The reservoir is surrounded on three sides by large globular cells each independently opening via a small duct into the main reservoir (Figure 1D). The reservoir opens to the outside through a small opening in the upper third of the inner surface of the mandible (Figure 1C, arrow).

Glands were reduced in size in males collected just prior to the time when they usually ended their territorial activities (compare Figure 1B and C). Reduction in size was largely due to collapse of the reservoir and to some extent to a decrease in the size of the globular cells. Diameter of these cells from males collected just after territorial initiation averaged 0.1 mm (0.8–0.12,

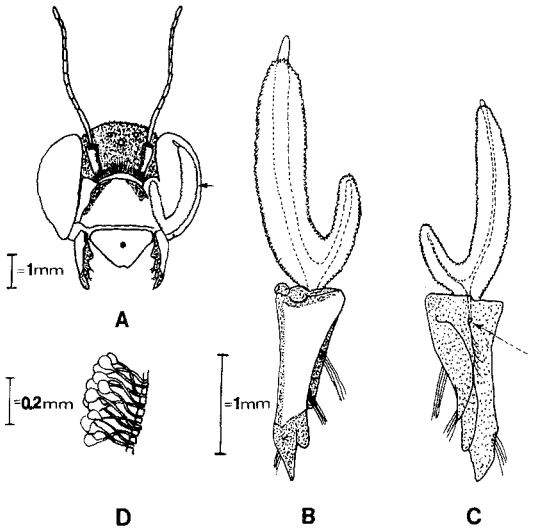

FIG. 1. (A) The head of a male *Centris adani* showing the positions of the mandibular gland (right-hand arrow) in the head capsule. (B) The mandibular gland dissected from a male just after initiation of territorial marking. The position of the gland with respect to the outer margin of the mandible is diagramed. (C) The mandibular gland dissected from a male 2½ hr after territorial marking. The inner margin of the mandible is diagramed. Note smaller gland size and opening in mandible (arrow) where secretion is released. (D) Diagramatic representation showing the glandular cells and their ducts entering the cuticle of the gland reservoir. The diagram was copied from sections of glands removed from males 2½ hr after territorial marking.

$N = 20$), while cells measured from glands removed from males 2.5 hr after territory initiation averaged 0.05 mm (0.03–0.06, $N = 25$).

Chemical Analysis of Volatiles from Heads and Mandibular Glands. Chemical identification of the 1980 pooled samples of both heads and mandibular glands dissected free from the head yielded similar results with five compounds shown in Table 1, along with the percent of the total volatiles each represents. Geraniol and geranyl acetate were the major constituents of the mandibular gland. Since geranyl acetate gives no parent peak on MS, comparison of the NMR spectrum of a sample collected by preparative GC with that of a commercial sample was used to confirm its identification. Table 2 presents the major mass-spectral data. In addition to the monterpenoids identified in the 1980 samples, the 1976 samples contained ethyl laurate as the major component and minor amounts of geranial, geranic acid, and nerolic acid. Lack of refrigeration over extended storage periods was a serious problem in our early field studies.

Grass Samples. Grass samples collected within 2 min after marking by

TABLE 2. MASS-SPECTRAL DATA (MAP) OF VOLATILE COMPOUNDS ISOLATED FROM MANDIBULAR GLANDS OF *Centris adani*

Compound	Parent ion	1	2	3	4	5	6	7
Neral	152 (1)	69 (100)	41 (65)	39 (44)	91 (35)	67 (34)	84 (26)	53 (25)
Geranyl acetate		69 (100)	41 (99)	43 (94)	93 (80)	68 (58)	67 (38)	39 (34)
Nerol	154 (0.4)	41 (100)	69 (85)	93 (59)	67 (31)	39 (30)	91 (26)	79 (24)
Geraniol	154 (0.6)	69 (100)	41 (90)	93 (73)	39 (47)	67 (34)	91 (30)	53 (27)
Ethyl laurate	228 (6)	88 (100)	101 (41)	43 (26)	41 (25)	55 (21)	73 (19)	69 (18)

males were subjected to the same analysis as the insect glands. The analysis showed monoterpenes present in approximately the same ratios as in the glands. Marked grass samples left in the field for 24 hr prior to collection revealed only trace amounts of ethyl laurate. Control grass samples had none of the identified components present.

DISCUSSION

Males of *C. adani* established territories in grassy sites. These territories (see Frankie et al., 1980, for details) were delineated and maintained by scent-marking behavior of males. Marking was exhibited by a short vibrational forward movement similar to the waggle dance performed by returning worker honey bees (Von Frisch, 1954). The grass stem thus marked possess a faint geraniol-like odor. During the marking process the mandibles were closed and closely pressed to the substrate as the males moved from side to side and forward. The scent-marking behavior of *C. adani* is similar to that described for *Bombus* (Kullenberg et al., 1973). We did not find evidence from direct observations or from movies made of marking behavior that the males chewed the grass stems or had their mandibles open during scent marking as discussed for male bees by Haas (1952) and as described for *Centris decolorata* by Raw (1975).

When males were collected, a scent similar to that detected on the grass was detected in the heads of the males. Dissection of male *C. adani* heads revealed enlarged mandibular glands (Figure 1) similar in size and shape to those described for male *Centris dirrhoda* by Raw (1975). These glands had a strong odor similar to that detected on the grass stems.

Chemical analysis of extracts of mandibular glands excised from males revealed similar compounds and ratios of compounds to those detected in extracts of whole bee heads. These results show that mandibular glands are the main source of scent-marking compounds in males. That mandibular glands decrease in size during territorial maintenance further supports the view that mandibular glands are the source of scent marking compounds.

The dominant monoterpenes identified from mandibular glands of *C. adani* have been reported from the mandibular gland of *Colletes* bees from Sweden (Bergström and Tengö, 1978). Hefetz et al. (1979) identified linalool, neral, and geranial in an approximate ratio of 3:1:1, respectively, from the mandibular glands of three species of male and female *Colletes* bees collected in Maryland. These compounds appear to serve as an aggregation pheromone. Both neral and geranial have been reported from *Trigona* spp. where they serve as trail and alarm pheromones (Blum et al., 1970), from honey bees where they are found in the Nasonov gland and attract foragers (Butler and Calam 1969), and from the mandibular glands of *Andrena* spp. where they

may function as an aggregation pheromone (Tengö and Bergström, 1977). However, unlike the species reported by other workers, *C. adani* lacked geranial but contained predominately geraniol.

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INHIBITION OF OVIPOSITION BY VOLATILES OF CERTAIN PLANTS AND CHEMICALS IN THE LEAFHOPPER *Amrasca devastans* (DISTANT)[1]

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Abstract—Oviposition by the leafhopper *Amrasca devastans* (Distant) on its susceptible host plant, cotton (*Gossypium hirsutum* var. PS-10), was inhibited by the volatiles of certain plants and by the vapors of some chemicals occurring in various plants when these were presented at a distance from the ovipositional substrate. The effectiveness of the volatiles of the plants for inhibiting the oviposition decreased in the order: eucalyptus > coriander = castor = tomato > lime, *Ocimum* being without effect. Among the volatile plant chemicals tested, the inhibitory effects decreased in the order: citral = carvacrol > citronellol = farnesol = geraniol = eucalyptus oil > neem oil = *Cymbopogan* oil. These chemicals served as volatile antiovipositants and did not reduce the arrival/stay of the insects on the host plants. Carvacrol had a slight toxic effect on the nymphs, but none of the volatiles was toxic to the adults.

Key Words—Volatile ovipositional inhibitors, citral, citronellol, farnesol, geraniol, plant/chemical volatiles, leafhopper, *Amrasca devastans*, Homoptera, Cicadellidae, *Gossypium hirsutum*.

INTRODUCTION

Oviposition by an insect constitutes an important step in its reproduction and, hence, in determining the magnitude of its population in a locality. In view of this, inhibition of oviposition by an insect pest can reduce its population and, thus, help in its management. A number of workers have reported that some chemicals, including those occurring in certain plants, inhibit oviposition by a

[1] Homoptera: Cicadellidae.

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few insects (Jermy, 1965; Lundgren, 1975; Robert, 1976; Muschinek et al., 1976; Flint et al., 1977; Rothschild and Schoonhoven, 1977; Jermy and Szentesi, 1978). The chemicals reported by these workers to inhibit the oviposition do so on contact with the insects at the ovipositional sites. Such contact ovipositional inhibitors may have the following limitations in their practical application: (1) They need to be applied to all the ovipositional sites in an area to prevent the insects from laying eggs on untreated sites, and it is difficult to obtain 100% coverage of these sites by the chemicals. (2) The persistence of the chemicals may render the treated materials, particularly the edible ones, unfit for use. In these respects, volatile materials whose vapors can inhibit oviposition by insects would be advantageous: (1) the vapors of these materials, placed at one or more places away from the ovipositional sites, can diffuse throughout the area so as to surround these sites where the insects may be inhibited from laying eggs; (2) the vapors, even if adsorbed by the ovipositional sites, may not persist for as long a time as the nonvolatile contact inhibitors.

However, at present we have little information on whether vapors of any volatile material can inhibit oviposition by insects. The study of this aspect has, therefore, been taken up by us with reference to the leafhopper *Amrasca devastans* (Distant) which is a serious pest of cotton, okra, eggplant, etc. The first phase of this study involves the laboratory screening and comparison of the potential relative effectiveness of various volatile materials so as to identify a few promising ones for detailed investigations in the subsequent phases of this study. The results of the first phase of our study are given here.

METHODS AND MATERIALS

Nymphs of *Amrasca devastans* (Distant) were collected from the fields of okra [*Abelmoschus esculentus* (Linnaeus) Moench] or eggplant (*Solanum melongena* Linnaeus) and reared on fresh, unripe, okra fruits at 28° ± 1°C under a 13-hr photophase. The adults emerging from the nymphs were continued under the same conditions on okra fruits which were replenished on alternate days. When the insects attained the age required for different studies, they were tested for their responses to one of the highly susceptible host plants, i.e., the cotton *Gossypium hirsutum* var. PS-10, in the presence or absence of the vapors of the desired volatile materials.

All the tests were carried out in clear plastic chambers, each consisting of two removable cylindrical compartments (7 cm high × 7 cm diameter each), one above the other. The upper compartment had a detachable lid of nylon net (40 mesh/cm) and a fixed nylon net bottom which was supported on the top open end of the lower compartment having a plastic bottom. These test

chambers were newly constructed, washed thoroughly with the detergent Teepol, rinsed, dried, and were free from the test volatiles.

For each test, a freshly excised leaf of 4- to 8-week-old cotton plants had its lamina trimmed to form a 5 × 3-cm rectangle and its 2.5-cm petiole was immersed in water contained in a vial (2.5 cm high, 3.5 cm diameter). The leaf rectangle was placed in the upper compartment of the test chamber. In the lower compartment was placed one of the test volatile materials which included the leaves of certain plants (Table 2) or certain volatile chemicals (Table 3) occurring in various plants. The volatiles of these materials would diffuse into the upper compartment through its nylon net bottom. For testing the volatiles of the plants, 10 g freshly excised leaves were filled in the lower compartment. For testing the vapor of a chemical, its desired volume in liquid paraffin was taken in a glass cup (1 cm high × 1 cm diameter) which was placed in the lower compartment of the test chamber. The chemicals tested were obtained from different sources (Table 3) in pure form (90–99% purity) or as mixtures in the oils extracted from certain plants. Then 0.004 ml of each chemical in 0.4 ml liquid paraffin was placed in a chamber since this dose was found in the preliminary trials to be suitable for comparing the effects of the test chemicals.

For each test, 10 females with or without as many males or 10 nymphs in the stage required for various studies were introduced into the upper compartment of the test chamber. The chamber was kept at a temperature varying from 29° to 33° C under a 13-hr photophase with an illumination of about 550–800 lux from above by two daylight fluorescent tubes of 20 W each. The tests with one or more volatiles were accompanied by a control in which the insects were offered the leaf rectangle without the test volatile source. The duration of each test and the response measured varied according to the aspect studied, as described below.

Each test was replicated 3–5 times with different batches of insects. The data on their responses in the presence or absence of different volatile materials were statistically processed and compared by the usual procedures involving the analysis of variance (ANOVA) and tests of significance (Snedecor and Cochran, 1967). If the F test following the ANOVA was significant, the least significant difference (lsd) between the means was calculated for testing the statistical significance of the differences between them. A significant difference between the responses of the insects in the presence of a volatile test material and those in its absence would reflect the volatile's effect.

Tests for Oviposition. Since Saxena and Saxena (1971) have observed that the okra-fed adults of the leafhopper show maximum egg-laying when 10 days old, males and females of this age were taken for the present study. The insects were given access to the leaf rectangle in the test chamber, as described

above, for 24 hr. Thereafter, the leaf rectangle was taken out and treated with lactophenol reagent (Carlson and Hibbs, 1962) for about 2–4 weeks to render it almost transparent and stain the eggs, if laid, in the veins to be readily visible. The number of eggs laid on the leaf rectangle was recorded.

Tests for Effects of Volatiles on Arrival and Stay on Host Leaves. For this test, 10 females of the same age as those for the oviposition tests were used for each of the five replicates. These insects were introduced into the upper compartment of the test chamber containing the leaf rectangle, as described before. The percentages of the insects present on the leaf rectangle at the end of 1, 2, and 24 hr was recorded. These percentages would differ according to arrival and subsequent stay of the insects on the leaf rectangle in the presence or absence of different volatiles.

Tests for Effects of Volatiles on Survival and Growth. For each of the three replicates, 10 newly emerged adults or 2nd instar nymphs were kept on okra fruits in the presence or absence of the test volatiles in the cylindrical chamber, as described above. The okra fruits were replenished daily. The percentages of the males and females surviving at the end of 7 days, when the females would begin to lay eggs (Saxena and Saxena, 1971), were recorded. For the nymphs, the percentages of those completing their development to the adult stage and the periods required to do so were recorded.

RESULTS

Effects of Volatiles on Oviposition. In this study, every test with different volatiles was accompanied by a control in which the leafhoppers were given the leaf rectangle without any test volatile. Consequently, while the tests with different volatiles were replicated five times, the number of controls was as high as 55. In order to check if the egg-laying in these different controls was statistically identical, they were arranged at random into 11 batches of five replicates each using the Table of Random Numbers (Fisher and Yates, 1963). The mean numbers of eggs in these 11 batches were statistically identical at the 5% probability level (Table 1). In view of this, batch D of the insects, having a mean (15.6) closest to the average of the means of all 11 batches, was taken for statistical comparison of egg-laying in the control with that in the tests in the presence of the volatiles of different plants (Table 2) and chemicals (Table 3).

Effects of Volatiles of Certain Plants. In the presence of the volatiles of each of the test plants except *Ocimum*, the number of eggs laid by the leafhoppers was significantly less than that in the control (Table 2). Maximum reduction in the egg-laying occurred in the presence of the volatiles of the leaves of *Eucalyptus*, the number of eggs laid being about one third of that in the control and being very highly significant at the $P = 0.001$ level. The volatiles of castor, coriander, and tomato leaves reduced egg-laying to about

TABLE 1. OVIPOSITIONAL RESPONSES OF DIFFERENT BATCHES OF *Amrasca devastans* FEMALES[a] TO COTTON *Gossypium hirsutum* VAR. PS-10 LEAVES IN ABSENCE OF TEST VOLATILES

Batches of insects	No. eggs/24 hr[b] (mean ± SE)[c]
A	14.8 ± 1.4
B	16.2 ± 1.6
C	15.8 ± 1.9
D	15.6 ± 1.0
E	16.6 ± 1.7
F	15.2 ± 1.7
G	16.0 ± 1.9
H	14.4 ± 0.9
I	14.8 ± 1.7
J	14.8 ± 2.4
K	16.4 ± 1.9
lsd at $P = 0.05$	5.0

[a]10-day-old females kept with the males since emergence on okra fruits.
[b]Eggs laid by 10 females per replicate on a leaf rectangle offered singly.
[c]Each mean value based on five replicates.

half of that in the control, the differences being highly significant at the $P = 0.01$ level. The reduction in egg-laying caused by the lime volatiles was still less, the number of eggs laid being about two thirds of that in the control and the difference being significant at the $P = 0.02$ level only.

Effects of Vapors of Certain Plant Chemicals. The results (Table 3) show that the vapors of citral and carvacrol caused maximum reduction in the egg-laying by the leafhoppers, the number of eggs laid being about one third to one half of that in the control. Next in order were citronellol, farnesol, geraniol, and eucalyptus oil, reducing the egg-laying to about two third of that in the control. *Cymbopogan* and neem oils caused the least reduction in the egg-laying, whereas the remaining chemicals did not reduce this response significantly.

Of the above-mentioned oviposition inhibitors, carvacrol, citral, farnesol, and geraniol were tested for other effects on the insects, as described below.

Effects of Volatiles on Arrival and Stay on Host Leaves. The insects must first arrive and stay on a plant before they can lay eggs on it. The arrival and stay of *A. devastans* adults on a plant has been reported before (Saxena et al., 1974; Saxena and Saxena, 1974, 1975) to be mainly the result of their

TABLE 2. OVIPOSITIONAL RESPONSES OF *Amrasca devastans* FEMALES[a] TO COTTON *Gossypium hirsutum* VAR. PS-10 LEAVES IN PRESENCE OF VOLATILES OF CERTAIN NONHOST PLANTS

Plants[b]	No. eggs/24 hr (mean ± SE)
Citrus limettioides Tanaka (lime)	9.6 ± 0.9*[d]
Ricinus communis Linnaeus (castor)	8.6 ± 1.0**
Coriandrum sativum Linnaeus (coriander)	7.6 ± 0.8**
Lycopersicum esculentum Mill. (tomato)	8.8 ± 1.3**
Eucalyptus citriodora Hooker (eucalyptus)	6.6 ± 0.5***
Ocimum sanctum Linnaeus (basil)	12.2 ± 1.5 (NS)
Nil (control)[c]	15.6 ± 1.0

[a]See footnotes *a–c*, Table 1.
[b]10 g freshly excised leaves of each plant served as the source of volatiles.
[c]Batch D of Table 1, having the mean closest to the average of the means of all the 11 batches (A-K), used as the control here.
[d]NS-not significantly different from the control. Asterisks indicate that the difference between the number of eggs laid in the presence of a test volatile and that in the control is significant at $P = 0.02$ (*), 0.01 (**), or 0.001 (***).

orientational response to its visual, olfactory and contact stimuli. Whether or not the arrival and stay of the insects on the host plant leaves were interfered with by the vapors of the above-mentioned chemicals was examined in the present work. The percentages of 10-day-old, okra-fed, females present on the leaf rectangles at the end of 1, 2, and 24 hr reflected their arrival followed by stay on the latter. These percentages were statistically almost identical in the presence or absence of the test volatiles (Table 4). Thus, these volatiles had no effect on the arrival and stay of the leafhoppers on the host leaves.

Effects of Volatiles on Survival and Growth. Of the newly emerged adults kept since emergence on the okra fruits in the presence of the vapors of the test chemicals, the percentages of those surviving through the observation period were statistically as high as those in the controls (Table 5). In view of such a low mortality, the vapors were not toxic to the males and females.

Of the nymphs kept on okra fruits since the 2nd instar, those exposed to the vapors of citral, farnesol, and geraniol matured as adults in statistically as

TABLE 3. OVIPOSITIONAL RESPONSES OF *Amrasca devastans* FEMALES[a] TO COTTON *Gossypium hirsutum* VAR. PS-10 LEAVES IN PRESENCE OF VAPORS OF CERTAIN CHEMICALS OR PLANT EXTRACTS

Chemicals[b]	Source of chemicals[c]	No. eggs/24 hr (mean ± SE)
Camphene	BDH	17.8 ± 0.7 (NS)[d]
Carvacrol	KK	8.2 ± 0.9***
Cineole	GS	12.6 ± 1.6 (NS)
Citral	KK	6.4 ± 0.6***
Citronellal	KK	15.6 ± 1.7 (NS)
Citronellol	KK	10.0 ± 1.7**
Farnesol	KK	9.6 ± 0.9**
Geraniol	KK	9.8 ± 1.2**
Linalool	KK	14.2 ± 1.8 (NS)
Clove oil	VMF	12.0 ± 1.2 (NS)
Cymbopogan jwarancusa oil	SD	11.0 ± 1.1*
Eucalyptus citriodora oil	SD	9.2 ± 0.9**
Ocimum sanctum oil	SD	13.2 ± 1.6 (NS)
Mustard oil	local	15.6 ± 1.0
Neem oil	local	10.8 ± 1.2*
Nil (control)		15.6 ± 1.0

[a]See footnotes *a–c*, Table 1.
[b]0.004 ml chemical in 0.4 ml liquid paraffin.
[c]BDH: BDH Chemicals Ltd., England; GS: Goldensun Manufacturing Co., Bombay, India; KK: K & K Labs Division, ICN Pharmceuticals, Inc., U.S.A.; SD: Courtesy of Dr. Sukh Dev, Malti-Chem Research Centre, Baroda, India; VMF: V. Mane Fils, Grasse, France.
[d]NS, asterisks: as in Table 2.

TABLE 4. ARRIVAL AND STAY OF *Amrasca devastans* FEMALES[a] ON COTTON *Gossypium hirsutum* VAR. PS-10 LEAVES IN ABSENCE OR PRESENCE OF VAPORS OF CERTAIN PLANT CHEMICALS

	% insects present on leaf rectangle at different time intervals[b] (mean ± SE)[c]		
Chemicals	1 hr	2 hr	24 hr
Carvacrol	42 ± 4.9	60 ± 4.5	82 ± 5.8
Citral	50 ± 10.0	70 ± 7.1	90 ± 4.5
Farnesol	54 ± 6.8	76 ± 6.8	86 ± 2.5
Geraniol	52 ± 6.6	64 ± 6.0	86 ± 6.8
Nil (control)	40 ± 6.3	78 ± 3.7	92 ± 3.7
lsd at $P = 0.05$	21.6	16.8	14.9

[a]See footnote *a*, Table 1.
[b]In each test, 10 females were presented a single leaf rectangle.
[c]Each mean value based on three replicates.

TABLE 5. EFFECTS OF VAPORS OF CERTAIN PLANT CHEMICALS ON SURVIVAL AND NYMPH DEVELOPMENT OF *Amrasca devastans*[a]

Chemicals	Nymph development		% adults surviving[b] (mean ± SE)[c]	
	% developing to adult stage (mean ± SE)[c]	Average period (days) of development (mean ± SE)[c]	Males	Females
Carvacrol	50.0 ± 5.8[d]	5.8 ± 0.1	90 ± 5.8	93 ± 3.3
Citral	73.3 ± 6.7	6.5 ± 2.6	90 ± 5.8	90 ± 5.8
Farnesol	73.3 ± 8.8	6.0 ± 0.3	90 ± 5.8	90 ± 0
Geraniol	66.7 ± 3.3	5.5 ± 0.2	93 ± 3.3	83 ± 6.7
Nil (control)	86.7 ± 3.3	5.8 ± 0.2	96 ± 3.3	93 ± 6.7
lsd at $P = 0.05$	20.5	0.7	16.6	17.3

[a]For each test, 10 pairs of males and females or 2nd instar nymphs were kept from the time of their emergence on okra fruits in the presence or absence of the test chemicals.
[b]The percentage of adults surviving at the end of the 7-day period after which the females start laying eggs (Saxena and Saxena, 1971).
[c]Each mean value based on three replicates.
[d]Significantly lower than the control at $P = 0.05$.

high a percentage as the control individuals in the absence of these substances (Table 5). The periods of development in the presence or absence of these vapors were also almost identical. However, the vapors of carvacrol significantly reduced the percentage of nymphs reaching the adult stage to about three fifths of that in the control and thus had some toxic effect on them.

DISCUSSION

Inhibition of oviposition by insects infesting various crop plants or stored materials can serve as an important measure to reduce the populations of the pests for their management. One of the methods of inhibiting oviposition by the insects would be through appropriate chemicals. Most existing reports on this aspect deal with the substances which serve as contact antiovipositants and are applied to the ovipositional sites, e.g., plants or stored materials, where the insects coming in contact with the treated surface are prevented from laying eggs. Thus far, there is almost no information on the inhibition of oviposition by the vapors of volatile materials. The present work advances our knowledge of this aspect with reference to the leafhopper *Amrasca devastans* (Distant).

The number of eggs laid by the leafhopper on its highly susceptible host plant, cotton (*G. hirsutum* var. PS-10), is reduced to different degrees by the

volatiles or vapors of different plants or chemicals tested in this work. These differences in the reduction in the egg-laying in the presence of the volatiles or vapors under the controlled experimental conditions used in this work reflect only the potential relative effectiveness of the test chemicals. Among the plants tested, the volatiles of the leaves of the eucalyptus are most effective in inhibiting oviposition, followed by those of coriander, castor, tomato, and then by lime. Among the chemicals tested, the vapors of carvacrol and citral are most effective, followed by citronellol, farnesol, geraniol, eucalyptus oil, and then by *Cymbopogan* as well as neem oils.

The vapors of the volatile materials tested might reduce the number of eggs laid by the leafhoppers on their host plant leaves in one or both the following ways: (1) by interfering with the insects' behavior preceding oviposition, namely, their arrival and stay on the host leaves; and (2) by inhibiting actual deposition of eggs by the insects after their arrival on the plant leaves. The present work shows that the arrival and stay of the insects on the host plant leaves are not reduced by the volatiles and vapors tested. Hence, the reduction in the number of eggs laid by the leafhoppers in the presence of the volatiles and vapors would be caused by the inhibition of actual egg deposition by the insects after their arrival on the leaves. Thus, these plants and chemicals serve as sources of volatile antiovipositants.

Another aspect to be considered concerns the toxicity of the antiovipositants for the target insects. The present work shows that among the chemicals whose vapors reduce oviposition by the leafhopper, none is toxic to the adults since their survival is not affected. However, vapors of carvacrol are slightly toxic to the nymphs since the percentage of those developing to the adult stage in the presence of this chemical is less than that in the control.

In view of these observations, citral, being a highly effective volatile nontoxic ovipositional inhibitor, is a promising candidate for further tests for its use in interrupting oviposition by the leafhopper.

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CHEMISTRY AND FIELD EVALUATION OF THE SEX PHEROMONE OF WESTERN SPRUCE BUDWORM, *Choristoneura occidentalis*, FREEMAN

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Abstract—Chemical analyses and field bioassays showed a mixture of 92% (*E*)- and 8% (*Z*)-11-tetradecenal to be the sex attractant pheromone of the western spruce budworm, *Choristoneura occidentalis* Freeman. Females were also found to emit small amounts of the corresponding acetates and alcohols, but these components were not active in the bioassay. In contrast, in whole female tip extracts, (*E*)-11-tetradecenyl acetate predominates, and aldehyde pheromone components are present in lesser quantities, suggesting that final biosynthesis of pheromone takes place just prior to or during emission. At release rates approximating that of the female (2–4 ng/hr), a 92:8 *E*:*Z* blend of the synthetic aldehydes was at least as attractive as live females. Addition of the corresponding acetates or alcohols up to 50% of the aldehyde content did not significantly enhance or inhibit attraction. No major differences were apparent in pheromone production of females from a laboratory stock or from field collections from diverse geographic locations ranging from Colorado to British Columbia.

Key Words—Western spruce budworm, *Choristoneura occidentalis*, sex pheromone, attractants, moth behavior, chemical identification.

INTRODUCTION

The sex pheromone of the western spruce budworm, *Choristoneura occidentalis* Freeman, is known to be similar to that of the spruce budworm, *C. fumiferana* (Clem.) (Weatherston et al., 1971). The principal attractant component for males of both species is (*E*)-11-tetradecenal. Interspecific

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attraction was strong between the two species, but there were sufficient differences in response of *C. occidentalis* and *C. fumiferana* males to suggest the presence of different "secondary" pheromone components (Sanders et al., 1977).

This study was undertaken to clarify the *C. occidentalis* pheromone system, with particular attention to potential effects of the secondary components which may optimize or inhibit attraction. Female moths from selected geographic locations were evaluated for their pheromone content. All bioassays for attractiveness were conducted under field conditions with baited traps.

METHODS AND MATERIALS

Our study approach was to collect late instar or pupal stage *C. occidentalis* from selected geographic locations, and subject them to standard pheromone extraction and analytical procedures. A nondiapause laboratory stock was also evaluated. Once determined chemically, blends of synthetic pheromone components were compared to live females for their relative attractiveness in field bioassays.

Pheromone Extraction. Initially, the method of pheromone extraction consisted of dipping the tip of the female abdomen in hexane for 10–20 sec or soaking excised abdominal tips in hexane for an extended period. Conditioning the moths for the dip or soak extractions consisted of holding them in a 16-hr light:8-hr dark regime with the extractions taking place 13–15 hr following the onset of illumination. The specific techniques of removing abdominal tips for dipping in the solvent were basically those of Sower et al. (1973).

A system of air collection in Porapak-Q® was begun when a clear difference was revealed between the chemicals within or rinsed from the surface of the pheromone gland. Freshly emerged female moths in groups of 8–10 were placed in a 200-ml glass chamber fitted with an air-intake port at the base and channeled to a narrow outflow tube at the top. Compressed air was fed into the base of the chamber through a Porapak-Q column to ensure purity. Air moved through the chamber at a constant flow rate of 250 ml/min and was routed out the top through a second Porapak-Q column which trapped the pheromone. The glass chamber and glass columns were treated with silanizing reagent and thoroughly rinsed with solvent before usage. The amount of Porapak-Q used to absorb the pheromone was approximately 1 ml in bed volume. The moths were held under these conditions for 48 hr at a constant temperature of 21 ± 2° C and a light regime of 16 hr light: 8 hr dark. After 48 hr the moths were discarded, and the outflow Porapak-Q column was eluted with 1–2 ml of hexane to wash out the female moth effluvia. Prior to use

for pheromone collections the Porapak-Q columns were washed thoroughly with methanol and hexane. All extracts were held in a freezer until processed for chemical analysis.

A slow-release polyvinyl chloride formulation containing 0.1% *E*-11-tetradecenal was also checked for release rate using the same air collection technique. Pellets identical to those used for trap baits in field bioassays but longer in length (20 mm vs. 5 mm) were held in the collection apparatus for 48 hr at 26° C (three replications).

Insect Supply Sources. Six different sources of female *C. occidentalis* were subjected to the hexane-soak method of pheromone extraction. These included a nondiapause laboratory culture, reared on a wheatgerm-base artificial diet (Peterson, 1978), and field collections of pupae taken near Taos, New Mexico; McCall, Idaho; Missoula, Montana; Kamloops, British Columbia; and Warm Springs, Oregon.

The "dip" (gland wash) method of pheromone extraction was applied to two sources of insects, the nondiapause laboratory culture and the McCall, Idaho, collection.

Pheromone extracts by the air collection technique were limited to four populations: the nondiapause laboratory strain, and field collections from Twisp, Washington; Warm Springs, Oregon; and Fort Collins, Colorado.

Chemical Analyses. The sensitivity of two analytical techniques permitted the analysis of extracts derived from small numbers of insects (less than 20 females) without preparative treatment. The extracts were reduced in volume in Reactivials® by careful evaporation at room temperature under a slow stream of nitrogen to a volume of ca. 50 μl, and analyzed as follows:

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

Our second technique combined the use of gas chromatography and mass spectrometry. A Varian Aerograph 2700 gas chromatograph equipped with a glass column (2 m × 2 mm ID) packed with 1.5% OV-101 on 100–200 mesh Gas Chrom Q® was operated isothermally at 170° C. Injector and detector temperatures were 250° C. The instrument was interfaced with a DuPont 21-491B mass spectrometer equipped with a 4-channel DuPont SMID

accessory for selected ion monitoring. The following characteristic fragment ions were monitored: *m/e* 192 (11-tetradecenal), *m/e* 194 (11-tetradecenyl acetate and 11-tetradecenol), and *m/e* 166 (ion common to all three compounds). Unlike the capillary GC system, the *E* and *Z* isomers were not separable on the packed column.

Bioassay Procedures. Following tentative identifications of pheromone components, synthetic blends of these materials were formulated into solid-plastic controlled-release trap baits (Daterman, 1974). The relative attractiveness of these baits was evaluated in the field by comparing the numbers of male moths captured in traps baited with live females versus those baited with the different blends and strengths of synthetics. Traps used in these evaluations were fashioned from 2-liter paper milk cartons formed into a triangular cross-section. The traps were open at each end, lined with Tanglefoot® adhesive, and had a maximum capture capacity of about 130 males. Baits were all 5 × 3-mm-diam pellets which contained 0.01, 0.1, or 3.0% total pheromone components by weight. Baits were suspended from the interior center of the trap on an insect pin. Female moths used as baits were 12–48 hr old when placed in the field and were confined singly within small cylinders (approximately 25 × 75 mm) of fiberglass screen. One female, caged in this way, was positioned in the center of each trap.

The first of four field bioassays reported here was conducted in 1976 and incorporated different proportions of *E*:*Z* 11-tetradecenals formulated in polyvinyl chloride bait pellets. All preparations contained 3% tetradecenals by weight, and the *E*:*Z* blends varied from 100% *E* isomer to a 60:40 *E*:*Z* ratio. The baits were tested for attractiveness to *C. occidentalis* in south central British Columbia and in south central Oregon, and to *C. Fumiferana* in southern Ontario. Each treatment blend of the *E:Z* isomers was replicated in 10 baited traps for each location. The results of this test are included here to demonstrate a basic diference in the pheromone response of *C. occidentalis* and *C. fumiferana.*

Three additional field experiments were conducted in 1979 and 1980 to evaluate the addition of secondary components. The first of these compared only synthetic bait preparations as follows: >99% pure (*E*)-11-tetradecenal; a commercially available blend approximating the known pheromone for *C. fumiferana* (Sanders and Weatherston, 1976, reported 94:6 *E*:*Z*; Silk et al., 1980, reported 95:5 *E*:*Z*) of 97:3 *E*:*Z* 11-tetradecenal; a 92:8 blend of *E*:*Z* 11-tetradecenal; and a 90:8:1.8:0.2 blend of (*E*)-11-tetradecenal:(*Z*)-11-tetradecenal:(*E*)-11-tetradecenyl acetate:(*Z*)-11-tetradecenyl acetate. The >99% pure (*E*)-11-tetradecenal was prepared by progressive ether-in-hexane elution of a 97:3 blend of *E*:*Z* 11-tetradecenal through a silicic acid–silver nitrate column. Bait preparations were formulated in polyvinyl chloride and contained 0.1% by weight of the candidate pheromone components. Six sets

or replications of the four baits were placed in the field for three days and nights, with the sequence of trap placement randomized within individual sets. All traps were hung on the ends of host tree branches 2–2.5 m above the ground, in such a way that the ends would remain open at all times. Within a replication, traps were placed 20–40 m apart, whereas the different trap lines or replicate sets were separated by 75 m or more. Host trees within the area were pole-sized and mature Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, and grand fir, *Abies grandis* (Dougl.) Lindl. Population density of the insect was apparently similar within the trapping locations and the surrounding stands as judged by the uniformly light degree of defoliation.

The next series of baited traps was basically the same except that they included female-baited traps and the synthetic baits were formulated to contain 0.01% of the pheromone components. Again, six replications of each bait treatment were exposed in the field with the sequence of trap placement randomized within a set of the five treatments. The traps were collected after three days and nights of exposure in the field. We estimate that the 0.01% polyvinyl chloride preparations of (*E*)-11-tetradecenal approximated the release rate of this compound from females. This estimate was based on Porapak-Q collections from bait pellets and live females. In making this comparison we arbitrarily assumed the females each emitted pheromone for about 2 hr/day in the collection chambers. Polyvinyl chloride samples containing 0.1% (*E*)-11-tetradecenal and held in the same apparatus as the females, released the *E*-aldehyde at a rate of 2 ± 0.6 μg/day. Projecting downward to the 5 × 3-mm-diam. bait size and 0.01% concentration, we get a release rate of 8 ng/hr at 26° C.

The final series of trap baits was designed to assess effects on male attraction when 11-tetradecenyl acetate and alcohol compounds were released in larger proportions in blends with the aldehyde. This was of interest because of the negative influences caused by the acetate and alcohol components associated with the *C. fumiferana* pheromone system (Sanders, et al., 1972; Sanders 1976; Weatherston and Maclean 1974). Also, the alcohols and acetates were expected to have lower release rates from the polyvinyl chloride baits, and field testing higher concentrations would partially compensate for this and provide greater opportunity to detect inhibiting or optimizing effects on attraction. Five combinations of synthetic aldehydes, acetates, and alcohols were tested and compared to one another and live female baits for attractiveness. Synthetic baits were formulated to contain 0.1% candidate pheromone components on a weight basis, and aldehyde, acetate and alcohol preparations were all 90–92% *E* and 8–10% *Z* isomer mixtures. The six treatments were as follows: live females (one per trap); 10 parts aldehyde only; 10 parts aldehyde plus 2 parts acetate; 10 parts aldehyde plus 2 parts acetate plus 2 parts alcohol; 10 parts aldehyde plus 5 parts acetate; and 10 parts

aldehyde plus 5 parts acetate plus 5 parts alcohol. Traps were exposed in the field for three days and nights. Each bait treatment was replicated 10 times with the sequence of placement randomized within a replicate set of the six different treatments.

RESULTS AND DISCUSSION

Chemical Analyses. Table 1 lists the amounts of pheromone compounds measured in the effluvia and abdominal tip extracts of *C. occidentalis* obtained from a number of sources. Certain trends evident from the table can be summarized as follows:

The effluvia contained principally (*E*)-11-tetradecenal with lesser amounts of (*Z*)-11-tetradecenal, these isomers being present in the ratio 92:8. Small amounts of (*E*)-11-tetradecenyl acetate were present in some of the samples at about 2% of the amounts of 11-tetradecenal. No alcohols were detected in our air collection extracts. In contrast, 11-tetradecenyl acetate was the predominant pheromone-related constituent in all of the hexane-soak gland extracts, with smaller amounts of (*E*)-11-tetradecenal and 11-tetradecenol also present. The average value for the *E*-to-*Z* acetate stereoisomer ratio was 90:10 in these extracts.

Hexane-wash (dip extracts) of pheromone glands showed a clear difference in relative quantities of aldehydes and acetates found outside versus inside (soak extracts) the gland. Results were inconsistent, however, concerning the quantities of alcohols recovered in proportion to aldehydes. The proportion of 11-tetradecenol varied from 0 to 30% of the quantity of 11-tetradecenal (Table 1), and this caused us to consider the possibility that the dip technique was also extracting material from inside the gland. This skepticism was apparently unwarranted, however, since Silk and coworkers (1982) have shown that the alcohol is emitted in the *C. occidentalis* effluvia.

It is possible that some pheromone-related components in the effluvia, particularly small quantities of alcohols, were lost on the glass surfaces of our air collection apparatus. This factor could partially account for some of the discrepancies between our determination of acetate and alcohol content, and those of Silk et al. (1982).

As shown by the hexane-soak gland extracts, acetates are the major pheromone-related chemicals inside the gland, and they must be considered important precursors in biosynthesis. Conversion to aldehydes, the predominant chemicals released by females, apparently occurs at or very near the gland surface during the emission period.

Field Bioassays. Results of the 1976 experiment comparing the attraction of *E*: *Z* 11-tetradecenal blends to Oregon and British Columbia populations of *C. occidentalis* and an Ontario population of *C. fumiferana* showed a definite response difference between the species (Figure 1). The *C. fumiferana*

TABLE 1. MEASUREMENT OF PHEROMONE COMPOUNDS IN EFFLUVIA AND ABDOMINAL TIP HEXANE EXTRACTS OF *C. occidentalis*

	Females extracted[a]	Compound (ng/female moth)					
		11-tetradecenal		11-tetradecenyl acetate		11-tetradecenol	
		E	(*Z*)	*E*	(*Z*)	*E*	(*Z*)
Effluvia							
Lab stock	30	10	(–)	–	(–)	–	(–)
Warm Springs, OR	32, 18, 24	20	(1.8)	0.4	(–)	–	(–)
Fort Collins, CO	32	20	(1.8)	0.5	(–)	–	(–)
Twisp, WA	24	10	(0.9)	tr	(–)	–	(–)
Gland soak extract							
Lab stock	161, 158, 99, 40, 36	2	(–)	5	(0.5)	1	(–)
Idaho	73, 41, 29	2	(–)	10	(1)	–	(–)
Montana	58, 52, 41	2	(–)	6	(0.6)	–	(–)
New Mexico	25	2	(–)	8	(0.8)	–	(–)
British Columbia	27, 31, 30	2	(–)	12	(1.2)	4	(–)
Oregon	20, 30	2	(–)	10	(1)		
Gland wash ("dip")							
Lab stock	286, 554	1	(–)	0.6	(–)	0.3	(–)
Idaho	117	1	(–)	0.5	(–)	–	(–)

[a]The number of females extracted comprised the pool of specimens present in each analyzed sample.

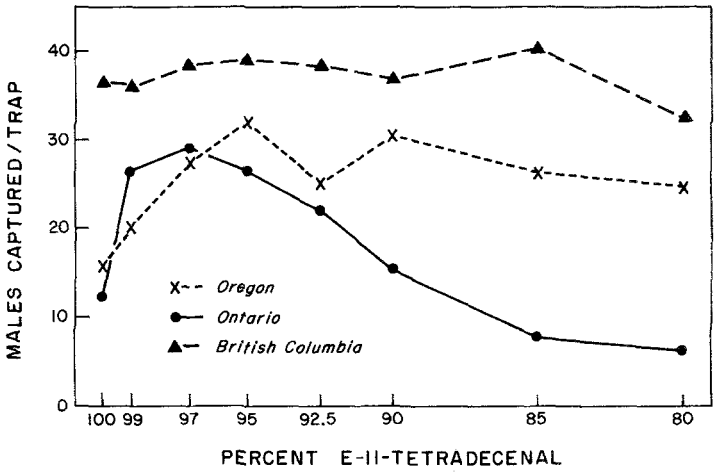

FIG. 1. Response of *C. occidentalis* and *C. fumiferana* (Ontario) males to synthetic trap baits containing different E:Z ratios of 11-tetradecenal.

males were more sensitive to changes in the *E*: *Z* isomer blends, displaying their strongest response and with no significant differences to blends composed of 92.5–99% *E* isomer. The Oregon *C. occidentalis*, in contrast, demonstrated maximum response with no significant differences to blends containing 80–97% *E* isomer. The British Columbia *C. occidentalis* were equally trapped by blends containing *E* isomer of 60–100%.

Subsequent testing of 0.1% synthetic baits to evaluate the attractant effects of (*Z*)-11-tetradecenal and 11-tetradecenyl acetate confirmed that the addition of (*Z*)-11-tetradecenal enhanced attraction (Table 2). The preparation from which all detectable (*Z*)-11-tetradecenal had been removed (>99%

TABLE 2. ATTRACTION OF SYNTHETIC PHEROMONE BAITS FOR *C. occidentalis* FORMULATED AT 0.1% CONCENTRATION IN PVC CONTROLLED-RELEASE PELLET

Trap baits	Males per trap
90:8 *E*:*Z* 11-TDAL[a] + 1.8:0.2 *E*:*Z* 11-TDAC	64.5a[b]
92:8 *E*:*Z* 11-TDAL	60.5a
97:3 *E*:*Z* 11-TDAL	51.7a
100 (*E*)-11-TDAL (>99% pure)	15.3

[a]TDAL and TDAC refer to tetradecenals and tetradecenyl acetates.
[b]Means followed by same letter are not significantly different at $P = 0.05$.

pure) caught significantly fewer male moths than preparations where the chemical was present. The attractiveness of the other three preparations, however, did not significantly differ. Thus, 0.1% bait preparations showed no apparent differences in the attractiveness of the 97:3 and the 92:8 blends of (*E*)-11-tetradecenal:(*Z*)-11-tetradecenal, or the mixture of 90:8:1.8:0.2 (*E*)-11-tetradecenal:(*Z*)-11-tetradecenal:(*E*)-11-tetradecenyl acetate:(*Z*)-11-tetradecenyl acetate.

The field test which compared live female attraction to various synthetic baits formulated at 0.01% concentration provided only a slightly different set of results (Table 3). Both synthetic preparations containing 8% of the (*Z*)-11-tetradecenal were again superior ($P \leqslant 0.05$) to the >99% pure (*E*)-11-tetradecenal, but we did not show significant differences among any of the other four treatments. The average trap catches by the 97:3 blend of *E*:*Z* 11-tetradecenal and the live female baits were only about half that of the traps baited with blends containing 8% of the *Z* isomer (Table 3), and it is possible that a more sensitive experimental design (more replication) would separate these groups. Addition of the 9:1, *E*:*Z* 11-tetradecenyl acetate to the optimal aldehyde mixture did not significantly improve attraction. On the basis of these results we can say that (*Z*)-11-tetradecenal is a necessary component of the pheromone that clearly enhances attraction. It is also evident that the 0.01% baits composed of 90 or 92% (*E*)- and 8% (*Z*)-11-tetradecenal were at least as attractive as the authentic pheromone produced by live females. The quantities of pheromone recovered from live females in the air-collection device (Table 1) suggest the average female emits pheromone at a rate of about 2–5 ng/hr. Thus, the 0.01% bait pellets were approximating the female release rate at 26°C or below.

The final field test evaluating possible enhancing or inhibitory effects of

TABLE 3. ATTRACTION OF LIVE FEMALES AND SYNTHETIC PHEROMONE BAITS FOR *C. occidentalis* FORMULATED AT 0.01% CONCENTRATION IN PVC CONTROLLED-RELEASE PELLET

Trap baits	Males per trap[b]
90:8 *E*:*Z* 11-TDAL[a] + 1.8:0.2 *E*:*Z* 11-TDAC	28.5a
92:8 *E*:*Z* 11-TDAL	24.2a
97:3 *E*:*Z* 11-TDAL	13.7a,b
Live females (one/trap)	12.7a,b
100 (*E*)-11-TDAL (>99% pure)	6.2b

[a]TDAL and TDAC refer to tetradecenals and tetradecenyl acetates.
[b]Means followed by same letter are not significantly different at $P \leqslant 0.05$.

11-tetradecenyl acetates and alcohols, when formulated at concentrations up to 50% of the aldehyde component, failed to show any such effects. Table 4 summarizes the results of this test which indicated no significant differences in attractiveness ($P \leqslant 0.05$) among the synthetic preparations. Two of the synthetic preparations, one containing only the *E* and *Z* aldehyde pheromone components and the other containing these plus the corresponding acetates and alcohols at 20% of the aldehyde concentration, were significantly more attractive ($P \leqslant 0.05$) than live females.

In a separate study of *C. occidentalis* pheromone chemistry, Silk et al. (1982) have reported larger quantities of the 11-tetradecenyl acetates plus 11-tetradecenyl alcohols in the female effluvia than we have reported here. The proportions of acetates and alcohols reported by Silk and coworkers are greater than the proportions used in any of our field bioassays, although the quantities used in this final evaluation begin to approach their ratios. Thus, if the acetates or alcohols were functional pheromone components and enhanced male attraction, such effects should have been evident in the results of this final bioassay. These results suggest that the acetates and alcohols are not necessary pheromone components for long-distance sex attraction.

Silk et al. (1980) have reported a parallel finding with *C. fumiferana*, where female emissions of acetate and alcohol components were apparently not active in the pheromone system. It is possible that the acohols or acetate play a role in short-distance communication between the sexes, and our trapping bioassay was not designed to detect this role. In any case, blends of 92% (*E*)-11-tetradecenal plus 8%(*Z*)-11-tetradecenal closely approximate the sex phreromone of *C. occidentalis*.

TABLE 4. EFFECTS ON ATTRACTION OF SYNTHETIC *C. occidentalis* SEX PHEROMONE BAITS (FORMULATED AT 0.1% CONCENTRATION) BY ADDITION OF LARGE QUANTITIES OF ALCOHOLS AND ACETATES TO THE OPTIMUM *E* AND *Z* BLEND OF ALDEHYDES

Trap baits[a]	Males per trap[b]
10 TDAL + 2 TDAC + Z TDOH	62.7a
10 parts TDAL	58.6a
10 TDAL + 5 TDAC + 5 TDOH	51.4a,b
10 TDAL + 2 TDAC	50.6a,b
10 TDAL + 5 TDAC	45.0a,b
Live females (one/trap)	29.7b

[a]TDAL, TDAC, and TDOH refer to tetradecenals, tetradecenyl acetates, and tetradecenyl alcohols, all of which were blended with 90–92% *E* and 8–10% *Z* isomers.
[b]Means followed by same letter are not significantly different at $P \leqslant 0.05$.

CONCLUSIONS

Chemical analyses and field bioassays of the *C. occidentalis* pheromone system showed the active sex attractant components to be (*E*)- and (*Z*)-11-tetradecenal in a ratio of ~ 92:8. Field tests with synthetic baits releasing these components at rates approximating that of female moths demonstrated they were at least as attractive as the females. Within the gland, these aldehyde components were present in relatively low quantities with the corresponding (*E*)-11-tetradecenyl acetate present in greater quantity. (*Z*)-11-Tetradecenyl acetate and (*E*)- and (*Z*)-11-tetradecenyl alcohols were also found within the gland in small quantities. In the effluvia, or material emitted by the females, we detected only the aldehyde and acetate. Female effluvia contained 90 parts (*E*)-11-tetradecenal:8 parts (*Z*)-11-tetradecenal:1.8 parts (*E*)-11-tetradecenyl acetate:0.2 parts (*Z*)-11-tetradecenyl acetate.

The (*E*)- and (*Z*)-11-tetradecenyl alcohols were recovered from some dip (gland-wash) extracts, and we cannot explain why air collection in Porapak-Q did not also detect alcohols in the female effluvia. In field bioassays we could not demonstrate any significant enhancing or inhibitory effects on attraction caused by either 11-tetradecenyl acetates or alcohols.

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SEX PHEROMONE CHEMISTRY OF THE WESTERN SPRUCE BUDWORM *Choristoneura occidentalis* FREE[1]

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Abstract—SCOT capillary chromatographic and SCOT capillary chromatographic–mass spectrometic analyses of gland washes and effluvia of virgin female *Choristoneura occidentalis* Free. have been conducted with both a diapausing and nondiapausing strain of this insect. The following compounds were identified in gland washes and effluvia in both strains: *E* and *Z*11-14:Ald, *E* and *Z*11-14:Ac, *E* and *Z*11-14:OH and 14:Ald, 14:Ac, and 14:OH. The average aldehyde:acetate:alcohol ratio found by analysis of single glands by virgin females (nondiapausing strain) was 1:7:0.73. Analysis of virgin female effluvia gave this ratio as 10:3:8 (diapausing strain: $\%Z = 8, 11, 15$, respectively) and 10:3:6 (nondiapausing strain: $\%Z = 8, 11, 12$, respectively). The saturated components were generally 1–2% of the *E* isomer in each case. Comparisons of EAG responses of both *C. occidentalis* and *C. fumiferana* to *E*11-14:Ald, *E*11-14:Ac and *E*11-14:OH were made. Correlations with both laboratory and field data previously published were also made between *C. fumiferana* and *C. occidentalis*.

Key Words—*Choristoneura occidentalis*, western spruce budworm, Lepidoptera, Tortricidae, pheromone chemistry, *Choristoneura fumiferana*, capillary GC-MS, (*E*/*Z*)-11-tetradecenal.

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INTRODUCTION

Field and laboratory studies of six closely related budworm moths have indicated that three species, *C. fumiferana* Clem., *C. occidentalis* Free., and *C. biennis* Free., appear to share similar sex pheromones (Sanders, 1971). Both *C. fumiferana* and *C. occidentalis* are attracted in the field to *E*/*Z* mixtures of 11-tetradecenal (Weatherston *et al.*, 1971).

The primary components of the sex pheromone of *C. fumiferana* have been identified as (*E*)-11-tetradecenal (*E*11-14:Ald) and (*Z*)-11-tetradecenal (*Z*11-14:Ald) in the ratio of 96 parts *E* to 4 parts *Z* (Sanders and Weatherston, 1976). (*E*)-11-tetradecenyl acetate (*E*11-14:Ac) and (*E*)-11-tetradecenol (*E*11-14:OH) have been shown to be potent inhibitors to the primary components (Sanders *et al.*, 1972). *E*11-14:OH was subsequently found in abdominal tips of virgin female *C. fumiferana* (Weatherston and MacLean, 1974), but no evidence was presented for the presence of these compounds in female effluvia. Recent chemical studies involving capillary GC and GC-MS analysis of virgin female glands and effluvia (Wiesner et al., 1979; Silk et al., 1980) have revealed a more complex pheromone blend in this insect. *E*11-14:Ac was shown to be the major component (30–40 ng/insect) with *E*11-14:Ald and *E*11-14:OH present at 1–3 ng/insect. Female effluvia contained four components: *E*11-14:Ald, *Z*11-14:Ald, 14:Ald, and *E*11-14:Ac (95:5:2:0.2). *E*11-14:OH could not be detected in the effluvia.

In the present study, we have applied high-resolution capillary GC and GC-MS techniques to study the sex pheromone chemistry of *C. occidentalis*. We present the analysis of gland extracts and effluvia from virgin females both in a diapausing and a nondiapausing strain. Correlations with published laboratory and field data (Cory et al., 1982; Sanders, 1971; Sanders et al., 1977; Silk et al., 1980) have been made between *C. fumiferana* and *C. occidentalis*.

METHODS AND MATERIALS

Insects. Insects were reared on a semisynthetic diet (McMorran, 1965; Grisdale, 1970) and isolated by sex as pupae. Extracts were collected from moths 2–3 days after emergence. Females collected in British Columbia were also examined. Insects were conditioned by holding in a 17-hr light–7-hr dark regime prior to extractions.

Extraction of Airborne Volatiles from Females and Female Glands. Effluvial extracts were obtained by entrainment on Porapak Q® using batches of approximately 100 virgin females as previously described (Silk et al., 1980). Glands, obtained by eversion and removal (1–2 hr prior to the onset of the dark cycle) using a transverse cut, were soaked in hexane (up to 2 hr) in groups

of 5–10 at a time or singly (1 min hexane rinse) and analyzed by capillary GC-FID directly. All effluvial extracts were submitted to silica gel column fractionation prior to GC analyses (Buser and Arn, 1975; Silk *et al.*, 1980). Extracts were eluted into three fractions: hexane (containing hydrocarbons), hexane–8% ether (containing aldehydes and acetates), and ether (containing alcohols). Resolution of compound classes was excellent and recoveries of standard components were reproducible (90% for aldehydes and acetates; 60% for alcohols). The hydrocarbon fraction was not examined.

Instrumental Analysis. For packed and capillary column GC, a Varian 3700 gas chromatograph with an FID detector was used. The chromatograph was equipped with a split/splitless injector and was operated in the split mode (split ratio 1:25 for glands; 1:100 for effluvia). For capillary column analysis, hydrogen was used as a carrier gas at an inlet pressure of 1.5 kg/cm^2. The capillary column employed for GC-FID or GC-MS-EI was a 50-m SP1000 SCOT column (Chromatographic Specialties). This column (Neff 49,300; $k = 6.55$) was capable of partially resolving $\Delta 9$, $\Delta 10$, and $\Delta 11$ double-bond isomers. The column was operated at 150° C for 3 min and then at 5° C/min to 180° C. Injection volume was generally 1μl. Injector and detector temperature was 270° C.

Packed column analysis was performed (both on GC and GC-MS-EI) on a 6-ft column packed with 3% SE-30 on Chromosorb W® (80/100 mesh) and was operated isothermally at 160° C.

GC-MS-EI was performed on a Finnigan 3200F-100 EI system coupled with a 6110 data system. A Grob® injector was used for capillary SCOT column GC-MS analysis and was operated in the splitless mode with helium as carrier gas.

Capillary GC traces were quantitated by comparison of peak heights against internal and external standards. Replicate analyses were performed in each case, and corrections were made for procedural recoveries. Blanks of all materials taken through either the gland extraction procedure or the entrainment of virgin females showed no interferring peaks on capillary GC.

Components were identified by retention time both on packed and capillary column GC and by their EI-MS spectra compared with synthetic material. Microozonolysis of extracts and standards was carried out using a syringe-type ozonizer (Beroza and Bierl, 1967). Ozonides were cleaved with dimethyl sulfide and the products analyzed by packed column GC.

Chemicals. The source and purity of synthetic standards has been reported previously (Silk et al., 1980); undecan-1-ol (11:OH) was obtained from PolyScience Inc.

Electroantennograms. Electroantennograms (EAGs) were obtained by Ag/AgCl electrodes from laboratory-reared *C. fumiferana* and *C. occidentalis* (nondiapausing strain) budworms, 1–4 days old. Test chemicals

were dissolved in hexane and delivered to the antennae as previously described (Grant et al., 1972). Only the unsaturated components of the pheromone were tested; the acetates and alcohols with 10 ng at source, the aldehydes with 1 ng at source. Data for each species was transformed by $\sqrt{(x + 0.5)}$ and subjected to an analysis of variance and a Student-Newman-Keuls' test to separate significantly different means at $P = 0.05$.

RESULTS

Gland Analysis. The results of the analysis of gland washes from the nondiapausing and diapausing laboratory strains as well as feral females of *C. occidentalis* are presented in Table 1. In the nondiapausing strain *E*11–14:Ac is the major component of the gland ($\bar{X} = 30.8$ ng/gland, $\%Z\ \bar{X} = 9$) with *E*11–14:Ald ($\bar{X} = 2.59$ ng/gland, $\%Z\ \bar{X} = 5$) and *E*11–14:OH ($\bar{X} = 3.49$ ng/gland) being minor components. In the diapausing strain *E*11–14:Ac is again the major component ($\bar{X} = 8.19$ ng/gland, $\%Z\ \bar{X} = 8.5$) with *E*11–14:Ald ($\bar{X} = 1.31$ ng/gland, $\%Z\ \bar{X} = 6$) and *E*11–14:OH ($\bar{X}$ not quantitated, GC interferences) as minor components.

The results of single gland analyses for virgin females of the nondiapausing strain are presented in Table 2. The quantities of aldehyde, acetate, and alcohol were significantly different ($P < 0.05$; 1-way ANOVA) with acetate > aldehyde > alcohol ($P < 0.05$; paired t test). Average quantities were as follows: *E*11–14:Ald $\bar{X} = 1.4$ ng/gland, $\%Z\ \bar{X} = 5.6$; *E*11–14:Ac $\bar{X} = 10.2$ ng/gland, $\%Z\ \bar{X} = 7.9$; 14:Ac $\bar{X} = 2.8\%$ (of *E*11–14:Ac); *E*11–14:OH $\bar{X} = 1.07$ ng/gland. Of the three batches of analyses shown (batches 1–3), the *E*11–14:Ald and *E*11–14:Ac levels were not significantly different between batches ($P < 0.05$; $F_{2,12} = 1.82$, $F_{2,12} = 1.5$). The levels of *E*11–14:OH were, however, significantly different ($P < 0.05$; $F_{2,9} = 5.87$). 14:Ald and 14:OH were also present in the glands and, although interferences prevented accurate quantitation, they were generally at ~2–3% of the corresponding *E*11 components. A typical chromatogram of a single gland analysis by capillary GC-FID is shown in Figure 1.

Effluvia Analysis. The results obtained from analysis of effluvial extracts of various batches of insects, received on different dates, are shown in Table 3. The table includes the analysis of batches of virgin females (~100 per batch) from the diapausing and nondiapausing strains. The levels of aldehyde, acetate, and alcohol in the effluvia of the diapausing strain are not significantly different ($P < 0.05$; 1-way ANOVA, paired t test). The levels of the same components in the effluvia of the nondiapausing strain were, however, significantly different ($P < 0.05$; 1-way ANOVA), e.g., average ng/FNE (female night equivalent): aldehyde > alcohol > acetate ($P < 0.05$;

TABLE 1. *Choristoneura occidentalis* GLAND WASHES

No. of glands in wash	*E*11-14:Ald			*E*11-14:Ac				*E*11-14:OH	
	Total[a] NG	NG/gland	% *Z*	Total NG	NG/gland	% *Z*	%Sat	Total NG	NG/gland
Nondisapausing strain									
5	9	1.8		60	12	10	1.4	5.4	1.08
5	4.4	0.88		41.4	8.28	8	1	Trace	Trace
3	5.6	1.87		24.4	8.13	10	7	3.6	1.2
3	5	1.67		35.8	11.9	9	0.6		
3	18	6	5	137	45.7	9	1.5	8	2.67
2	8	4		56	28	9	1.6	Trace	Trace
2	0.4	0.2		4.4	2.2	8			
1	5.6	5.6	5	153	153	10	1.1	16	16
1	1.3	1.3		8.2	8.2				
Average		2.59			30.8				3.49
N		9			9				6
SEM		0.70			15.9				2.53
Diapausing strain									
5[b]	7	1.4		11.4	2.3	10	1.5	*c*	*c*
7[b]	2.4	0.34		11	1.57	10	Trace		
7[b]	16.6	2.37	6	15.6	2.23	8	Trace		
19[b]	6.6	0.35	5	20.6	1.08	8	Trace		
3[b]	5	1.67		18.2	6.1	8			
4[b]	3	0.75		12.8	3.2	9	0.5		
10	16.4	1.64	5	222	22.2	8	1		
10	7.6	0.76		144.6	14.5	8	1.7		
10	25	2.5		205	20.5	8	1		
Average		1.31			8.19	8.5	0.7		
N		9			9				
SEM		0.27			2.84				

[a]NG = nanograms (10^{-9} g).
[b]Feral females from British Columbia.
[c]Not quantitated due to interferences.

TABLE 2. *Choristoneura occidentalis* GLAND WASH: ONE GLAND REMOVED FROM EACH INSECT

Animal number	*E*11-14:A1d			*E*11-14:Ac			*E*11-14:OH		Remarks
	NG/gland	% *Z*	% Sat	NG/gland	% *Z*	% Sat	NG/gland	% *Z*	
Nondiapausing strain									
1	0.36			2.28			0.2		Sept. 2-5
2	0.82			20.7	8	5.5	1.12		Batch 1
3	0.30			3.23	5		0.32		
4	1.78			14.7	8	1.1	Trace		
5	1.18	8		3.15	8		0.25		
6	3.15	5		14.9	10	1.3	2.97		
1	3.38	5		12.7	8	1.8	2.51		Sept. 5-8
2	3.55	5		48.2	8	3.6	2.81		Batch 2
3	0.15			0.80					
4	0.29			0.33					
5	4.37	5		22.4	8	3.6	2.06		
1	0.70			2.45	8		0.07		Sept. 8-11
2	Trace			0.68					Batch 3
3	0.73			1.75	8		0.09		
4	1.21			4.62	8		0.57		
Average	1.46[a]	5.6		10.2[a]	7.9	2.8	1.07[b]		
N	15			15			12		
SEM	0.37			3.35			0.33		

[a]Three sets of data not significantly different ($P < 0.05$; $F_{2,\ 12} = 1.82$; $F_{2,\ 12} = 1.50$, respectively).
[b]Three sets of data significantly different ($P < 0.05$, $F_{2,\ 9} = 5.87$).

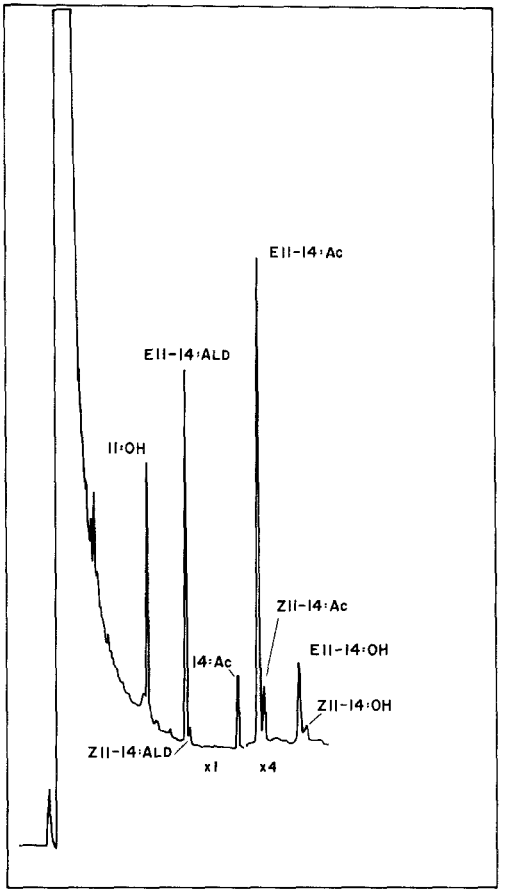

FIG. 1. *C. occidentalis* single gland analysis. 50-m SCOT capillary chromatogram; Varian 3700 GC.

paired *t* test). The *E*/*Z* ratios are also presented in Table 3. The amount of *Z* isomer relative to the *E* isomer for the aldehyde, acetate, and alcohol was $\bar{X} = 8\%$, 11%, and 15%, respectively, for the diapausing strain and $\bar{X} = 8\%$, 11%, and 12%, respectively, for the nondiapausing strain.

A typical GC-MS capillary effluvium analysis of the acetate–aldehyde fraction (8% ether–hexane) is illustrated in Figure 2. *E*/*Z*11–14:Ald, 14:Ald, and *E*/*Z*11–14:Ac are clearly seen; other peaks shown did not repeatedly occur and were ignored (11:OH was a marker/internal standard added after silica gel fractionation). *E*/*Z*11–14:OH do not appear in this chromatogram since they elute in the ether fraction from silica gel chromatography.

Compound Identification. GC retention times (t_Rs) on both packed and capillary columns, as well as characteristic fragmentation patterns in EI-MS,

TABLE 3. *Choristoneura occidentalis*: EFFLUVIA ANALYSIS

Pumping dates	*E*11-14:Ald			*E*11-14:Ac			*E*11-14:OH	
	NG/FNE[a]	% *Z*	% Sat	NG/FNE	% *Z*	% Sat	NG/FNE	% *Z*
Original diapausing strain								
Jan. 21-25	5.49	8		1.21			1.92	17
Feb. 26-28	5.38							
Sept. 2-8	12.99	8	1.8	2.16	10		3.81	17
Sept. 8-11	13.97	6	2.1	4.26	12		4.01	13
Sept. 12-15	1.43	8	1.9	1.29		1.9	9.16	15
Sept. 15-19	0.67	9	3.9	0.43		2.6	8.61	13[d]
Sept. 15-19	1.43	7	2.1	0.81			1.09	13[d]
Average	5.91[b]	8	2.4	1.69[c]	11	2.2	4.77[f]	15
N	7			6			6	
SEM	2.09			0.56			1.38	
Nondiapausing strain								
Aug. 1-6	12.46	8	<0.5	5.83	13	<1	11.65	13
Sept. 2-5	14.74	8	1.8	3.22	10		4.70	8[e]
Sept. 5-8	15.91	7	1.5	6.43	12		13.82	13[e]
Sept. 8-11	11.03	8	2.9	2.91	10		4.76	12[e]
Average	13.54[b]	8	1.6	4.60[c]	11		8.73[f]	12
N	4			4			4	
SEM	1.10			0.90			2.35	

[a]FNE = female night equivalent.
[b]SD ($P < 0.05; t = 2.60$).
[c]SD ($P < 0.05; t = 2.90$).
[d,e]Same batch of pupae.
[f]NS ($P < 0.05; t = 1.56$).

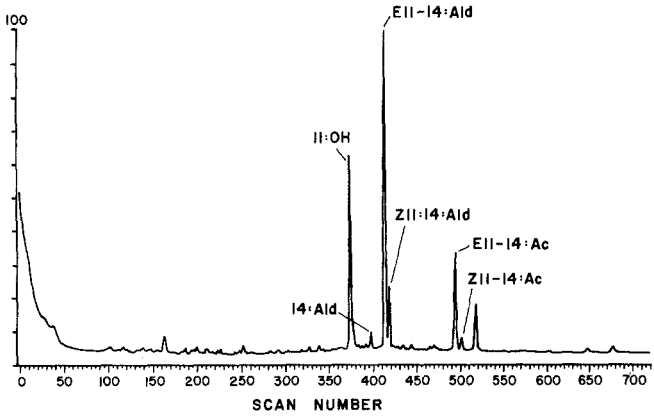

FIG. 2. *C. occidentalis* effluvia analysis. 50 m SCOT SP1000 capillary column: Finnigan 3200F-100 GC-MS. 8% Ether fraction washes Sept. 2–8.

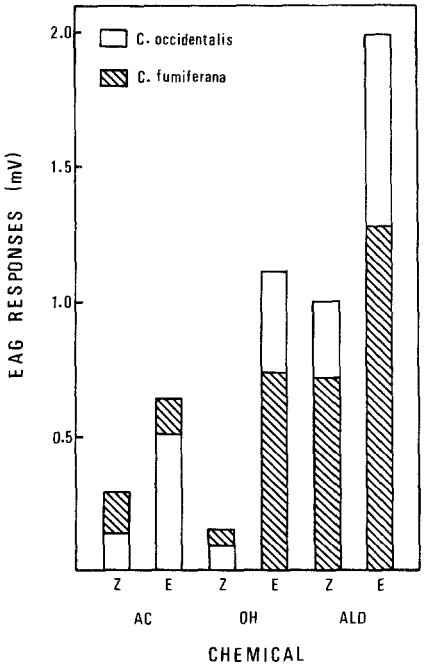

FIG. 3. EAG responses of *C. fumiferana* ($N = 10$) and *C. occidentalis* ($N = 8$) to unsaturated components found in effluvia of *C. occidentalis*. Quantites of *E* and *Z*11–14: Ac and *E* and *Z*11–14: OH tested were 10 ng; quantities of *E* and *Z*11–14: Ald were 1 ng.

were used to identify components. MS and t_Rs of authentic synthetic materials exactly matched those of the biologically derived materials.

Ozonolysis of several gland extracts (after silica gel fractionation; 8% ether–hexane fraction) gave the olefin position as $\Delta 11$ in *E*11–14:Ac since 11-acetoxy undecanal was the only major product observed. Olefin position in the other components was inferred from capillary SCOT t_Rs and their retention time characteristics on other capillary columns (60 m WCOT, UCON 50 HB 5100 and 60 m WCOT, SP1000) used in a similar study with *C. fumiferana* (Silk et al., 1980).

Electroantennograms. The results of the EAG tests are illustrated in Figure 3. The figure shows the EAG responses of *C. fumiferana* ($N = 10$) and *C. occidentalis* ($N = 8$) to unsaturated components found in the effluvia of *C. occidentalis*.

DISCUSSION

It has been known for some time that the sex pheromone of *C. occidentalis* involves *E*/*Z*11–14:Ald (Weatherston et al., 1971), and this study confirms the presence of these components in the effluvia of virgin females (8%Z). Cory et al. (1982) have obtained similar results. In contrast to Cory et al., we report the presence of significant quantities of *E*/*Z*11–14:Ac and *E*/*Z*11–14:OH in the effluvia (Table 3). However, field testing of all three components by these authors does not indicate any effect of acetate or alcohol on aldehyde attractancy.

C. fumiferana males, in contrast to *C. occidentalis*, are strongly inhibited (trap catch) when aldehyde lures are used which contain a high proportion of acetate and/or alcohol (Sanders *et al.*, 1971; Silk *et al.*, 1980). Male *C. fumiferana* are attracted to virgin female *C. occidentalis* in both laboratory bioassays and to baited traps but the response in both cases is only about 50% of that to conspecific females (Sanders, 1971). Since female *C. occidentalis* release both acetate and alcohol (Table 3), this reduction in attractiveness may be accounted for by the presence of these compounds.

Comparison of the EAG responses to *E*11–14:Ald, *E*11–14:Ac, and *E*11–14:OH were made. The EAG profile for both species was similar (Figure 3); the *E* isomers of each compound (except for the acetates with *C. occidentalis*) were significantly more stimulating than the corresponding *Z* isomers, and *E*11–14:Ald was the most stimulating of all the compounds tested. *E*11–14:OH was significantly more stimulating than the corresponding acetate for *C. occidentalis* but was only slightly more stimulating for *C. fumiferana*. Since *E*11–14:OH is present in substantial amounts in the effluvia of *C. occidentalis* and absent from that of *C. fumiferana*, there may be some behavioral significance to this difference.

The possible role of *E*/*Z*11–14: Ac and *E*/*Z*11–14: OH and the saturated components is not known although Cory et al. (1982) speculate that they may play a role in short-range communication (not demonstrated by trap catch). One might also speculate that the sex pheromone chemistry of all the coniferophagous *Choristoneura* species involves blends of *E*/*Z*11–14: Ald, *E*/*Z*11–14: Ac, and *E*/*Z*11–14: OH (and their corresponding saturated analogs) with different ratios, *E*/*Z* ratios, and release rates and which together account for the interspecific inhibition and attraction among them. However, confirmation of this will have to await more detailed chemical and behavioral investigations.

In contrast to *C. fumiferana* (Silk et al., 1980) *E*/*Z* ratios between aldehyde, acetate, and alcohol in gland extracts are significantly different, and these ratios are different again in the effluvia. These results suggest a rather complex biosynthesis involved in pheromone production. The variability in the ratios of all components found in both gland and effluvial extracts is striking. This variability does not appear to be an analytical artifact. However, the greater variability in the data for the diapausing strain compared with the nondiapausing variety may be accounted for by the fact that the nondiapausing strain was free of microsporidia, and hence, tended to be slightly larger resembling the wild type. There appear to be some differences between gland analyses obtained for the diapausing strain (laboratory stock) and those obtained for *C. occidentalis* collected in the field in British Columbia (Table 1). However, within the variability of the data, these differences are not significant.

It does appear that only the aldehyde components are necessary to elicit long-range anemotaxis to traps and that the acetate and alcohol have no effect, at least in the ratios tested to date (Cory et al., 1982).

Although the ratios of the aldehyde, acetate, and alcohol components are variable, the *E*/*Z* ratio of the emitted aldehydes is more precisely defined by this insect, and this is also true for *C. fumiferana* (Silk et al., 1980).

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MALE-SPECIFIC CONVERSION OF THE HOST PLANT COMPOUND, MYRCENE, TO THE PHEROMONE, (+)-IPSDIENOL, IN THE BARK BEETLE, *Dendroctonus brevicomis*[1]

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Abstract—When both sexes of the bark beetle, *Dendroctonus brevicomis* LeConte, were exposed to vapors of myrcene from ponderosa pine, only the male produced (+)-ipsdienol. In the field, racemic ipsdienol significantly reduced the attraction of both sexes in flight to a mixture of myrcene and the aggregation pheromones, *exo*-brevicomin and frontalin. This suggests that ipsdienol may be involved in regulating colonization density of *D. brevicomis*. The implications of the biosynthesis of various enantiomers of ipsdienol by *D. brevicomis* and the cohabitating bark beetles, *Ips paraconfusus* and *I. pini*, in relation to their behavioral responses are discussed in regard to reducing interspecific competition.

Key Words—*Dendroctonus brevicomis*, Coleoptera, Scolytidae, *Pinus ponderosa*, pheromone biosynthesis, bark beetle, myrcene, ipsdienol, *exo*-brevicomin, frontalin, attractants, pheromones, competition.

INTRODUCTION

The process of aggregation of the western pine beetle, *D. brevicomis* LeConte (Coleoptera: Scolytidae), on a ponderosa pine, *Pinus ponderosa* Laws., begins when a female initiates the entrance hole and begins excavating a nuptial chamber and tunnel in the phloem tissue. One component of the aggregation pheromone, *exo*-brevicomin, is synthesized only in the female during boring and feeding in the tree, is released by defecation, and causes a low-level attraction of females and males (Silverstein et al., 1968). A male

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joins the female in the nuptial chamber and immediately releases frontalin which is synergistically attractive with *exo*-brevicomin (Kinzer et al., 1969; Pitman et al., 1969; Bedard et al., 1970; Wood et al., 1976). Myrcene, a host monoterpene, presumably is released primarily at the entrance hole from exuding oleoresin and beetle frass, and it enhances the attraction of *D. brevicomis* to the pheromones (Bedard et al., 1969, 1970).

Hughes (1973) reported that males of *D. brevicomis* produce a "large quantity" of ipsdienol when exposed to myrcene vapors (concentration undetermined). The production of ipsdienol is probably the result of a simple hydroxylation of myrcene at carbon 4. However, Hughes did not mention whether females had been exposed to myrcene. Furthermore, he indicated it was not certain that myrcene (96%) was converted to ipsdienol since it was "not possible to determine whether closely related monoterpenes cause the formation of different quantities of the same products or if the small amounts of other terpenes present as impurities are the cause." Since ipsdienol was not known to have a behavioral effect on *D. brevicomis* and sex-specificity in production was not reported, the finding until now has not been regarded as significant.

The present study was undertaken to confirm the report of Hughes and to determine whether females could synthesize ipsdienol. The quantities and chirality of the ipsdienol produced in beetles after exposure to known vapor concentrations of purified myrcene were determined. In the field, the effect of ipsdienol on the response of *D. brevicomis* in flight to a mixture of its aggregation pheromones and myrcene was tested.

METHODS AND MATERIALS

D. brevicomis adults were reared from bark removed from naturally infested ponderosa pine trees in the Sierra National Forest, California, at about 1000 m elevation (Browne, 1972). Both sexes of *D. brevicomis* were exposed to various concentrations of myrcene vapor (GLC purified >99.8%) or no vapor in bottles for 18 ± 0.5 hr at 21 ± 1.7° C (Byers et al., 1979) on several occasions under natural photoperiods (Table 1). The myrcene concentrations were determined near the end of the exposure periods by withdrawing headspace air from each bottle with a gas-tight syringe for quantification by GLC analysis on a 1.8-m × 2-mm ID glass column of 3% Apiezon L on 100/120 Gas Chrom Q at 100° C and N_2 flow of 12 ml/min. The mid- and hindguts of beetles from each bottle (Table 1) were excised and extracted with 0.2 ml diethyl ether. The amounts of ipsdienol present in these gut extracts were analyzed by GLC (3.6-m × 2-mm ID glass column of Ultrabond II on 100/120 mesh at 100° C and N_2 flow of 30 ml/min; and the Apiezon L column described above). Authentic ipsdienol (GLC purified,

TABLE 1. QUANTITIES OF IPSDIENOL IN MALE AND FEMALE *D. brevicomis* AFTER AN 18-HOUR EXPOSURE TO MYRCENE VAPOR OR NO VAPOR.

Date	Sex	No. exposed	Myrcene concentration (10^{-6} g/ml)[a]	Ipsdienol per beetle ($\times 10^{-8}$ g)
Oct. 13, 1977	♂	20	4.4 ± 0.3	310
	♂	20	None	16
	♀	20	4.4 ± 0.3	<1[d]
	♀	20	None	<1[d]
Nov. 16, 1977	♂	20	3.9 ± 0.6	210
	♀	20	3.9 ± 0.6	<1[d]
May 3, 1980	♂	30[b]	2.0 ± 0.4	152 ± 5
	♂	30[b]	None	12 ± 3
	♀	30[b]	2.0 ± 0.4	<0.5[d]
	♀	30[b]	None	<0.5[d]
May 10, 1980	♂	800[c]	2.1 ± 0.1	169 ± 9

[a]Values represent average ±SEM for all groups exposed on the same date.
[b]Groups of 15 per bottle.
[c]Groups of 100 per bottle.
[d]None detected at GLC sensitivity employed.

>99.5%, Chem Sample Co.) was compared to gut extracts by GLC for retention time and peak area to quantify the production in the beetles.

Eight groups of 100 males were exposed to myrcene (Table 1) as described above and the guts of each group extracted with 0.4 ml diethyl ether so that sufficient amounts of ipsdienol could be obtained for determination of its optical rotation. The ipsdienol produced by these males was isolated by injecting about 100 μl of gut extract for each GLC run and collecting the compound from the 50:1 stream-split effluent from a 2-m × 4.5-mm ID Teflon column of 3% Apiezon L on 100/120 Gas Chrom Q at 100° C and N_2 flow of 100 ml/min. A glass U tube, 150-mm × 4-mm ID, filled 1 cm deep with Glasperline glass beads (0.45–0.5 mm diam) and immersed in liquid N_2 was used to condense the ipsdienol from the effluent gas. After collection of ipsdienol, the U tube was washed with ethanol and the sample rechromatographed, collected, and washed from the U tube with a total of 300 μl of ethanol. This sample was analyzed by GLC to determine the final concentration of the ipsdienol collected (5.02×10^{-7} g/μl). The rotations of the ipsdienol produced by male *D. brevicomis* and the authentic ipsdienol used in the field tests were determined with an electro balancing polarimeter (Autopol III). The ipsdienol from males and the authentic ipsdienol were further analyzed by GCMS on a SCOT 40-m capillary column of OV-101 at 115° C. Mass spectra (EI) were obtained with a V. G. Micromass 7070F mass spectrometer with computerized data system.

Six trap pairs in the Sierra National Forest at about 1000 m elevation were used to determine if ipsdienol (GLC purified >99.5%) released from one trap of each pair had any effect on the attraction of *D. brevicomis* to *exo*-brevicomin, frontalin, and myrcene released at approximately equal rates from both traps. The trap consisted of a 6-mm mesh metal-screen cylinder (19 cm diam × 30.5 cm high) coated with Stickem Special® (Bedard and Browne, 1969) placed 1.2 m above the ground. The traps of each pair were spaced 9–10 m apart and at least 100 m away from other pairs. Treatment and check were assigned at random within each pair each day. *exo*-Brevicomin, frontalin, and myrcene were each released at about 2 mg/day from glass tubes inside a glass salt shaker (Byers and Wood, 1980). Ipsdienol was released at about 0.6 mg/day from another salt shaker containing two 4- × 65-mm glass tubes each filled with 30 mg ipsdienol (GLC >99.5%). The trap catches on July 1–6, 1980, of *Enoclerus lecontei* (Wolcott) (Coleoptera: Cleridae), *Temnochila chlorodia* (Mannerheim) (Coleoptera: Trogositidae), and both sexes of *D. brevicomis* on each trap pair were compared with Wilcoxon signed rank tests (Lehmann, 1975). Sex ratio comparisons of *D. brevicomis* for the treatment and check were performed by chi-square tests.

RESULTS

Female *D. brevicomis* did not contain detectable quantities of ipsdienol ($<1 \times 10^{-8}$ g/female) after exposure to myrcene vapors while males contained at least 1.5×10^{-6} g ipsdienol per male after similar treatment (Table 1). Females unexposed to myrcene also did not contain ipsdienol; however, unexposed males appeared to have as much as 10% of the amount of ipsdienol that myrcene-exposed males contained (verified by GLC only). Ipsenol was not detected in either sex. The rotation of the ipsdienol isolated by GLC from males exposed to myrcene had a specific rotation of $[\alpha]_D^{21} = +12 \pm 4°$ (ethanol) which is similar to $[\alpha]_D^{20} = +10 \pm 0.9°$ reported by Silverstein et al. (1967) for ipsdienol isolated from *Ips paraconfusus* Lanier (Coleoptera: Scolytidae) [95% (+) enantiomer, Plummer et al., 1976].

The GC-MS fragmentation pattern of the purified ipsdienol obtained from vapor-exposed males matched the mass spectra of authentic ipsdienol. The masses of the following major fragments are listed with their percentages of the base peak because they differ quantitatively from those published earlier (Silverstein et al., 1967): 32:4.1, 39:3.0, 41:8.1, 53:2.8, 55:4.9, 65:2.8, 67:7.7, 68:4.9, 69:2.4, 79:5.0, 85:100.0 (base peak), 91:8.2, 109:4.6, 119:6.5, 134:5.1, 152:2.7. The quantitative differences in the spectra might be the result of some thermal decomposition of ipsdienol on the GLC column used by Silverstein et al.

The number of *D. brevicomis* caught on traps releasing aggregation

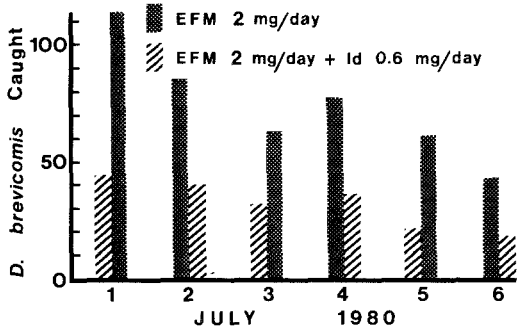

FIG. 1. The reduction in the attraction of *D. brevicomis* in flight to *exo*-brevicomin (E), frontalin (F), and myrcene (M) by ipsdienol (Id) as shown by the comparison of daily catch totals on 6 pairs of traps from July 1 to 6, 1980, in the Sierra National Forest, California. The catch totals for treatment and check are shown alternating each day to indicate random placement of ipsdienol.

components and ipsdienol (191, 93 male : 98 female) was significantly less than the number attracted to traps with aggregation components alone (443, 198 male : 245 female) (Figure 1). The response of both sexes to the aggregation pheromones appears to be inhibited (significantly lowered catch) by ipsdienol ($P < 0.001$ in each case), and there was no significant difference between the responses of the sexes as indicated by the ratio of catch by sex on the treatment and check traps ($P > 0.1$). The attraction of *T. chlorodia*, a predator, to the aggregation components was not effected by release of ipsdienol (75 vs. 67 on ipsdienol, $P > 0.1$). However, the catch of another predator, *E. lecontei*, was significantly increased at traps with ipsdienol (31 vs. 5, $P < 0.001$) which is consistent with their response to ipsdienol alone (Wood et al., 1966). The rotation of the ipsdienol used in the field tests was $[\alpha]_D^{21} = -0.35°$ (4% in ethanol) or racemic [52% (−) : 48% (+)].

DISCUSSION

Myrcene appears to play a role in several important ecological functions for *D. brevicomis*. The release rate of myrcene from the tree is increased by the boring activity of beetles which use the compound in combination with aggregation pheromones to locate its host and breeding sites. In the present study, myrcene was converted to (*S*)-(+)-ipsdienol only in the male beetle, and racemic ipsdienol, released at rates comparable to the aggregation pheromones, inhibited the attraction of both sexes in the field. This suggests that ipsdienol may function in regulating the density of attack and/or terminating the attack in an area depending on the compound's release rate in relation to

release rates of other behavioral chemicals during the colonization period. The precise function of ipsdienol remains to be elucidated. Another role that myrcene may play in the chemical ecology of *D. brevicomis* was shown by Byers and Wood (1981a) in which at least one of the myrcene-derived pheromones of a cohabitating bark beetle, *I. paraconfusus*, ipsenol and ipsdienol (Hughes, 1974; Byers et al., 1979; Hendry et al., 1980), synergized with another pheromonal component, *cis*-verbenol, to inhibit the response of *D. brevicomis* to its pheromones in the laboratory. The response of *D. brevicomis* to pheromone from naturally infested logs was inhibited by the presence of logs infested with *I. paraconfusus* males (Byers and Wood, 1980). These results indicated that ipsdienol/ipsenol plus *cis*-verbenol from *I. paraconfusus* may function to reduce interspecific competition for food and space.

In California, *I. pini* (Say), *I. paraconfusus*, and *D. brevicomis* may all compete for food and space on the same host tree. *I. pini* produces (−)-ipsdienol as its primary pheromone and this inhibits the response of *I. paraconfusus* to its pheromones (Light and Birch, 1979). On the other hand, *I. paraconfusus* produces (+)-ipsdienol (Plummer et al., 1976), and this inhibits the response of *I. pini* to its pheromone (Light and Birch, 1979). *D. brevicomis* probably produces (+)-ipsdienol under natural conditions because it feeds on phloem containing myrcene (Byers, 1981). Therefore, it remains to be established if a sufficient quantity of (+)-ipsdienol is released by *D. brevicomis* to inhibit *I. pini* response and thus could function to reduce interspecific competition. Release of (+)-ipsdienol from *D. brevicomis* would not inhibit *I. paraconfusus* since this beetle uses the enantiomer as one component of its aggregation pheromone. However, Byers and Wood (1981a) showed that verbenone from male *D. brevicomis* was very effective in inhibiting the response of *I. paraconfusus* to its pheromones.

It appears that only specific host monoterpenes can be converted to specific pheromones in most bark beetles studied. For example, myrcene is not converted to *cis*-verbenol in *I. paraconfusus* (Byers et al., 1979), and another major monoterpene found in ponderosa pine, α-pinene, is not converted to ipsdienol or ipsenol in the same insect (Renwick et al., 1976; Byers, 1981). α-Pinene is converted to the pheromones *cis*-verbenol in *I. paraconfusus* (Brand et al., 1975; Renwick et al., 1976; Byers, 1981) and *trans*-verbenol in *D. ponderosae* (Pitman, 1971; Hughes, 1973). Exposure of *I. paraconfusus* to Δ-3-carene, β-pinene, and limonene did not result in synthesis of any of the above pheromones, but other specific compounds (unidentified) were produced (Byers, unpublished). Similar exposure of *D. frontalis* to host monoterpenes resulted in the production of compounds specific to "a particular terpene" (Hughes, 1973).

One reason that these bark beetle species, as well as *D. brevicomis* (and

possibly *I. pini*), have evolved to use myrcene and α-pinene as precursors for certain of their pheromones may be due to the variation of monoterpenes in ponderosa pine. For example, Smith (1964) reported that the minimum percentages of myrcene and α-pinene in the oleoresin of 64 ponderosa pines in the central Sierra Nevada of California were higher (Myrcene the highest) than the sometimes trace amounts of Δ-3-carene, β-pinene, and limonene. In a similar study, 369 trees located at 27 sites from southern to northern California had much more narrow ranges of percentages of myrcene and α-pinene than the other three major monoterpenes (Smith et al., 1969). Thus, there would appear to be a selective advantage for those insects that utilize host monoterpenes which are less variable in their distribution for the conversion to pheromones so crucial for bark beetle survival.

Byers and Wood (1981b) found that the antibiotic, streptomycin, inhibited the conversion of myrcene to ipsdienol and ipsenol in male *I. paraconfusus*, which suggests a symbiotic microorganism within the intestine of the beetle. The biosynthetic system in *D. brevicomis* appears to be different in that only ipsdienol is produced, and it is not reduced to ipsenol. Further work is needed to determine if microorganisms play a role in *D. brevicomis*.

Unequivocal proof that ipsdienol is a pheromone of *D. brevicomis* can only be presented when quantitative comparisons are made between the release of the chemical from naturally infested trees and the amounts needed to effect behavior. It appears that ipsdienol could be useful in protecting pines from *D. brevicomis* attack or in mass release applications to "confuse" the beetle. Release of ipsdienol and verbenone (Byers and Wood, 1980) would have the advantage of inhibiting the responses of *D. brevicomis*, *I. paraconfusus*, and *I. pini* to any pine substrate infested by any one of these species in order to suppress movement of the infestation to surrounding pines or reduce the attack rate sufficiently for the tree to overcome the beetles with resin. Further work is needed to determine if these inhibitors of attractive response might even repel beetles away from a place where susceptible trees are growing.

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INFLUENCE OF OPPOSITE SEX ON ATTRACTION PRODUCED BY PIONEER SEX OF FOUR BARK BEETLE SPECIES COHABITING PINE IN THE SOUTHERN UNITED STATES

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Abstract—Catches of *Dendroctonus frontalis* and *Ips avulsus* on traps surrounding bolts infested with both sexes of each species in the gallery were not significantly different from catches at bolts infested only with the sex which normally pioneers the host colonization process. In contrast, the presence of the second sex in the gallery significantly reduced catches of *I. grandicollis* and *I. calligraphus* and, additionally, the presence of females in the male galleries of *I. calligraphus* significantly reduced the catch of *I. avulsus* females as compared with their response to bolts infested with the male *I. calligraphus* only.

Key Words—*Dendroctonus frontalis*, *Ips calligraphus*, *I. grandicollis*, *I. avulsus*, Coleoptera, Scolytidae, mating, attraction.

INTRODUCTION

The chemically mediated behavior of four cohabiting bark beetle species, *Dendroctonus frontalis* Zimmerman, *Ips avulsus* (Eichhoff), *I. calligraphus* (Germar), and *I. grandicollis* (Eichhoff), which colonize loblolly pines, *Pinus taeda* L., was described by Birch et al. (1980). These four species often colonize the same tree in characteristic temporal and spatial patterns (Birch and Švihra, 1979; Švihra et al., 1980; Paine et al., 1981). However, little is known about the role of the sexes of each of these four species in the patterns of colonization. Coulson (1979) delineated five phases in host-tree colonization by *Dendroctonus* species: (1) host selection and gallery initiation by pioneer beetles, (2) concentration of responding sex (females), (3) production of additional pheromones and response of both sexes, (4) mating and oviposi-

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tion, and (5) reemergence of parent adults. This chronology does not explain whether or not the arrival of the second sex and subsequent mating in galleries influence the attraction or termination of host colonization by that or other species. The presence of males (the second sex) in the galleries of some species of *Dendroctonus* may enhance attractiveness (Coster and Vité, 1972), or may cause an abrupt decline in attractiveness of flying beetles (McMullen and Atkins, 1962; Rudinsky, 1969). However, in *Ips*, where males are the pioneer sex, male attractiveness progressively declines as the male mates with more females (Borden, 1967; Werner, 1972).

Preliminary observations in previous work by Švihra et al. (1980) indicated that interactions between males and females may influence not only the behavior of the same species, but also the in-flight and landing behavior of other sympatric species which colonize the same pine tree. These experiments were conducted to demonstrate whether or not the presence of the second sex affected the inter- and intraspecific attraction generated by the pioneering sex.

METHODS AND MATERIALS

All tests were conducted between May and June 1977 in pine forests near Nacogdoches, in east Texas. Their basic design was similar to that described by Birch et al. (1980). In each test, three bolts (30 cm long and 10–15 cm diameter) were cut from apparently healthy loblolly pine, *P. taeda*, and allowed to age in the laboratory for two days after felling. Twenty-five holes were drilled through the bark of each bolt at an angle of about 30° to the long axis. Twenty-five males of either one of the three *Ips* species or 25 females of *D. frontalis* were introduced (head first) into the preformed holes in two bolts, and the holes were covered with fine mesh metal screening to prevent escape. The third bolt was left empty as a control. All three bolts were left for 24 hr in the laboratory, then the mesh screenings were removed from the holes on one bolt and beetles of the second (or opposite) sex were introduced into each hole (two females for *Ips* species; one male for *D. frontalis*). Thus each experiment tested three treatments: (1) the bolt with the pioneering sex alone, (2) the bolt with males and females together in each hole, and (3) the control bolt with drilled holes but no beetles. The mesh was again stapled over each hole, and the bolts were entirely wrapped in fine mesh screening to prevent additional colonization by responding beetles during the field tests.

Attractiveness of the bolts was tested in the field by placing them in sticky traps (Bedard and Browne, 1969; Birch et al., 1980). Traps were placed 15 meters apart in a line and their positions rotated systematically as described by Birch et al. (1980). The duration of each experiment and the number of replications in each are indicated in the tables. After the experiment was terminated, the bark was peeled from each bolt to confirm the sex and

mortality of introduced beetles and to establish that no attack by other species had occurred.

Differences in the response of *D. frontalis*, *I. calligraphus*, *I. grandicollis*, and *I. avulsus* to the attractive sources were analyzed by the nonparametric Friedman test for multiple comparisons. The sex-specific responses were measured by comparing the sex ratio of each treatment by the chi-square goodness-of-fit test, with an expected response sex ratio 1:1.

RESULTS AND DISCUSSION

The response of *D. frontalis* was not significantly reduced, nor was the sex ratio changed in either of the two experiments when males were present with females in the galleries (Table 1). Insufficient numbers of *Ips* species were attracted to either of the *Dendroctonus* treatments to assess the effects of the presence of *D. frontalis* males on the cross-attraction of *Ips* species. Similar results were found after *I. avulsus* females were introduced to the males in the gallery (Table 2). Their presence apparently had no significant effect on the number of either sex of *I. avulsus* trapped.

In contrast, the presence of *I. calligraphus* females with male *I. calligraphus*, and *I. grandicollis* females with male *I. grandicollis*, significantly decreased the catch of conspecifics without any change in sex ratios (Tables 3 and 4). In addition, male *I. calligraphus* attracted large numbers of both *I. grandicollis* and *I. avulsus*. However, with the addition of female *I. calligraphus* to the gallery, the response of *I. avulsus* females was significantly reduced.

TABLE 1. RESPONSE (TRAP CATCH) TO BOLTS CONTAINING 25 FEMALE *D. frontalis*; 25 FEMALE *D. frontalis* PLUS 25 MALE *D. frontalis* (IN THE SAME HOLES); AND TO A CONTROL BOLT CONTAINING NO BEETLES[a]

Test	Treatment (bolt)	Response[b]			
		D. frontalis	*I. avulsus*	*I. calligraphus*	*I. grandicollis*
A	*D. frontalis* (♀♀)	23[a] (1:0.9)	5 (1:0.25)	0	4 (1:0)
	D. frontalis (♀♀ & ♂♂)	17[a] (1:0.7)	4 (1:0.3)	3 (0:1)	5 (1:0.7)
	Control	2[b] (0:2)	2 (1:1.0)	0	0
B	*D. frontalis* (♀♀)	27[a] (1:0.5)	3 (1:0)	0	6 (0:1)
	D. frontalis (♀♀ & ♂♂)	16[a] (1:0.8)	2 (0:1)	0	6 (1:5)
	Control	2[b] (1:1.0)	2 (1:0)	0	0

[a]East Texas. Test A: April 29-30, 1977, 16 × 0.75-hr replications of all treatments. Test B: May 18-20, 1977, 30 × 0.75-hr replications of all treatments. Numbers are the sum caught in each test and sex ratio (m:f).

[b]Letters within columns in a test denote homogeneous subsets, Friedman's tests, $P \leq 0.05$.

TABLE 2. RESPONSE (TRAP CATCH) TO BOLTS CONTAINING 25 MALE *I. avulsus*; 25 MALE *I. avulsus* AND 48 FEMALE *I. avulsus* (IN THE SAME HOLES); AND TO A CONTROL BOLT CONTAINING NO BEETLES[a]

	Response[b]			
Treatment (bolt)	*D. frontalis*	*I. avulsus*	*I. calligraphus*	*I. grandicollis*
I. avulsus (♂♂)	0	123[a] (1:0.9)	9 (1:0.5)	6 (1:0.5)
I. avulsus (♂♂ & ♀♀)	0	109[a] (1:1.1)	1 (1:1)	5 (1:0.2)
Control	0	1[b] (1:0)	1 (0:1)	0

[a]East Texas, June 9-11, 1977. Numbers are sum of 24 1.5-hr replications of all treatments and sex ratio (m:f).
[b]Letters with columns in a test denote homogeneous subsets, Friedman's tests, $P \leq 0.05$.

By trapping beetles as they arrived, these experiments excluded subsequent natural activity of responding beetles on the host, and the manner in which the second sex was added to the pioneering sex in the laboratory may not occur as quickly under natural conditions. However, under these experimental conditions, the effects of the second sex were: (1) the intraspecific responses of *D. frontalis* and *I. avulsus* were not significantly affected, (2) intraspecific attraction of *I. calligraphus* and *I. grandicollis* was apparently reduced, and (3) the cross-attraction of *I. avulsus* females to *I. calligraphus* was reduced.

These experiments failed to demonstrate that the addition of the male to the female increased attraction of *D. frontalis* as Coster and Vité (1972)

TABLE 3. RESPONSE (TRAP CATCH) TO BOLTS CONTAINING 25 MALE *I. calligraphus*, 25 MALE PLUS 50 FEMALE *I. calligraphus* (IN THE SAME HOLES); AND A CONTROL BOLT WITH NO BEETLES[a]

	Response[b]			
Treatment (bolt)	*D. frontalis*	*I. avulsus*	*I. calligraphus*	*I. grandicollis*
I. calligraphus (♂♂)	0	207[a] (1:1.6)*	471[a] (1:3.0)	47[a] (1:1.4)
I. calligraphus (♂♂ & ♀♀)	0	79[b] (1:0.7)*	128[b] (1:2.9)	33[a] (1:1.2)
Control	0	0	0	0

[a]East Texas, June 7-9, 1977. Numbers are sum of 24 1.5-hr replications of all treatments and sex ratio (m:f).
[b]Letters within columns in a test denote homogeneous subsets, Friedman's tests, $P \leq 0.05$. Asterisks denote significant ($P \leq 0.05$) deviation from 1:1 sex ratio response; χ^2 goodness-of-fit-test.

TABLE 4. RESPONSE (TRAP CATCH) TO BOLTS CONTAINING 25 MALE *I. grandicollis*, 25 MALE PLUS 50 FEMALE *I. grandicollis* (IN THE SAME HOLES), AND TO A CONTROL BOLT CONTAINING NO BEETLES[a]

Test	Treatment (bolt)	Response[b]			
		D. frontalis	*I. avulsus*	*I. calligraphus*	*I. grandicollis*
A	*I. grandicollis* (♂♂)	0	2 (0:1)	0	143[a] (1:2.7)
	I. grandicollis (♂♂ & ♀♀)	2 (1:1)	7 (1:0.4)	5 (1:4)	78[b] (1:2.2)
	Control	0	0	0	7[c] (1:2.5)
B	*I. grandicollis* (♂♂)	1 (1:0)	2 (1:1)	1 (1:0)	147[a] (1:1.5)
	I. grandicollis (♂♂ & ♀♀)	0	2 (1:0)	1 (0:1)	86[b] (1:1.1)
	Control	0	0	0	0

[a]East Texas. Test A: May 9-11, 1977. Test B: May 13-15, 1977. Numbers in both tests are sum of 30 0.75-hr replications of all treatments and sex ratio (m:f).
[b]Letters within columns in a test denote homogeneous subsets, Friedman's tests, $P \leq 0.05$.

reported. Also, these data indicate that the interpretation of host colonization by *D. frontalis* proposed by Vité and Francke (1976) is perhaps oversimplified. They suggested that females release the attractant and males carry inhibitors which terminate the colonization process. The results presented here imply that there may be factors other than the presence of males that influence colonization in this species.

When *D. frontalis* attacks apparently healthy pines, a copious resin flow is usually triggered, which may influence aggregation. While it was not possible in these experiments to simulate host conditions of standing trees under attack, it appears that, at least for three days, the colonization process of *D. frontalis* and *I. avulsus* is not curtailed by the presence of both sexes and their subsequent mating in the galleries. After the bark was peeled off those logs, the 2- to 4-cm-long egg galleries were found to contain eggs, which is sufficient evidence that mating took place.

Mason (1970) considered *I. avulsus* an aggressive species able to attack healthy pines. Vité et al. (1972) stated that some aggressive bark beetle species, such as *D. frontalis* and *I. avulsus*, aggregate on the host using "contact pheromones," which are produced after arrival but before the pioneering species begin to feed in the host. The secondary species, such as *I. calligraphus* and *I. grandicollis*, tend to produce "frass pheromones" only after feeding and gallery construction have been initiated. The response of the less aggressive species, *I. calligraphus* and *I. grandicollis*, was significantly reduced after the second sex had been added to the pioneering one. Nevertheless, in these significantly reduced beetle responses, the responding males could enter the bark and initiate new sources of attraction.

The reduced cross-attraction of *I. avulsus* to the bolt with both sexes of *I. calligraphus* in the gallery is of particular interest since Birch et al. (1980) found that the presence of *I. avulsus* males in the same bolt with *I. calligraphus* males significantly reduced the responses of *I. calligraphus*. Similar interspecific relationships did not appear in other species as may have been expected. Thus, oversimplified models of colonization by these four sympatric species may be misleading if they imply that the pioneering sex carried the attractants and that the mates or the mating process inhibits this phenomenon.

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AVERSION SUBSTANCE(S) OF THE RAT COAGULATING GLANDS

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Abstract—The aversive substance(s) present in adult male urine were not found in castrate rat urine. Removal of the coagulating glands also resulted in a loss of the aversion compounds. The aversion substances were restored to the urine after androgen treatment of the castrate rats.

Key Words—Coagulating gland, aversive compounds, testosterone, rat.

INTRODUCTION

The relationship of urine to rodent behavior has been studied in the past, with most of the investigations having been done on mice (Bronson, 1971; Bronson et al., 1973; Jones and Nowell, 1973; Mugford and Nowell, 1970). In 1973, Bronson et al. obtained evidence for mouse urinary marking substances by an ultraviolet visualization technique. Aggression-promoting compounds occurred in male mouse urine, while in females aggression-inhibiting chemicals were reported (Mugford and Nowell, 1970; Bronson, 1971). Male mouse urine apparently contains an aversive substance from the coagulating glands that is under androgen control, because castration abolished the aversive effects but testosterone replacement after castration restored the effects (Jones and Nowell, 1973). Gawienowski et al. (1976) demonstrated that adult rats produce androgen-dependent marking compounds which are soluble in ether. We decided to investigate the source of the aversive compounds in male rats in order to determine if they require an intact coagulating gland.

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METHODS AND MATERIALS

Animals. The behavior of four groups of male Sprague-Dawley rats (10 per group) was evaluated in a test chamber. All were at least 8 months old. Three Sprague-Dawley males were randomly assigned to one of four treatment groups at 5 months of age to serve as urine donors. Group I consisted of sham-operated controls, group II was castrated, group III was castrated followed by testosterone replacement, and in group IV the coagulating glands were removed, in addition to castration, and was followed by testosterone replacement. Replacement of testosterone consisted of 2.4 mg testosterone propionate in sesame oil given every third day beginning the first day after surgery. All injections were administered within an hour after the beginning of the dark period, and controls were treated similarly with only the vehicle. All rats, donors and test rats, were housed individually and received food (Purina Rat Chow) and water ad libitum. Donors were housed in separate rooms from test rats and maintained on a reversed light cycle (light–dark, 12:12) with the temperature at 24° C.

Urine Collection. Urine was collected daily within the first hour of the dark period, stored in an ice bath, pooled, and used within 2 hr. Long-standing urine at room temperature was not found to be effective.

Olfactory Procedures. A metal cage (61 × 61 × 35.5 cm) with paper covering the floor served as the olfactory test area (Gawienowski et al., 1976). The floor was divided in half by a pencil mark, and each half was spotted with 1.5 ml of different samples in 20 equidistant spots. Opposite sides were treated on alternate days of testing to avoid locational preferences. The timing was begun when the animal, which was placed on the pencil line, moved into either area. A rat was not considered to be in an area until all four feet were on one half of the paper. Accumulated times in either half were recorded for a 5-min period using a 25-watt red light bulb for viewing. After testing each rat, the chamber was washed and the paper replaced. Testing was done during the first hour of the dark period.

RESULTS AND DISCUSSION

The results, as presented in Table 1, revealed that the test rats spent significantly ($P < .05$) more time in the control half of the olfactory test chamber when urine from either intact males (group I) or castrate males treated with testosterone replacement (group III) was presented. However, no preference for either half of the chamber was observed when the urine from group II or group IV was introduced. These groups contained the castrates and the rats without coagulating glands.

Previously Gawienowski et al. (1976) reported that there were aversion

TABLE 1. MALE RAT OLFACTORY PREFERENCE

Urine donors		Mean time (seconds ± SD)		
Group	Treatment	Control area	Treated area	P^a
I	Control	201.6 ± 17.8	98.4 ± 17.8	<0.02
II	Castrate	161.8 ± 20.5	138.2 ± 20.5	NS
III	Castrate + TP[b]	207.0 ± 22.4	93.0 ± 22.4	<0.05
IV	Castrate + TP-CG	153.0 ± 18.2	146.8 ± 18.2	NS

[a]Computed according to paired t test (Steel and Torrie, 1960).
[b]TP: testosterone proprionate; CG: coagulating glands.

semiochemicals in voided rat urine and that they were not present in the urine of castrates. This aversion effect is androgen-dependent and was observed in castrates receiving testosterone treatment (group III). Replacement levels of the testosterone were adequate to overcome the effects of castration. These semiochemical effects indicate the need for an adequate endogenous androgen level for normal maintenance of the coagulation gland. Whether or not other mammals have a coagulating gland aversion compound(s) remains to be seen by future research.

As our study indicated, fresh urine collected in a chilled reservoir was needed for maximum aversion effect. This indicates that the compound(s) are volatile and unstable in warm urine. As our previous studies showed, the aversive compound(s) are lipids. Lipid analysis of the coagulating glands of male rats revealed cholesterol (56.3%), fatty acids (26.3%), cholesterol esters (9.6%), and triglycerides (7.8%) (Gawienowski and Snyder, 1981) as the main components.

The removal of the coagulating glands results in the elimination of the aversion compounds, indicating that they play an important role in the release of the lipid compounds, but studies with mice (Jones and Nowell, 1973) indicate that only the coagulating glands are involved.

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SEX PHEROMONE OF THE FALL WEBWORM MOTH, *Hyphantria cunea*[1,2]

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Abstract—Three compounds have been identified as sex pheromone components produced by female fall webworm moths, *Hyphantria cunea* (Drury). These compounds are: (*Z,Z*)-9,12-octadecadienal (I), (*Z,Z,Z*)-9,12,15-octadecatrienal (II), and (*Z,Z*)-3,6-*cis*-9,10-epoxyheneicosadiene (III). The ratio of these compounds was approximately 5:6:13, respectively, in female tip extracts prepared from U.S.S.R. insects and approximately 1:8:21, respectively, in extracts from U.S. insects. The ratio in female effluvia trapped from U.S. insects was 1:6:27, respectively. Compound III plus either I or II is effective in eliciting upwind flight in a wind tunnel. Compounds I, II, and III are also components of the sex pheromone system of the saltmarsh caterpillar moth, *Estigmene acrea* (Drury).

Key Words—Insect sex pheromone, fall webworm moth, American white moth, *Hyphantria cunea*, salt march caterpillar moth, *Estigmene acrea*, Lepidoptera, Arctiidae, (*Z,Z*)-9,12-octadecadienal, linolealdehyde, (*Z,Z,Z*)-9,12,15-octadecatrienal, linolenaldehyde, (*Z,Z*)-3,6-*cis*-9,10-epoxyheneicosadiene.

[1]Lepidoptera: Arctiidae.

[2]Part of this work was carried out under the sponsorship of the U.S.A.-U.S.S.R. Scientific Exchange Program and the U.S.S.R. Ministry of Agriculture. It was also supported in part by National Science Foundation grant PCM78-13241. These results were first disclosed at the EUCHEM Conference on Chemistry of Insects, held at Borgholm, Sweden, on August 13–17, 1979. We sadly note the passing away of our distinguished colleague and friend, Dr. A.S. Hill.

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INTRODUCTION

The fall webworm moth, *Hyphantria cunea* (Drury), which is known as the American white moth in the U.S.S.R., is a widely distributed species throughout the northern hemisphere. It has been found feeding on over 100 species of deciduous trees (Metcalf et al., 1951). Its presence becomes obvious during the spring and summer months when it forms communal webs or tents on its hosts. These webs become larger in size as the larvae mature and can cover an entire tree during a heavy infestation. The larvae feed within the entire web and return to the central portion during periods when they are not feeding. This species overwinters as diapausing pupae, either under debris or underground, and the adults emerge in the spring. It has one or more generations per year, depending upon its geographical location. At Geneva, New York (U.S.A.), there is one generation per year, and at Kishinev (U.S.S.R.) there are two generations per year.

Two different races of *H. cunea* have been described, a "black-headed" race and a "red-headed" (or "orange") race (Oliver, 1964; Warren and Tadic, 1970; Hidaka, 1977). Only the "black-headed" race has been recognized in Japan, eastern Europe and western Asia, including the U.S.S.R. (Tchuraev, 1953; Umnov and Tchuraev, 1955). It is believed to be native to North America and to have been introduced from there into the other regions. Both races have been recognized in North America, where they can occur in the same localities. The races cross-mate readily under laboratory conditions and the F_1 and F_2 progeny are viable. However, no evidence of cross-breeding in nature has been reported (Oliver, 1964). Further, Oliver (1964) and Hidaka (1977) have reported that there are behavioral as well as morphological differences between the races. Dr. Makio Takeda[5] has examined larval specimens from the Geneva, New York, area and has concluded that they are of the "red-headed" race.

The mating behavior of *H. cunea* has been observed in the field in Japan (Hidaka, 1972; Hasegawa and Ito, 1967), and in the U.S.A. (Calcote and Gentry, 1973). Males of both races have been observed in the field to engage in mate-seeking behavior only during a short period of time (<1 hr) just before sunrise. Females exhibit a concurrent "calling" behavior, the most obvious sign of which is raising their wings to an almost vertical position.

In the U.S.A. *H. cunea* is not considered to be a pest of economic importance. In the U.S.S.R. *H. cunea* is an important pest of mulberry, which is used to feed the larvae of the domesticated silkmoth, *Bombyx mori*, which in turn produces silk for cloth. For some time scientists in the U.S.S.R. have been interested in devising methods for controlling pests of mulberry that are

[5] Private communication. Dr. Takeda is at the University of Delaware, Newark, Delaware 19711.

an improvement on the methods presently used and which might be more compatible with its eventual use as a larval food. In 1968, at Kishinev, Republic of Moldavia, U.S.S.R., studies were initiated to identify the sex pheromone system of *H. cunea* with the eventual goal of using the sex pheromone in a control program. In late 1976 this became a cooperative U.S.A.–U.S.S.R. project as part of the scientific exchange program between the two countries.

METHODS AND MATERIALS

U.S.S.R. Female tip extracts are of U.S.S.R. *H. cunea* ("black-headed" race), unless stated otherwise. In the U.S.S.R., last instar *H. cunea* larvae were collected in the Kishinev area during June and July and were fed maple leaves until they formed pupae. Pupae were kept outdoors in cages until they emerged later in the summer. Normally there are two generations of *H. cunea* in the Kishinev area. For laboratory rearing, the adults were placed in glass or plastic jars, where they mated and the females laid eggs on folded filter paper placed in the jars. The larvae were fed an artificial diet (Starets, 1973). Under these conditions, three successive generations could be raised, after which the fourth generation pupae lapsed into diapause and had to be kept at ca. −5° C for four months to complete diapause. Adults started emerging about 15 days after the pupae were placed in a chamber at ca. 20° C.

Female moths were collected after emergence, and their abdominal tips were extruded and excised sometime during the first day after collection. The tips were left in ethyl acetate–benzene (1 : 1) for about one week in a freezer, after which the tips and other solids were removed by filtration. The extract was stored in a freezer until used or was sealed in glass under nitrogen and sent to the U.S.A., where it was stored in a freezer until it was used.

The traps used for field tests were flat pieces of gray cardboard (ca. 20 × 25 cm) with a ca. 5-cm-diameter hole in the middle portion and were coated on both sides with partially polymerized butenes. A piece of filter paper (folded accordion-style and ca. 5 × 5 cm) impregnated with an *H. cunea* tip extract of 8–14 female equivalents (FE) was suspended in the opening by a piece of wire. The traps were hung on tree branches at a height of ca. 2 m.

The route used to synthesize a racemic sample of III is outlined in Figure 1 and will be described in detail elsewhere.

U.S.A. H. cunea larvae were collected in the Geneva, New York, area in August and early September of 1978 and 1979 from a variety of trees, although by far the largest number of webs was found on walnut trees. In the Geneva, New York, area there is usually only one generation of *H. cunea* each year. The larvae were fed mostly apple and walnut leaves. Pupae were removed from their cocoon webbing and segregated by sex. Approximately

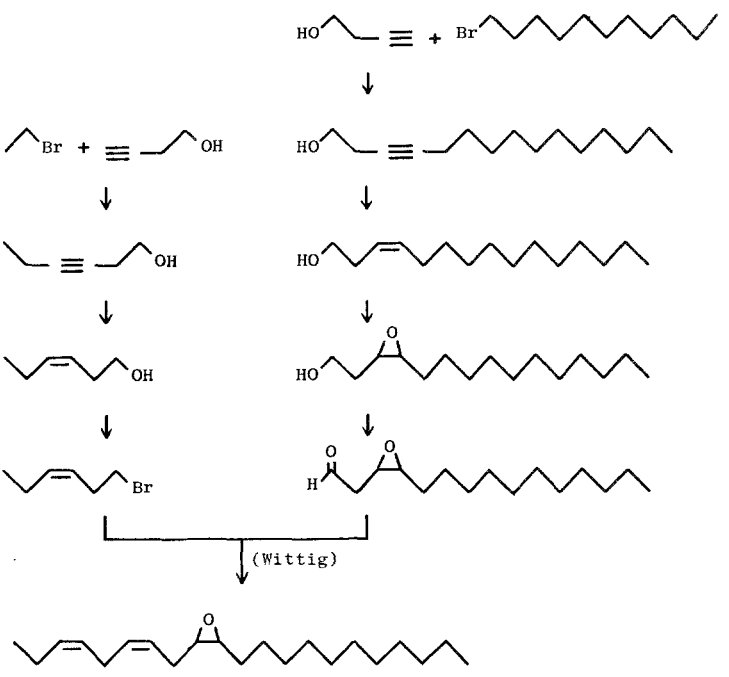


FIG. 1. General outline of the synthetic route used by Dr. Kovalev in synthesizing (*Z,Z*)-3,6-*cis*-9,10-epoxyheneicosadiene.

half the pupae were kept at 30° C. The other half were kept at 5–10° for 3.5 months, after which they were placed in the 30° chamber. Unrefrigerated pupae started to eclose about one month after pupation and continued emerging for 4 months after that. The refrigerated pupae started to eclose after about 1 month at 30°.

At 1–3 days after eclosion, ovipositors were protruded, excised, and kept in methylene chloride in a freezer until used. Airborne effluvia from female moths 2–3 days old were trapped on Porapak Q. Air was pulled through a glass tube (8 cm ID × 40 cm) containing 20 female moths and then through a Porapak Q column (1.8 cm ID × 13 cm) with a model 3 Dyna pump at a rate of ca. 1–2 liters/min for 8 hr of scotophase. The Porapak was rinsed several times with Skelly B and the solvent then evaporated under reduced pressure. GLC analyses of the trapped volatiles were carried out with the OV-101 column. Males were held 1–5 days at ca. 20° C, 16:8 light–dark cycle. Bioassays were carried out in a sustained flight tunnel (Miller and Roelofs, 1978) at 7–8 hr into the moth's scotophase and at ca. 3 lux (white light).

Electroantennograms (EAGs) were carried out as described by Roelofs (1977, and references therein). Methylene chloride, carbon disulfide and Skellysolve B (petroleum ether 60–68°) (Skelly B) were purified by distillation.

The analytical instruments used, the chemical transformations carried out, the sources of chemicals, and the preparation of authentic reference compounds are the same as described by Hill and Roelofs (1981). In addition, *n*-nonanal and *n*-dodecanal were purchased from Aldrich Chemical Company. One additional microreaction was carried out. Cleavage of an epoxide group into the two aldehydes was accomplished by treatment of the epoxide with periodic acid (HIO_4) in methylene chloride at room temperature for ca. 10 min. The products could be analyzed directly by GC (Bierl et al., 1971).

RESULTS

When a crude *H. cunea* female tip extract was collected from OV-101, 210°, in fractions timed to coincide with the visible GC peaks, two of the fractions collected (3–4 min and 7–9 min) were found to be EAG active (1.5 mV and 3.0 mV, respectively, compared to 0.5 mV for other fractions). Each of these active fractions was recovered and collected from Hi-Eff, 210°, in the same manner as from the OV-101 column. The earlier of the two fractions contained two EAG-active materials and the later fraction contained one (Figure 2). The three materials were designated I, II, and III, in their order of elution from the Hi-Eff column (Figure 2). The compounds were found in a 5:6:13 ratio, respectively, with female tip extracts from the U.S.S.R., in a

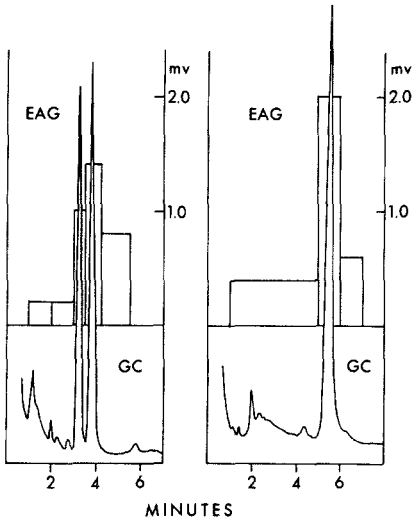


FIG. 2. *H. cunea* female tip extract collected from OV-101 in two EAG-active fractions and each fraction reinjected on Hi-Eff at 210° C to give two EAG-active components between 3 and 4 min (compounds I and II) from the first fraction and one EAG-active component between 5 and 6 min (compound III) from the second fraction.

1:8:21 ratio with extracts from U.S. insects, and in a 1:6:27 ratio with female effluvia trapped from U.S. insects. The glands contained approximately 1 μg of the major component (III).

Identification of I as (Z,Z)-*9,12-Octadecadienal* (*Linolealdehyde or Linoleyl Aldehyde, CAS Registry No. 2541-61-9*). Samples of I were purified by collection from an OV-101 (or OV-1) GC column followed by collection from a Hi-Eff (or XF-1150) GC column.

A Fourier transform–infrared (FT-IR) spectrum of I (Figure 3) showed absorptions at ca. 2710 cm^{-1}, and 1725 cm^{-1}, which are characteristic absorptions for aldehydes, and at ca. 3010 cm^{-1}, which is characteristic for

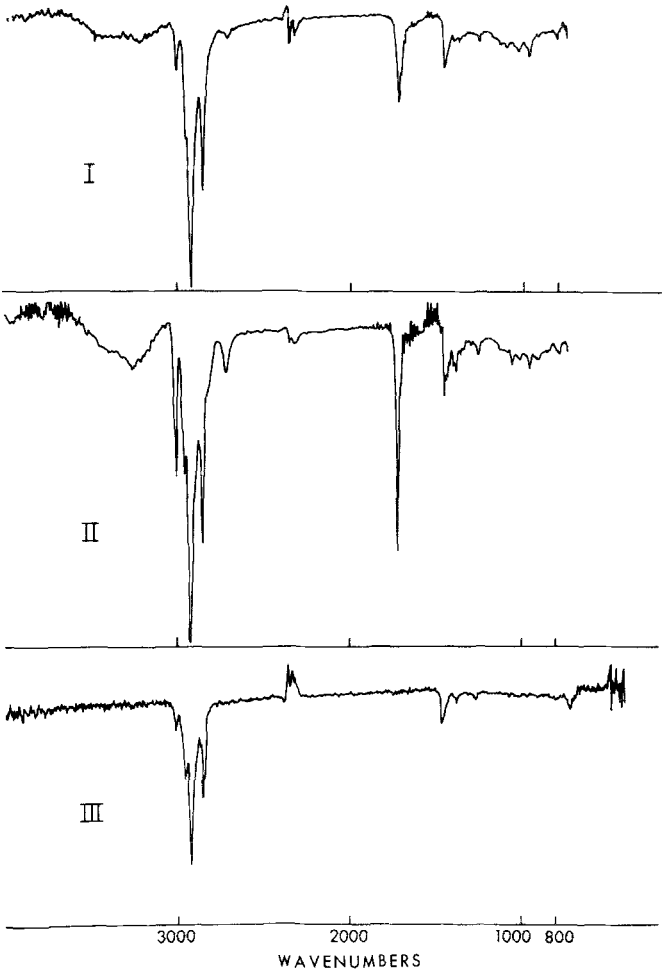


FIG. 3. FT-IR spectra of compounds I, II, and III (1–10 μg each).

alkenes. Comparison with the FT-IR spectrum obtained for (*E*)-9-docosene indicated that the small peak at 967 cm^{-1} in the spectrum of I is considerably weaker than that expected for an *E* double bond. This, as well as the relatively intense absorption at ca. 3010 cm^{-1}, which is usual for *Z* but not for *E* isolated double bonds, led us to conclude that I has only *Z* double bonds. This, however, does not exclude the possibility that small amounts of an *E* isomer may be present.

Catalytic hydrogenation of I produced *n*-octadecanal, as determined from comparison of its GC retention times on OV-101, 100°, and Hi-Eff, 200° (7.0 and 5.9 min, respectively), with those for *n*-octadecanal under the same conditions. Chemical ionization–mass spectra (CI-MS) (isobutane) of hydrogenated I and authentic *n*-octadecanal were the same, and had ions at *m/e* 269 (MH^+), 325 (MH + 56), 326 (MH + 57), and 000 (M − 18).

A CI-MS (isobutane) of I had ions at *m/e* 265 (MH^+), 263 (M − 1) and 264 (M^+) as well as at *m/e* 321 (M + 57) and 247 (MH − 18). This was virtually the same CI-MS (isobutane) as obtained for authentic linolealdehyde. The addition of four protons with hydrogenation established that I has two double bonds.

Treatment of I with lithium aluminum hydride (LAH), followed by reaction with acetyl chloride (AcCl), produced a compound having the same retention time on OV-101, 210°, and Hi-Eff, 210° (6.65 and 4.7 min, respectively) as linoleyl acetate (6.65 and 4.75 min, respectively). The acetate thus obtained from I was collected from OV-101 and ozonized. The ozonolysis products identified were *n*-hexanal and 9-acetoxynonanal. *n*-Hexanal was identified by its retention times on OV-101, 45° (3.35 min) and XF-1150, 60° (3.7 min), which were comparable to those seen for authentic *n*-hexanal (3.35 and 3.65 min, respectively). Authentic 9-acetoxynonanal was prepared by ozonolysis of (*Z*)-9-tetradecen-1-yl acetate, and had the same retention times on OV-101, 150°, and Hi-Eff, 170° (4.0 and 4.55 min, respectively) as those obtained from the ozonized acetate of I (4.0 and 4.65 min, respectively). In addition, the CI-MS (isobutane) of the two acetoxy aldehyde samples were identical: each exhibited only one major ion at *m/e* 201 (MH^+), with small ions at *m/e* 200 (M^+), 202 (M + 2), 239 (M + 39), 123 (M − 77), and 141 (M − 59).

The GC retention times of I on OV-101, 195°, Hi-Eff, 211°, and XF-1150, 195° (9.15, 3.8, and 4.95 min, respectively) were identical to those for authentic linolealdehyde.

Identification of II as (Z,Z,Z,)-*9,12,15-Octadecatrienal* (*Linolenaldehyde or Linolenyl Aldehyde, CAS Registry No. 2423-13-4*). This component was purified by sequential collections from two GC columns: OV-101 (or OV-1) followed by Hi-Eff (or XF-1150).

An FT-IR spectrum of II (Figure 3) showed absorption peaks at 2716 cm^{-1} and 1731 cm^{-1}, which established that an aldehyde group is present in II,

and at 3012 cm^{-1}, which is a characteristic absorption for double bonds. As in the case of I, a small peak at 967 cm^{-1} was present, but it was not judged to be due to an *E* double bond in the major component of II because of the relative intensities of the peaks at 3012 cm^{-1} and at 967 cm^{-1}, which are usually the reverse of those seen for II when the double bond configuration is *E*.

Catalytic hydrogenation of II produced *n*-octadecanal, which was identified by its GC retention times on OV-101, 200°, and on Hi-Eff, 200° (7.0 and 5.9 min, respectively), and by its CI-MS (isobutane). These data were coincident for those obtained with an authentic sample of *n*-octadecanal, and which are described in the previous section.

A CI-MS (isobutane) of II had ions at *m/e* 263 (MH^+) and 261 (M − 1), as well as at 319 (M + 57) and 245 (MH − 18). This spectrum was the same, for all practical purposes, as the CI-MS (isobutane) obtained for authentic linolenaldehyde. Comparison of the MS of II with that of hydrogenated II established that II has three double bonds.

A sample of II was treated with LAH to produce the alcohol, which was treated with AcCl to produce the corresponding acetate. The GC retentions of the alcohol and acetate produced from II coincided with those of authentic linolenyl alcohol and linolenyl acetate on OV-101, 210° (4.55 min for both alcohol samples and 6.65 for both acetate samples) and Hi-Eff, 210° (6.0 min both alcohols and 5.6 min for both acetates). The OV-101-collected acetate of II was ozonized to produce 9-acetoxynonanal, as established by comparison of its GC retention times with those of the authentic acetoxy aldehyde produced by ozonolysis of (*Z*,)-9-tetradecen-1-yl acetate, on OV-101, 150° (3.95 vs. 4.0 min, respectively), and on Hi-Eff, 170° (4.45 vs. 4.55 min, respectively). The CI-MS (isobutane) of the acetoxy aldehyde from II and from (*Z*,)-9-tetradecen-1-yl acetate were identical and have been described in the previous section.

Ozonolysis of II produced propanal, which was identified by its GC retention times on Tenax, 110°C (2.4 compared to 2.55 min for *n*-propanal produced by ozonolysis of linolenal). These data established that two of the three double bonds are at positions 9 and 15 on the 18-carbon straight-chain aldehyde.

The GC retention times of II and of authentic linolenaldehyde on OV-101, 195° (9.25 min in both cases), on Hi-Eff, 210° (3.5 vs. 3.45 min, respectively), and on XF-1150, 195° (6.05 vs. 6.1 min, respectively), were essentially identical.

Identification of III as (Z,Z)-*3,6*-cis-*9,10-Epoxyheneicosadiene*. Samples of III were purified by collection from an OV-101 (or OV-1) GC column followed by collection from a Hi-Eff column. In some cases the crude *H. cunea* extract was first chromatographed on Florisil, and the active fraction (determined by GC-EAG analyses of the fractions and found to elute with 5–10% diethyl ether in Skelly B) was then further purified by collection from

the GC columns. The GC retention times of III on OV-101, Hi-Eff, and XF-1150 were identical to those obtained for the epoxide produced by *E. acrea* and had the following equivalent carbon numbers (compared to *n*-hydrocarbons; to obtain approximate Kovats index, multiply by 100): 22.25 on OV-101, 190°, and 25.40 on Hi-Eff, 190°. At the time that identification of III was initiated, one of the sex pheromone components from *E. acrea* was known to be a *cis*-9,10-epoxyheneicosadiene, with the two double bonds postulated to be at the 3 and 6 positions (Hill and Roelofs, 1981). In addition, III was shown to produce as good an EAG response on a male *E. acrea* antenna as the epoxide from *E. acrea* females, and both materials had identical GC retention times on OV-101 and Hi-Eff columns. Consequently, the identification procedure for III was directed at establishing identity or nonidentity with the *E. acrea* epoxide, and the procedures outlined below often included direct comparisons of the two materials.

An FT-IR of III (Figure 3) showed absorption at ca. 3010 cm^{-1} attributable to vinyl hydrogen stretching. No peak was seen at ca. 970 cm^{-1}, which indicated that the double bonds present are of the *Z* configuration. No absorptions for other functional groups were seen. This spectrum was very similar to that obtained for the *E. acrea* epoxide.

A CI-MS (methane) of III produced a large ion at *m/e* 307 (MH^+) and the next most prominent ion was *m/e* 289 (MH − 18). This indicated the same molecular weight for III as was found for the *E. acrea* epoxide, i.e., 306.

Catalytic hydrogenation of III produced a material with the same GC retention times as *cis*-9,10-epoxyheneicosane on a UCON capillary column (temperature programed; 17.4 min for *cis*, 16.5 min for *trans*, with hydrogenated III cochromatographing with the *cis* isomer) and on Hi-Eff, 170° (25.2 min for both, compared to 24.0 min for the *trans* isomer). A CI-MS (methane) of hydrogenated III had a base ion at *m/e* 311 (MH^+) and an ion at 293 (MH − 18). This was similar to the CI-MS (methane or isobutane) of authentic *cis*-9,10-epoxyheneicosane.

The epoxide group was found to be located at the 9,10 position by two methods. The first was the same as that used for the *E. acrea* epoxide and involved the following sequence of reactions: (1) catalytic hydrogenation to produce the saturated epoxide, (2) treatment with dilute sulfuric acid in aqueous tetrahydrofuran to produce the diol, and (3) trimethylsilation of the diol with hexamethyldisilazane, chlorotrimethylsilane, and pyridine. The electron impact mass spectrum (EI-MS) of this trimethylsilated diol showed a base ion at *m/e* 73 due to $(CH_3)_3Si^+$ (TMS) and only two other prominent ions, at *m/e* 215 and 257, which are the $C_9H_{18}O(TMS)^+$ and the $C_{12}H_{24}O(TMS)^+$ ions, respectively (Capella and Zorzut, 1968). The second method involved only two reactions: catalytic hydrogenation followed by oxidative cleavage of the epoxide group with periodic acid in methylene chloride to produce the two aldehydes *n*-nonanal and *n*-dodecanal, which

were identified by comparison of their GC retention times with those of authentic samples of the aldehydes on OV-1, temperature programed from 35° to 190° at the rate of 15°/min (8.75 and 11.95 min in both cases, respectively).

The foregoing data are all in agreement with the structure reported for the *E. acrea* epoxide. That is, III is a 21-carbon, straight-chain compound with a *cis*-epoxide group at the 9,10 position and two double bonds with *Z* configurations. These two double bonds were found to be at the same positions on the chain as in the *E. acrea* epoxide, i.e., 3 and 6, by two separate ozonolysis procedures. Ozonolysis of III produced *n*-propanal, which had identical GC retention times on Tenax, 100° (5.35 min) and TCEPE, 40° (2.2 min) as those found under the same conditions for authentic propanal. That the other double bond is at position 6 was established by ozonolysis of III after reductive removal of the epoxide group by the following sequence of reactions: (1) treatment with LAH to produce a mixture of the 9 and 10 position alcohols, (2) conversion of these alcohols to the two corresponding bromides by treatment with triphenylphosphine dibromide in methylene chloride, (3) reductive removal of the bromide with LAH to produce the diunsaturated hydrocarbon, and (4) ozonolysis. The ozonolysis product, *n*-pentadecanal, was identified by comparison of its GC retention times with those of authentic *n*-pentadecanal on OV-1, 160° (9.0 vs. 8.95 min, respectively) and on Hi-Eff, 160° (6.95 min for both).

In addition, a UV spectrum of III in cyclohexane failed to reveal any absorption peaks in the region 220–240 nm, which would have indicated the presence of conjugated double bonds.

An FT-NMR (60 MHz) of III showed essentially the same features as described for the epoxide from the *E. acrea* (Hill and Roelofs, 1981).

EAG Responses of Male H. cunea *Antennae to* (+)-, (−)- *and* (±)-*Disparlure.* The sex pheromone (Bierl et al., 1970) of the gypsy moth, *Lymantria dispar* (L.), also is a long-chain hydrocarbon with an epoxide functionality near the center of the chain. The pheromone, 2-methyl-*cis*-7,8-epoxyoctadecane (named disparlure) possesses an asymmetric center and thus can exist as (−)- and (+)-disparlure. Studies with the gypsy moth have shown that (+)-disparlure is the active isomer (Miller et al., 1977; Carde et al., 1977). Since there is a similarity in structures between disparlure and compound III, samples of (+)-, (−)-, and racemic disparlure (100 μg each) were tested for EAG responses with *H. cunea* male antenna. The highest EAG responses obtained with male *H. cunea* antennae were with the (−) isomer (1.4 mV). The (−) isomer consistently elicited 3–7 times the response produced by the (+) isomer. The racemic sample elicited responses somewhat lower (ca. 1 mV), but comparable to those obtained with the (−) isomer. Component III (at a much lower, undetermined concentration) produced EAG responses ca. 2–3 times

TABLE 1. RESPONSES OF MALE *H. cunea* MOTHS IN A FLIGHT TUNNEL TO I, II, III, AND COMBINATIONS THEREOF

Sample[a]	No. of observations	No. of males responding (% of total)[b] Fanning	Flying to source
H. cunea, ♀ tip extract	9	6 (67) abc	3 (33) bc
E. acrea, ♀ tip extract	12	9 (75) abc	5 (42) bc
III (♀) + I (♀) + II (♀)	5	5 (100) a	5 (100) a
I + II	9	3 (33) bc	0 (0) d
III (♀)	33	10 (30) c	0 (0) d
III (♀) + I	13	10 (77) abc	8 (62) bc
III (♀) + II	9	9 (100) a	9 (100) a
III (♀) + I + II	3	3 (100) a	3 (100) a

[a]Samples are synthetic unless indicated to be from ♀♀. Samples from moths were purified by GC collection (ca. 10 FE). Samples were placed on filter paper at the rate of 5 μg each for I and II and 10 μg for racemic III.

[b]Numbers followed by the same letter in each column are not significantly different ($P < 0.05$) (Ryan, 1960).

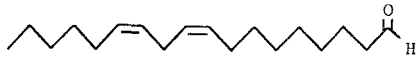
higher than the (−) isomer, but antennal recovery to baseline ("stickiness" of the sample) appeared to about equal that of (−)-disparlure.

Flight Responses of Male H. cunea *("Red-Headed" Race) to I, II, and III in a Sustained Flight Tunnel in the U.S.A. H. cunea* males flew equally well to sources of *H. cunea* and *E. acrea* tip extract (Table 1). The response was better to combinations of the isolated components or synthetics that included compound III with either I or II. There was no flight to the source of either III alone or to a combination of I and II.

Field Trapping of Male H. cunea *in the U.S.S.R. Using Female* H. cunea *Extracts. H. cunea* female extracts were used as bait in traps at five different concentrations (5, 10, 15, 20, and 25 FE of extract) for seven consecutive nights in May 1973 (unreplicated). The average catches of *H. cunea* were 5.7, 10.7, 17.0, 28.0, and 36.9 males per trap per night, respectively. These results show that all components required for trap catch are present in the female-tip extracts.

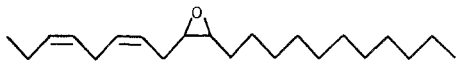
DISCUSSION

Compounds I, II, and III (Figure 4) have been identified as components of the sex pheromone system of *H. cunea* as well as of *E. acrea* (Hill and Roelofs, 1981). Compound III was not previously known. Compounds I and II, although known previously (CAS registry numbers 2541-61-9 and 2423-13-



I. (Z,Z)-9,12-octadecadienal

II. (Z,Z,Z)-9,12,15-octadecatrienal



III. (Z,Z)-3,6-cis-9,10-epoxyheneicosadiene

FIG. 4. Structures of I, II, and III.

4, respectively) had not been discovered until now to be sex pheromone components.

Compounds I and II are the aldehyde analogs of linoleic and linolenic acids, respectively. These acids, although widely distributed in nature, are essentially dietary components for most animals, including insects. Thus, they are biosynthesized almost exclusively by plants and not by animals. Should *H. cunea* and *E. acrea* be incapable of biosynthesizing linoleic and linolenic acids, as has been the case for those insects studied to date (Downer, 1978), then the question arises, are I and II synthesized de novo by these insects or are they modified dietary constituents? The same question would arise for III, since its structural relationship to II suggests strongly that it could be biosynthesized from II by addition of three carbons at the aldehyde end of the molecule.

As in the case of *E. acrea*, the *H. cunea* antennal acceptor sites for III appear to have chiral character and are able to discriminate between (+)- and (−)-disparlure. With an antenna of either species, a stronger EAG signal is registered with the (−) isomer than with the (+) isomer. The same correlations made for *E. acrea* (Hill and Roelofs, 1981) can be made for *H. cunea*, and these lead to the prediction that III should be predominantly, or exclusively, the 9*S*,10*R* isomer. EAG analyses with the two enantiomers of III (10 μg/cartridge, supplied by K. Mori) showed that the 9*S*,10*R* isomer elicits a male antennal response 2–3 times greater than the 9*R*,10*S* with *H. cunea* and with *E. acrea*.

The extracts from U.S.S.R. *H. cunea* females ("black-headed" race) are very similar to those from U.S.A. *H. cunea* females ("red-headed" race) with respect to the relative proportions of I, II, and III. This appears to indicate little or no pheromonal divergence of either population from their common ancestral North American population. However, more information con-

cerning the two populations will be obtained when the three chemicals are tested for attractancy in field trapping tests with the two populations. Such tests are in progress.

In a prior report (Lee et al., 1978), the sex pheromone of *H. cunea* was identified as a mixture of tetradecane and tetradecan-1-ol. These identifications were not supported by any chemical, biological, or behavioral tests and appear to be incorrect.

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COMPOUNDS MODIFYING THE ACTIVITY OF TWO SEX ATTRACTANTS FOR MALES OF THE PEA MOTH, *Cydia nigricana* (F.)

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Abstract—(*E*)-10-Dodecen-1-yl acetate (*E*10–12:Ac) and (*E,E*)-8,10-dodecadien-1-yl acetate (*E,E*8,10–12:Ac) are sex attractants for males of the pea moth, *Cydia nigricana* (F.). Thirty-two structurally related compounds with chain lengths of 9–14 carbon atoms were exposed with *E*10–12:Ac or *E,E*8,10–12:Ac in traps in the field to investigate their influence on the activity of the attractants. Only alchols and acetates unsaturated at C-8, -9, or -10 greatly influenced moth captures. (*Z*) and (*E*)-8-dodecen-1-ol were weak synergists for *E*10–12:Ac but no synergists for *E,E*8,10–12:Ac were found. (*Z*) and (*E*)-8-dodecen-1-yl acetate and (*E,E*)-8,10-dodecadien-1-ol inhibited both *E*10–12:Ac and *E,E*8,10–12:Ac while (*E*)-10-dodecen-1-ol, 10-dodecyn-1-ol, (*Z*) and (*E*)-9-dodecen-1-yl acetate, (*Z*)-10-dodecen-1-yl acetate, and undecyl acetate inhibited only the former attractant.

Key Words—Pea moth, *Cydia nigricana* (F.), Lepidoptera, Olethreutidae, sex attractant, (*E*)-10-dodecen-1-yl acetate, (*E,E*)-8,10-dodecadien-1-yl acetate, inhibitor, synergist, lure.

INTRODUCTION

Males of the pea moth, *Cydia nigricana* (F.) (Lepidoptera: Tortricidae), are attracted by (*E,E*)-8,10-dodecadien-1-ol (*E,E*8,10–12:OH) (Lewis et al., 1975), (*E*)-10-dodecen-1-yl acetate (*E*10–12:Ac) and (*E,E*)-8,10-dodecadien-1-yl acetate (*E,E*8,10–12:Ac) (Wall et al., 1976), *E,E*8,10–12:OH is the sex pheromone of the closely related codling moth, *Cydia pomonella* (L.) (Roelofs et al., 1971; Beroza et al., 1974), and is a weak attractant for pea moth (Wall et al., 1976). The related acetate, *E,E*8,10–12:Ac, has been identified as

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the pea moth female sex pheromone and is the most potent attractant when fresh, but it is rapidly degraded to an inhibitor (Greenway and Wall, 1980, 1981). *E*10–12:Ac, while not as attractive as *E,E*8,10–12:Ac, can be formulated as lures which are stable for several months in the field and suitable for practical monitoring of pea moth populations (Wall and Greenway, 1981). In field tests, Wall et al. (1976) showed that simultaneous evaporation of certain related compounds could either synergize or inhibit the attractiveness of *E,E*8,10–12:OH for pea moth, so it was of interest to investigate the effects of such compounds on the activity of the two more potent attractants. Such phenomena are well known in other moth species, for example in *Argyrotaenia velutinana* (Walker) (Roelofs and Comeau, 1971), *Adoxophyes orana* (Fischer von Roslerstamm) (Voerman et al., 1974), and *Ostrinia nubilalis* (Hubner) (Klun and Robinson, 1972) and, in *A. orana*, pheromone communication between the sexes could be disrupted by inhibitors (Minks et al., 1976). Synergists might be valuable for mass trapping or in population monitoring, especially to increase trap catches in sparse populations and to enhance the competitiveness of lures in dense populations.

The structure of behaviorally active compounds, whether attractants, synergists, or inhibitors, is significant in the wider context of the mechanisms underlying sex-pheromone perception in insects. They provide the opportunity to investigate these mechanisms at both the electrophysiological and behavioral levels. In fact, *E,E*8,10–12:OH can act both as an attractant, when on its own, and as an inhibitor of both *E*10–12:Ac and *E,E*8,10–12:Ac (Wall et al., 1976), and an explanation for this effect would be of considerable interest.

It is also useful to know whether any compounds which might occur as impurities in the synthetic attractants modify their activity so that appropriate synthetic routes can be chosen and specific impurities removed if necessary.

METHODS AND MATERIALS

Chemical Formulation. Attractant lures were prepared by applying *E*10–12:Ac (10^3 μg) or *E,E*8,10–12:Ac (10^2 μg) in redistilled dichloromethane (10^2 μl) to rubber serum stoppers (Greenway and Wall, 1981). Test chemicals (commercial or synthesized here) were purified to at least 98% and formulated in the same way in separate rubber stoppers (for amounts see Tables 1 and 2). The solvent was allowed to evaporate while the attractant or test chemical was absorbed, and the dosed stoppers were then stored in screw-cap glass bottles in the dark at −15° less than 14 days before use.

Field Tests. Experiments were done on farms in east and southeast England, in pea fields or in wheat fields after peas. Triangular aluminum traps (Lewis and Macaulay, 1976) with removable inserts coated with Tangletrap

(The Tanglefoot Company, Grand Rapids, Michigan) were placed on stands at crop height in wheat or on the ground in peas. Each trap contained an attractant lure, either alone or with a test chemical; the rubber stoppers were suspended within the traps. In the earlier experiments (Table 1), test chemicals (up to 9 per experiment) at a 1:1 ratio with either *E*10–12:Ac or *E,E*8,10–12:Ac were evaluated for their effects upon the activity of the attractants. Chemicals which showed a large modifying effect were tested further at lower doses with ratios of 1:10 and 1:100 to the appropriate attractant (Table 2). Traps were examined regularly, usually each morning before the flight period; captured moths were counted and removed, and the sticky inserts replaced if necessary.

In the earlier experiments (Table 1), traps were set out in rows (blocks) and treatments assigned to traps randomly within each block; the distance between traps within blocks was never less than 20 m. Treatments involving attractants alone were replicated thrice within each block. At least three blocks were used, either simultaneously at different positions in the same field or sequentially at the same position. In later experiments (Table 2), Latin-square designs (Perry et al., 1980) were used after interactions between attractant traps for pea moth had been shown to be significant (Wall and Perry 1978). This involved allocating each of the t treatments to one of t sites, usually at least 150 m apart within a locality. After each trapping period (usually one or two days), the treatments were reallocated to sites so that after t trapping periods each treatment had occupied each site once; standard randomization procedures were used.

Transformation of the data to natural logarithms aided additivity and equalized error variance. An analysis of variance of the transformed counts was done for each experiment. Since the effects of individual chemicals were usually tested in experiments with several other compounds, some values within the same column of Tables 1 and 2 are not statistically independent.

RESULTS AND DISCUSSION

The results of experiments in which all of the test chemicals were evaluated at a 1:1 ratio with either *E*10–12:Ac or *E,E*8,10–12:Ac were expressed as the mean logarithmically transformed catch per trap for "attractant + test chemical" minus the corresponding value for the attractant alone (Table 1). Compounds were evaluated either because they showed substantial EAG activity (Wall et al., 1976), were structurally related to active compounds, or were potential impurities in the synthetic attractants. Some of the chemicals (Nos. 4, 7–11, 13, 22–26, and 28) had already been tested in traps on their own without any evidence of attractive properties (Wall et al., 1976), and of those tested alone in these experiments (12, 16, 17, 19–21, 27, 30–32) only (*E,E*)-8, 10-dodecadienal (32), attracted moths.

TABLE 1. ATTRACTANCY OF MALE *C. nigricana* TO TRAPS CONTAINING *E*10-12:Ac OR *E,E*8,10-12:Ac AND AN EQUAL AMOUNT OF TEST CHEMICAL

Test Chemical			Attractancy[a] with	
No.	Name	Abbreviation	*E*10-12:Ac	*E,E*8,10-12:Ac
1	Nonanol	9:OH	−0.596	
2	Decanol	10:OH	−0.447	
3	Undecanol	11:OH	−0.078	
4	Dodecanol	12:OH	0.205	−0.097
5	Tridecanol	13:OH	−0.002	
6	Tetradecanol	14:OH	−0.276	
7	10-Undecen-1-ol	10-11:OH	−0.104	
8	(*Z*)-8-Dodecen-1-ol	*Z*8-12:OH	*0.401*	−0.078
9	(*E*)-8-Dodecen-1-ol	*E*8-12:OH	*0.562*	−0.288
10	(*Z*)-9-Dodecen-1-ol	*Z*9-12:OH	−0.071	0.389
11	(*E*)-9-Dodecen-1-ol	*E*9-12:OH	0.120	−0.553
12	(*Z*)-10-Dodecen-1-ol	*Z*10-12:OH	0.350	0.371
13	(*E*)-10-Dodecen-1-ol	*E*10-12:OH	*−1.073*	0.626
14	10-Dodecyn-1-ol	10YNE-12:OH	*−0.776*	0.023
15	(*E,E*)-8,10-Dodecadien-1-ol	*E,E*8,10-12:OH	*−2.604*	*−3.032*
16	Nonyl acetate	9:Ac	0.336	0.364
17	Decyl acetate	10:Ac	−0.090	0.357
18	Undecyl acetate	11:Ac	*−0.716*	0.350
19	Dodecyl acetate	12:Ac	−0.401	0.385
20	Tridecyl acetate	13:Ac	−0.352	0.062
21	Tetradecyl acetate	14:Ac	−0.286	0.352
22	10-Undecen-1-yl acetate	10-11:Ac	0.180	
23	(*Z*)-8-Dodecen-1-yl acetate	*Z*8-12:Ac	*−3.376*	*−2.508*
24	(*E*)-8-Dodecen-1-yl acetate	*E*8-12:Ac	*−1.667*	*−2.874*
25	(*Z*)-9-Dodecen-1-yl acetate	*Z*9-12:Ac	*−2.683*	−0.594

26	(*E*)-9-Dodecen-1-yl acetate	*E*9–12 : Ac	*−0.631*	−0.279
27	(*Z*)-10-Dodecen-1-yl acetate	*Z*10–12 : Ac	*−4.621*	−0.175
28	11-Dodecen-1-yl acetate	11–12 : Ac	0.106	0.065
29	10-Dodecyn-1-yl acetate	10YNE–12 : Ac	−0.543	0.230
30	(*Z*)-10-Dodecenal	*Z*10–12 : ALD	0.048	0.044
31	(*E*)-10-Dodecenal	*E*10–12 : ALD	−0.136	−0.249
32	(*E*,*E*)-8,10-Dodecadienal	*E*,*E*8,10–12 : ALD	−0.419	0.099

[a]Results are expressed as the mean $\log_e$ (catch per trap) for "attractant + test chemical" minus the corresponding value for the attractant. To convert such a difference, d, to a percentage increase in catch, p, due to test chemical, use: $p = (e^d - 1) \times 100$ (a negative value of p indicates a percentage decrease in catch). Figures in italics indicate treatments (attractant + test chemical) whose catch was found to be significantly different at the 5% level from that of *E*10–12 : Ac (or *E*,*E*8,10–12 : Ac) after an analysis of variance. This implies that the corresponding chemicals are synergists (positive values) or inhibitors (negative values).

TABLE 2. MEAN LOG CATCHES OF MALE *C. nigricana* IN TRAPS CONTAINING *E*10–12:Ac OR *E*,*E*8,10–12:Ac AND DIFFERENT AMOUNTS OF TEST CHEMICAL[a]

Test chemical			Attractant							
			*E*10–12:Ac(10^3 μg)				*E*,*E*8,10–12:Ac(10^2 μg)			
			Mean log catch				Mean log catch			
No.	Abbreviation	Dose (μg)	Attractant + test chemical	Attractant alone	SED	*df*	Attractant + test chemical	Attractant alone	SED	*df*
1	*Z*8–12:OH	10^2					3.258	3.336	0.414	24
		10^3	*4.333*	*3.933*	0.141	24				
2	*E*8–12:OH	10^2					3.049	3.336	0.414	24
		10^3	*3.937*	*3.376*	0.216	24				
13	*E*10–12:OH	10^2					3.963	3.336	0.414	24
		10^3	*2.303*	*3.376*	0.216	24				
14	10YNE–12:OH	10^2					2.577	2.554	0.458	24
		10^3	*2.809*	*3.585*	0.268	24				
15	*E*,*E*8,10–12:OH	1					*3.429*	*3.857*	0.097	6
		10	1.250	3.150	0.572	2	*3.910*	*5.655*	0.167	6
		10^2	*0*	*2.121*	0.348	12	*0*	*3.033*	0.552	8
		10^3	*1.025*	*3.629*	0.281	12				
18	11:Ac	10^2					2.904	3.336	0.414	24
		10^3	*2.869*	*3.585*	0.268	24				
23	*Z*8–12:Ac	1					3.850	3.857	0.097	6
		10	1.879	2.121	0.348	12	*4.741*	*5.655*	0.167	6
		10^2	*1.930*	*3.716*	0.351	12	*0.829*	*3.336*	0.414	24
		10^3	*0*	*3.376*	0.216	24				

24	*E*8-12:Ac	1					3.792	3.857	0.097	6
		10	1.405	2.121	0.348	12	*4.094*	*5.655*	0.167	6
		10^2	*1.996*	*3.716*	0.351	12	*0.463*	*3.336*	0.414	24
		10^3	*1.709*	*3.376*	0.216	24				
25	*Z*9-12:Ac	10	*0.679*	*2.121*	0.348	12				
		10^2	*2.653*	*3.716*	0.351	12	2.742	3.336	0.414	24
		10^3	*0.693*	*3.376*	0.216	24				
26	*E*9-12:Ac	10^2					3.058	3.336	0.414	24
		10^3	*2.745*	*3.376*	0.216	24				
27	*Z*10-12:Ac	10^2	3.339	3.716	0.351	12	2.443	2.618	0.337	20
		10^3	*0.230*	*4.852*	0.245	6				

[a]Results are expressed as mean $\log_e$ (catch per trap). To convert the difference, d, between result for (attractant + test chemical) minus result for attractant alone, to a percentage increase in catch, p, due to test chemical, use: $p = (e^d - 1) \times 100$ (a negative value of p indicates a percentage decrease in catch). Figures in italics indicate combinations (test chemical + attractant) whose catch was found to be significantly different at the 5% level from that of *E*10-12:Ac (or *E*,*E*8,10-12:Ac) after an analysis of variance. This implies that the corresponding chemicals are synergists (positive differences) or inhibitors (negative differences). SED = standard error of difference between means; df = error degrees of freedom.

Table 2 shows the effects of various proportions (1 : 1, 1 : 10, or 1 : 100) of each of the test chemicals shown to be behaviorally active in Table 1 on the activity of the attractants. Some standard errors are relatively high, either due to a patchy distribution of responding insects (Taylor et al., 1978) or, more probably, as the result of trap interactions (Wall and Perry, 1978). The later experiments, using Latin-square designs to allow for these sources of variability, are more efficient so that differences between treatments are easier to detect (Perry et al., 1980).

Table 1 shows that none of the compounds synergized *E,E*8,10–12:Ac, although *Z*8–12:OH and *E*8–12:OH (8 and 9) synergized *E*10–12:Ac weakly. However, several compounds inhibited one or both attractants: *E*10–12:Ac was inhibited strongly (attractancy reduced by >80%) by *E,E*8,10–12:OH (15), *Z*8–12:Ac (23), *E*8–12:Ac (24), *Z*9–12:Ac (25), and *Z*10–12:Ac (27), while *E*10–12:OH (13), 10YNE–12:OH (14), 11:Ac (18), and *E*9–12:Ac (26) were less effective inhibitors. *E,E*8,10–12:Ac was inhibited strongly by *E,E*8,10–12:OH (15), *Z*8–12:Ac (23), and *E*8–12:Ac (24). No other compounds, except *E,E*8,10–12:ALD (32, see above), had any obvious effects upon moth captures. These results are similar to those obtained with other Lepidoptera (Arn et al., 1974; Voerman et al., 1974) in that compounds which influence sex pheromone perception have common structural features. In some caes, e.g., *A. velutinana* (Roelofs and Comeau, 1971) and *Pectinophora gossypiella* (Saunders) (Beroza et al., 1971; McLaughlin et al., 1972), straight-chain saturated compounds had synergistic or inhibitory properties and 12:Ac was later found to be a pheromone component in *A. velutinana* (Roelofs et al., 1975) but only 11:Ac (18) of the saturated compounds had any effect on pea moth behavior.

Figure 1 shows the structural formulae of compounds with synergistic or inhibitory activities for comparison with those of the attractants, *E*10–12:Ac and *E,E*8,10–12:Ac. Both polar (alcohol) and less polar (acetate) classes of compounds contain both attractants and inhibitors as well as inactive compounds and, in the range of saturated compounds studied (C_9–C_{14}) no peak of activity was obvious at an optimum carbon number (i.e., correlated with volatility). Thus, the effects are determined by factors other than simple physical properties. Preliminary attempts to correlate activity with the presence and position of functional groups has led to some tentative conclusions about the influence of these structural features: (1) All but one (11:Ac, 18) of the behaviorally active compounds are unsaturated. (2) There are more active acetates than alcohols. (3) Olefinic unsaturation is generally more important than acetylenic. (4) The position of double bonds appears to be influential but not in a clear-cut way. (5) A terminal methyl group (absent in 10–11:OH and 10–11:Ac) is essential for activity.

The complexity of the mode of action is also illustrated by comparing

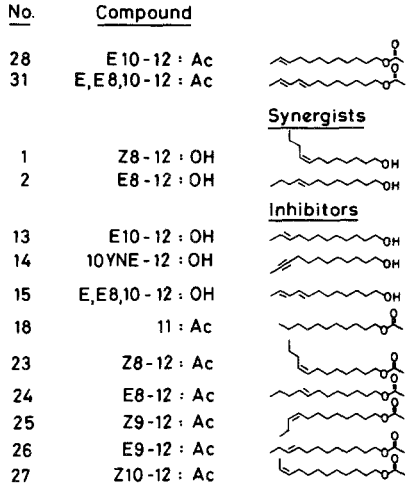

FIG. 1. Structures of *E*10–12:Ac and *E,E*8,10–12:Ac and their synergists and inhibitors.

these results (Table 1) with those of Wall et al. (1976) where *E,E*8,10–12:OH was the attractant, since this clearly shows that the behavioral activity of a given secondary compound depends on the attractant used. *Z*8–12:Ac (23) and *E*8–12:Ac (24) are two of the most powerful inhibitors (apart from *E,E*8,10–12:OH) of both *E*10–12:Ac and *E,E*8,10–12:Ac but had no effect on the attractiveness of *E,E*8,10–12:OH; similarly *Z*9–12:Ac (25) and *E*9–12:Ac (26) inhibited *E*10–12:Ac and *E,E*8,10–12:OH but did not affect *E,E*8,10–12:Ac. Also, *E*10–12:OH (13) inhibited *E*10–12:Ac but synergized *E,E*8,10–12:OH while *E*8–12:OH(9) had the opposite effect.

Further field tests (Table 2) investigated the effects of lower ratios (1:10 and 1:100) of those compounds which were strong inhibitors at 1:1 with either *E*10–12:Ac or *E,E*8,10–12:Ac. *E,E*8,10–12:OH(15), *Z*8–12:Ac (23), *Z*9–12:Ac(25), and *Z*10–12:Ac(27) were very inhibitory at 1:1 with *E*10–12:Ac, and *E*8–12:Ac(24) was only slightly less effective (Table 1). At lower proportions (Table 2), *E,E*8,10–12:OH and *Z*9–12:Ac retained considerable inhibitory power but the others lost activity—*Z*10–12:Ac at 1:10 and *Z*8–12:Ac and *E*8–12:Ac at 1:100. Similarly, *E,E*8,10–12:OH, *Z*8–12:Ac, and *E*8–12:Ac, at 1:1 with *E,E*8,10–12:Ac nearly inhibited all moth captures and were also active at 1:10, but only *E,E*8,10–12:OH was still inhibitory at 1:100 and then only weakly (Table 2).

*E*10–12:OH(13), IOYNE-12:OH(14), *E,E*8,10–12:OH(15), and *Z*10–12:Ac(27) are possible impurities in the synthetic attractants as precursors or potential side products and, because of this, their inhibitory effects might be significant. However, in practice, they are either unlikely to occur in amounts

sufficient to affect the potency of the attractant, as for *E*10-12:OH, 10YNE-12:OH, or *Z*10-12:Ac in *E*10-12:Ac, or are easily removed by chromatography in the case of *E,E*8,10-12:OH in *E,E*8,10-12:Ac.

The mode of action of the inhibitors and other active compounds described in this paper is not clear. The weight of evidence in Lepidoptera favors the idea of different receptor cell types responding differently to various compounds (Priesner, 1980; Boekh, 1980) rather than competition for the same receptor site (Roelofs and Comeau, 1971) and this is consistent with the complexity of the relationships between structure and activity discussed above. However, it may be significant that *E,E*8,10-12:Ac, the most potent pea moth attractant, was influenced by fewer compounds than was *E*10-12:Ac; perhaps these two attractants interact with the same receptor site and the stronger stimulus from the former is less easily modified by the action of secondary compounds. In some respects, the sex pheromone receptor system in *C. nigricana* seems to contrast with that in *C. pomonella*. Males of the latter species possess a receptor cell type for *E,E*8,10-12:Ac which is a strong behavioral inhibitor (Hathaway et al., 1974, 1979; George et al., 1975), although not present in the female sex pheromone, in addition to one for *E,E*8,10-12:OH, the natural pheromone (Preiss and Priesner, 1978). Also, as noted above, 11:Ac(18) is the only saturated inhibitor of a pea moth attractant while 11:OH inhibits the codling moth pheromone (Hathaway et al., 1974).

Thus, certain compounds structurally related to the pea moth sex attractants can influence their attractancy in the field; most of these are unsaturated at C-8, -9, or -10 and elicit large EAG responses. The effects range from mild synergism to strong inhibition and provide chemicals with which to investigate further the action of such compounds at the electrophysiological and behavioral levels.

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BIOLOGICAL ACTIVITIES OF *Trewia nudiflora* EXTRACTS AGAINST CERTAIN ECONOMICALLY IMPORTANT INSECT PESTS[1]

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Abstract—An ethanol extract of *Trewia nudiflora* (Euphorbiaceae) seed was tested as an agent for controlling several economically important insects. Results suggest that this plant extract acts as an antifeedant for the spotted cucumber beetle (*Diabrotica undecimpunctata howardi* Barber) and the European corn borer [*Ostrinia nubilalis* (Hübner)] but not for the other insects tested. Also indicated were morphogenic effects on the codling moth [*Laspeyresia pomonella* (L.)], disruption of the normal life cycle of the redbanded leafroller [*Argyrotaenia velutinana* (Walker)], and reduction in the progeny of the plum curculio [*Conotrachelus nenuphar* (Herbst)]. In addition, the extract was toxic to the striped cucumber beetle [*Acalymma vittatum* (F.)] and gave 100% control of the chicken body louse [*Menacanthus stramineus* (Nitzsch)] from 5 to 28 days. Fractionation of the extract was monitored by a bioassay using *O. nubilalis*. This fractionation yielded six pure compounds, the most abundant of which was trewiasine. Its LD_{50} was 7.4 ppm when incorporated into the diet of *O. nubilalis*. Dose–mortality relationships for the other compounds with *O. nubilalis* are presented.

Key Words—Pest control agent, plant extract, *Trewia nudiflora*, trewiasine, control agent, antifeedant, morphogenic agent.

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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INTRODUCTION

Ethanolic extracts of *Trewia nudiflora* (Euphorbiaceae) (false white teak) seed have shown significant activity in vitro against human carcinoma of the nasopharynx (KB) and in vivo against P388 lymphocytic leukemia (PS) (Powell et al., 1981). These extracts also inhibited initiation and growth of crown-gall tumors on potato disks (Galsky et al., 1980). In what appears to be the first insecticidal use of *T. nudiflora*, we observed previously that this extract produced high mortality when incorporated into the larval diet of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) (Freedman et al., 1979). We have now tested *T. nudiflora* extracts against a number of insects to determine their potential as a pest control agent. We have included several Lepidoptera and Coleoptera which are pests of fruit and vegetables, as well as the chicken body louse, *Menacanthus stramineus* (Nitzsch) (Mallophaga: Menoponidae). In addition, we report further testing with *O. nubilalis*.

Fractionation of the extract was guided by a dual monitoring arrangement; both antitumor (KB cell culture and PS leukemia) and *O. nubilalis* systems were employed. As detailed by Powell et al. (1981), this fractionation (Figure 1) culminated in the isolation of at least six new maytansinoid compounds; the structures of three of these are shown in Figure 2. The structures of the other three are not yet certain, although they are being investigated intensively. Results of these bioassays are presented in this paper.

METHODS AND MATERIALS

Trewia nudiflora *Extract.*

Extraction and fractionation of *T. nudiflora* seed has been described by Powell et al. (1981). The procedure used for the fractionation of the ethanol extract of this seed is summarized in Figure 1. Trewiasine is by far the most abundant of the maytansinoids in *T. nudiflora* and was purified in quantities sufficient for testing against *O. nubilalis* at several levels. Five less abundant congeners of trewiasine (demethyltrewiasine, dehydrotrewiasine, treflorine, trenudine, and *N*-methyltrenudone) have been purified in quantities sufficient only for more limited bioassays.

Insect Rearing and Testing

Codling Moth, Laspeyresia pomonella (*L.*) (*Lepidoptera*: *Olethreutidae*). Larvae were reared and tested at 26.6 ± 2°C, 60 ± 5% relative humidity, continuous light, on a modified Shorey's pinto bean diet (Shorey and Hale, 1965). *T. nudiflora* extract was combined with this diet to provide

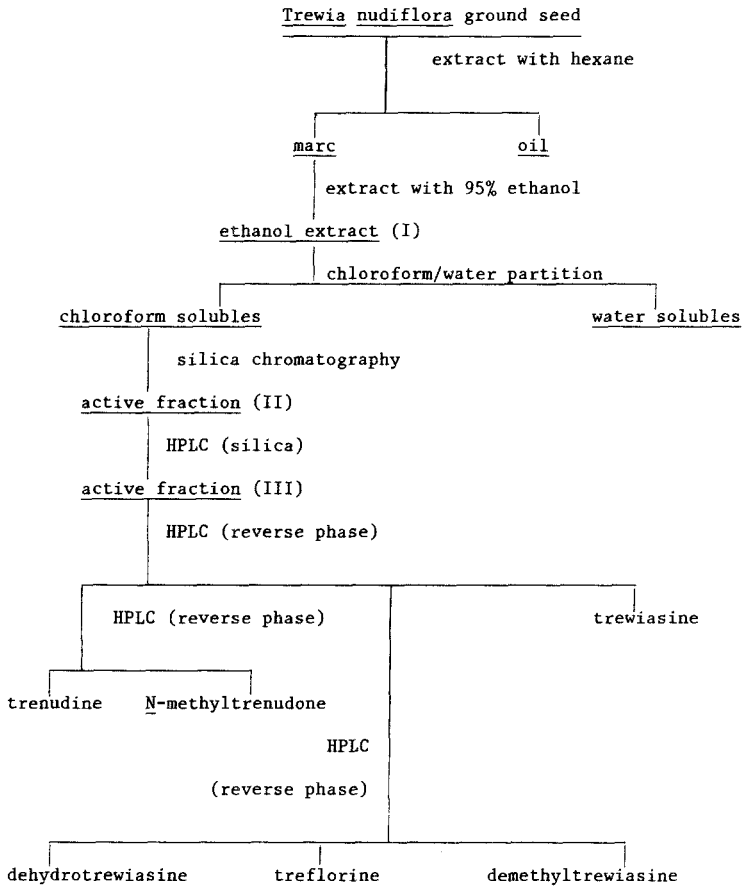
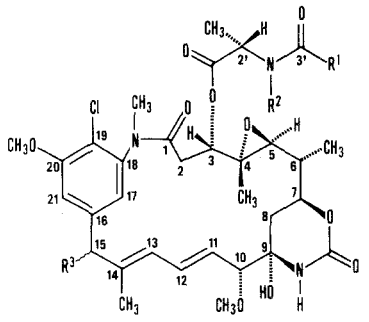


FIG. 1. Extraction and fractionation of *Trewia nudiflora*. Adapted from Powell et al. (1981).

concentrations of 0.1, 0.05, 0.01, and 0.005% by weight. The two higher concentrations were prepared by adding 40 mg and 20 mg of extract, respectively, to 2 ml of 95% ethanol, and emulsifying with a Brinkmann Polytron (PCR-2) for 5 sec. Each of the resulting suspensions was added to 40 g of hot diet and stirred thoroughly. To make the two lower concentrations, 10 mg of the extract was suspended in 10 ml of 95% ethanol, and 4 ml and 2 ml, respectively, of this suspension were added to 40 g of hot diet. The resulting hot diet mixtures were drawn up into a 5-mm-ID glass tube to harden. The diet was then expelled from the tubes, sectioned into 30- to 40-mm lengths, and evacuated at 17 mm of mercury for 15 min at 40° C to eliminate solvent. Two neonate *L. pomonella* larvae were placed onto each diet piece in a jelly cup with a plastic-coated cardboard cap. Each treatment was replicated 10



Trewiasine	$R^1 = CH(CH_3)_2$; $R^2 = CH_3$; $R^3 = OCH_3$
Dehydrotrewiasine	$R^1 = C(CH_3)=CH_2$; $R^2 = CH_3$; $R^3 = OCH_3$
Demethyltrewiasine	$R^1 = CH(CH_3)_2$; $R^2 = H$; $R^3 = OCH_3$

FIG. 2. Structures of trewiasine, dehydrotrewiasine, and demethyltrewiasine.

times. Larvae were transferred to untreated diet after 2 days. Mortality determinations were made after 7, 9, and 11 days. Ten replicates of two larvae on untreated diet were used for controls.

Striped Cucumber Beetle, Acalymma vittatum (*Fabricus*) (*Coleoptera*: *Chrysomelidae*). Beetles were reared and tested at 29.5 ± 2°C, 60 ± 5% relative humidity, 15:9 light:dark cycle, on cantaloupe leaves. A 1% solution was prepared by adding 50 mg of extract to 1 ml acetone, emulsifying with a Polytron homogenizer, and diluting to 5 ml with distilled water containing 0.01% Tween 20. Disks from cantaloupe leaves ca. 20 mm in diameter were cut with a cork borer, dipped for 10 sec in the appropriate solution, air dried, and placed in a cotton-stoppered shell vial containing a single beetle. Ten adult beetles which had recently emerged, preferably female, were tested per treatment. Feeding deterrence was evaluated by comparing the amount of treated leaf consumed in comparison with untreated leaf. Mortality was determined after 1, 4, and 5 days. Controls replicated 10 times, with one beetle per test, were fed on disks dipped into formulation blank only.

Spotted Cucumber Beetle, Diabrotica undecimpunctata howardi *Barber* (*Coleoptera*: *Chrysomelidae*). Beetles were reared as described for *A. vittatum*. Testing was also done with treated cantaloupe leaf disks. To a Petri dish containing two treated and two untreated disks, five beetles were introduced. This was replicated twice. Both feeding deterrence and mortality were evaluated.

Redbanded Leafroller, Argyrotaenia velutinana (*Walker*) (*Lepidoptera*: *Tortricidae*). Larvae were reared and tested at 26.6 ± 2°C, 60 ± 5% relative humidity, continuous light, on an artificial alfalfa leaf meal diet (Redfern, 1964). The diet was swabbed with the test material, and 8 neonate larvae, one per cup, were tested for each treatment. Mortality for the treatments was determined after controls had emerged as adults.

Plum Curculio, Conotrachelus nenuphar (*Herbst*) (*Coleoptera: Curculionidae*). Beetles were reared and tested at 26.6 ± 2°C, 60 ± 5% relative humidity, and continuous light. Adults, 3 days after emergence, were tested on apples which had one treated and one untreated side. After adding five adult beetles to each, apples were checked periodically until death of adults and/or emergence of progeny. This procedure was replicated four times.

Chicken Body Louse, M. stramineus. These lice were tested by the procedure of Hoffman and Hogan (1972) except that two rather than four chickens were treated. Also, the extract was tested at only one concentration (1% in 100% ethanol).

European Corn Borer, O. nubilalis. Larvae were reared and tested as previously described (Freedman et al., 1979) except that mortality was determined after 5 days and 11 days only.

Method for Determining LD_{50} of Trewiasine for O. nubilalis. Estimations of the LD_{50} of trewiasine for *O. nubilalis* were made using the standard probit analysis of Daum (1970). Trewiasine in 6 doses was mixed with the larval diet. To each 4 g of treated diet, five 7-day-old larvae were added. This was replicated four times. In addition, each dose was replicated twice. Mortality was determined after 5 and 11 days. Abbott's formula was used to adjust for control mortality.

RESULTS

Biological Activity of T. nudiflora

L. pomonella (*Codling Moth*). Both the crude ethanol extract and chloroform solubles at the two higher doses showed 100% mortality (Table 1); however, the two lower doses of the chloroform solubles appear to be more effective, reflecting a higher concentration of the active materials in the chloroform solubles. These bioassays showed that a high level of control of *L. pomonella* larvae was possible at low doses even with relatively crude fractions of *T. nudiflora*. We also observed that the extract produced morphogenic changes in the larvae. This manifested itself in prolonged larval development which usually resulted in an abnormally high mortality of pupae or adults.

A. vittatum (*Striped Cucumber Beetle*). Neither the ethanol extract (I, Figure 1) nor the active fraction obtained after HPLC (III) were effective as antifeedants against *A. vittatum*. Consumption of cucumber leaves treated with the extract by these beetles did result in 90% mortality, but only at relatively high dose levels and only after 5 days (Table 2). *A. vittatum* thus showed greater tolerance to the extract than did *L. pomonella*.

D. undecimpunctata (*Spotted Cucumber Beetle*). A 0.5% solution of the extract was highly effective as an antifeedant against these beetles. A 79%

TABLE 1. TOXICITY OF *Trewia nudiflora* TO *Laspeyresia pomonella* LARVAE[a]

Concentration weight % in diet	% mortality[b]					
	Crude ethanol extract			Chloroform solubles		
	7 days	9 days	11 days	7 days	9 days	11 days
0.005	28	35	82	61	88	100
0.01	67	65[c]	94	89	100	100
0.05	100	100	100	100	100	100
0.1	100	100	100	100	100	100

[a]Each dose was mixed with artificial diet to which two neonate larvae were added. This was replicated 10 times.
[b]Data adjusted for control mortality by Abbott's formula. Mortality in controls after 7 days was 10%; after 9 days and 11 days, 15%.
[c]The apparent decrease in % mortality from 7 to 9 days is due to the fact that mortality among control larvae enters into computation of the values listed.

difference in consumption was observed between treatment and control. Of the fruit and vegetable insects examined in this study, *D. undecimpunctata* was the only insect against which *T. nudiflora* extract acted as an antifeedant. A 0.5% solution of the extract also resulted in 50% mortality of this insect after 1 day.

A. velutinana (*Redbanded Leafroller*). A 0.5% solution of the extract when applied to the diet of these larvae prevented all larvae on treated diets from becoming adults. Control diets in the same test permitted 75% of the larvae to emerge as adults.

C. nenuphar (*Plum curculio*). A 0.5% solution of the extract was ineffective as a feeding and oviposition deterrent on treated apples exposed to

TABLE 2. EFFECT OF *Trewia nudiflora* EXTRACT ON *Acalymma vittatum* ADULTS[a]

Dose, % solution (w/v)	% mortality[b]		
	1 day	4 days	5 days
0.125	0	60	60
0.25	0	50	70
0.5	10	60	90
1.0	10	60	90

[a]Each dose was tested with ten beetles. Extract was applied to beetles' diet.
[b]No dead beetles in controls during the test.

TABLE 3. MORTALITY OF *Ostrinia nubilalis* LARVAE TREATED WITH COMPOUNDS FROM *Trewia nudiflora*[a]

Compound	Dose (mg)	% mortality[b]	
		5 days	11 days
Trewiasine	0.15	13	87S[c]
	0.075	18	69S
	0.038	24G[d]	51S
Demethyltrewiasine	0.20	6	69S
	0.05	24G	54S
	0.0125	22G	34S
Treflorine	0.20	18	100S
	0.05	11	100S
	0.0125	7	40S
Dehydrotrewiasine	0.20	1	60S
	0.05	17	47S
	0.0125	10	21
N-methyltrenudone	0.20	30G	100S
	0.05	17	94S
	0.0125	22G	94S
Trenudine	0.20	95S	100S
	0.05	53S	100S
	0.0125	10	100S

[a]Each dose was mixed with 4 g diet to which five 7-day-old larvae (third instar) were added. This was replicated four times.
[b]Data adjusted for control mortality by Abbott's formula. Mortality in controls after 5 and 11 days was 5 and 11%, respectively.
[c]S indicates that the mean mortality for that treatment is significantly different from control at $P = 0.05$, as determined by a range test criterion (Freedman et al., 1979).
[d]G indicates that the mean mortality for that treatment is suggestive of being different from control at $P = 0.05$, as determined by least significant difference (Freedman et al., 1979).

C. nenuphar adults. It did reduce the progeny from those insects which ate treated apples, with only 32% of larvae emerging.

M. stramineus (*Chicken Body Louse*). A 1% solution of *T. nudiflora* extract when sprayed on chickens provided 90% control of the chicken body louse within a 5-day period and 100% control for 28 days.

Bioassay of Compounds from T. nudiflora *with* O. nubilalis. Table 3 shows the results of testing all six maytansinoid compounds, each at three dose levels. These data and those in Table 4 indicate that several of the compounds may be more active than trewiasine, the most abundant. Further investigation will be needed to clarify this point. *O. nubilalis* larvae exposed to diet containing *T. nudiflora* extract, derived fractions, or pure maytansinoids crawled away from the diet as though they were avoiding it. When live or dead

TABLE 4. MORTALITY OF *Ostrinia nubilalis* LARVAE TREATED WITH TREWIASINE[a]

Dose (mg)	% mortality[b]	
	5 days	11 days
0.009	17G[c]	28S[d]
0.019	17G	41S
0.038	18S	50S
0.075	18S	61S
0.15	21S	94S
0.3	25S	92S

[a]Each dose was mixed with 4 g diet to which five 7-day-old larvae were added. This was replicated four times. Each dose was replicated twice.
[b]Data adjusted for control mortality by Abbott's formula. Mortality in controls after 5 and 11 days was 1 and 3%, respectively.
[c]G indicates that the mean mortality for that treatment is suggestive of being different from control at $P = 0.05$, as determined by least significant difference.
[d]S indicates that the mean mortality for that treatment is significantly different from control at $P = 0.05$, as determined by a range test criterion.

larvae were detected after a number of days, they were invariably much smaller in size than controls, as if they had eaten little or none of the diet.

LD_{50} of Trewiasine. Table 4 shows the mortality of *O. nubilalis* larvae when treated with trewiasine. With almost every dose, considerably higher mortality was achieved after 11 days compared to 5 days, indicating that even pure trewiasine acts relatively slowly. On the basis of the data in Table 4, the LD_{50} of trewiasine was determined to be 0.0297 mg/4 g of diet, which is equivalent to 7.4 ppm. The 95% confidence interval of 0.0185–0.0424 mg corresponds to 4.6–10.6 ppm. The slope was 1.405.

DISCUSSION

Possible Antifeedant Action of T. nudiflora. The mechanism by which *T. nudiflora* causes mortality in *O. nubilalis* is not known. Our observations indicate that it may act as an antifeedant and/or repellent. This avoidance behavior contrasts with that noted with other toxic plant extracts where dead larvae were usually found within the diet (Freedman et al., 1979). In terms of their activity towards *O. nubilalis* larvae, there appear to be no very striking differences among these six maytansinoids despite certain structural variations. We previously found that neriifolin, a plant-derived compound, had an LD_{50} of 0.12 mg (30 ppm) against *O. nubilalis* (McLaughlin et al., 1980). Trewiasine is thus four times as active against *O. nubilalis* as is neriifolin,

making it the most potent naturally occurring compound we have so far discovered in our tests with this insect.

Comparisons of the Effect of T. nudiflora *on Different Insects.* The wide variety of activity exhibited by *T. nudiflora* makes it interesting as a potential pest control agent. Its effect on *L. pomonella* appears to be concentration dependent. At the doses shown in Table 1, it acts mainly as a toxicant; at lower doses, as a morphogenic agent. Unlike the antifeedant effect observed with *O. nubilalis*, no antifeedant effect was observed with *L. pomonella* even at the higher doses. This may partially explain why toxic effects appear more rapidly than with *O. nubilalis*, a greater amount of material being consumed immediately.

Several other biological effects, such as suppression of adult emergence with *A. velutinana* and reduction in progeny with *C. nenuphar*, were also noted. With *D. undecimpunctata*, the extract was an antifeedant, as these insects largely avoided eating diet treated with it. Analysis of the data presented in Tables 1, 2, and 3 reveals that *L. pomonella* may be more sensitive to *T. nudiflora* than *O. nubilalis* and is definitely affected by lower doses of the extract compared to *A. vittatum*. For both *A. vittatum* and *O. nubilalis*, 90% or greater mortality was not achieved until the insects had been exposed to treated diet for 5–11 days. This suggests the effects produced by the extract occur relatively slowly.

In summary, the active components of *T. nudiflora* act as antifeedants, reduce or suppress adult emergence or progeny, or produce morphogenic changes in several insect species. Further experimentation is needed to define these modes of action more precisely. This initial study indicates the extract may have potential for controlling a number of economically important insects; however, further testing, including ultimately field tests, will be required to establish this potential.

The exotic structure of the maytansinoids, together with their biological activity, has prompted intensive synthetic efforts from several groups; at least two of these have already culminated in success (Corey et al., 1980, Meyers et al., 1980). *Trewia nudiflora*, the source of our active material, appears to be fairly abundant in its native habitat (India). However, from the standpoint of supply, the most significant development may be the discovery by a group of Japanese workers (Asai et al., 1979) that maytansinoids can be produced by fermentation with a *Nocardia* sp. This development opens up possibilities for a relatively cheap method for producing the complex macrocyclic ring of maytansinoids; these compounds could then be modified by attachment of various ester sidechains.

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MISIDENTIFICATION BY WILD RABBITS, *Oryctolagus cuniculus*, OF GROUP MEMBERS CARRYING THE ODOR OF FOREIGN INGUINAL GLAND SECRETION I. Experiments with All-Male Groups

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Abstract—To demonstrate that the inguinal gland odor conveys information on the identity of individuals, 60 male, wild-type rabbits, *Oryctolagus cuniculus*, living in groups of three in outdoor pens, were used in 120 tests. Members of the groups smeared with the inguinal gland secretion from unfamiliar rabbits were attacked by their penmates. In contrast the odors of chin gland secretions and urine of strange rabbits and of a commercial perfume did not cause serious disruption to the social relationships within the groups.

Key Words—Wild rabbit, *Oryctolagus cuniculus*, male groups, inguinal glands, strange odors, misidentification.

INTRODUCTION

The wild rabbit, *Oryctolagus cuniculus*, possesses several types of odor-producing skin glands which function for the purpose of communication. By means of specialized marking behaviors, the secretions from the anal and chin glands are distributed throughout the area occupied by a rabbit, and it has been shown experimentally that the presence in the environment of the odors of these secretions is an important factor in maintaining an animal's confidence and ability to defend its home ground against strange conspecifics. On the other hand rabbits do not actively disperse the secretion from their inguinal glands and its presence in the environment does not enhance their territorial confidence (Mykytowycz et al., 1976; Hesterman et al., 1981).

Instead general observations indicated that the odor of the inguinal gland

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secretion is involved in the recognition of individuals or groups. In one of the earliest studies of the behavior of the rabbit under natural conditions, Southern (1948) described a characteristic form of behavior of rabbits (tail-flagging) in situations requiring individual recognition thus, ". . . the buck elevates his haunches so that he walks 'stiff-legged' and lays his tail flat along his back . . . The visual stimulus is undoubtedly important, but the possibility is not excluded of some scent stimulus from the inguinal glands being involved." Our own behavioral as well as histological and biochemical observations pointed to the same conclusion (Mykytowycz and Goodrich, 1974). Myers and Poole (1962) suggested, on the basis of observation on free-living rabbits, that the inguinal glands may also be involved in sexual recognition. Adequate experimental evidence to substantiate such views has been lacking, however. Separate studies were therefore needed to test these hypotheses.

This paper summarizes the results of one of a series of experiments designed to clarify the behavioral function of the inguinal gland secretion in the European rabbit. It describes responses to strange inguinal odors within all-male groups of rabbits.

METHODS AND MATERIALS

Ethological Basis and Rationale for Experiments

In planning this study, consideration was given to the fact that rabbits live in separate social groups consisting usually of 2–5 adult individuals and their progeny. Each member of a group acquires a sharply defined status within a linear social order. The existing social hierarchy is constantly reinforced by frequent threats by the dominant members and avoidance by subordinates, and severe aggression rarely occurs between members of a long-established group. Each member can recognize all others and thus fits into the social order with a minimum of friction. On the other hand strangers introduced into a social entity are attacked by all members (Mykytowycz, 1968).

General observations suggested that differences in the odor of the inguinal gland secretions of individual animals were the main clues used by rabbits to differentiate between familiar and strange conspecifics. To test this hypothesis inguinal gland secretions derived from foreign animals were applied to the bodies of selected members of established, all-male groups of rabbits and changes in the incidence of aggressive activity and other forms of behavior were measured. For comparison the effects of other sources of odor were also tested.

Animals

Sixty adult, male, wild-type rabbits were used. Most of them were captured from field populations but some were bred in captivity. They were kept, in groups of three individuals, in outdoor pens approximately 3 × 3 m in size constructed from rabbit-proof wire netting. A shelter box with a single entrance hole was attached externally to one side of each pen. The boxes were of a standard design, subdivided internally into four interconnected compartments. Each compartment was fitted with a separate hinged top to enable experimenters to gain access to the rabbits without entering the pen itself. Water and food, in the form of high-quality lucerne hay, were provided ad libitum.

Socially stable groups were an essential prerequisite for the study. The groups were formed by initial random matching of available individuals. Substitutions for animals which were vigorously rejected by their penmates or complete regrouping of incompatible combinations were continued until severe aggression was eliminated. This was assessed by frequent observation from a watchtower and by daily inspections of animals for injuries and general condition.

The records collected during these observations were also used to determine the dominance hierarchies. A linear order of dominance is usually quickly formed within any group of rabbits brought together and often involves physical contests. Once the order has been established, higher ranking individuals frequently reinforce their superior status by aggressively chasing or threatening subordinates. In a socially stable group a rabbit of lower rank will never attack, or resist an attack by, a dominant individual but will either avoid contact or adopt a submissive posture (Mykytowycz, 1958). The dominance hierarchies were therefore assessed from the numbers of aggressive and/or submissive interactions which occurred between the individuals. A group was judged to be socially stable when the pattern of such interactions, i.e., the position of each animal in the hierarchy, remained constant.

Groups were used for experimentation only after they had been stable for at least two months and the social order within them was firmly established. Visual identification by observers of individual rabbits within each group was made possible by marking their fur in distinctive patterns with a black dye.

Odor Preparation and Application

Six sources of odor were used in the experiments: secretions from the inguinal glands of the lowest ranking rabbits in each group; inguinal gland secretions from unfamiliar males and females, respectively; chin gland

secretion and urine from unfamiliar male rabbits; and a commercially available perfume.

Preliminary trials in which subordinates were smeared with the inguinal gland secretions from single strange rabbits showed that there were considerable variations in the reactions of the same individuals and groups towards the secretions from different donors. To eliminate this source of variation, all unfamiliar rabbit odors used in the experiments were derived from pooled collections from a number of animals.

Inguinal Gland Secretions. The inguinal glands secrete into pouches situated in the groin, the mixture of glandular secretions and desquamated epithelium forming a strongly odorous waxy sebum (Mykytowycz, 1966).

For the tests involving rabbits treated with their own inguinal secretion, the animal's sebum was collected onto a cottonwool swab and immediately rubbed into the fur on its hindquarters, flanks, and head.

In the tests involving strange inguinal odors, samples of secretions pooled from unfamiliar male and female rabbits were used. The sebum from at least 20 rabbits was collected, weighed, and ground up to form a suspension in water containing 0.01% sodium lauryl sulfate which acts as a wetting agent. The volume was adjusted to give a concentration of 20 mg sebum/ml. Aliquots of 0.5 ml of the suspension were sealed into glass ampoules and stored at −18° C until required.

The quantity of sebum used for each test (10 mg) was roughly equivalent to the amount most commonly present in the inguinal pouches of an individual rabbit, although in our experience this may vary considerably from time to time and between individuals, ranging from less than 1 mg to over 50 mg.

Chin Gland Secretions. Pooled samples of chin gland secretions collected from at least 20 male rabbits unfamiliar to the test animals were used in the experiments. The pooled sample was diluted with water to a concentration of 8 mg chin secretion/ml, sealed in 0.5-ml amounts in glass containers, and stored at −18° C until required. The amount (4 mg) used for each test was eight times greater than that which in an earlier study was found to exercise a significant effect on the territorial behavior of rabbits (Mykytowycz et al., 1976).

Urine. Samples of urine from unfamiliar male rabbits were pooled, thoroughly mixed, and 0.5-ml amounts sealed into glass containers and stored at −18° C until required.

Perfume. A proprietary brand cologne lotion (Old Spice, Musk for Men, Shulton Inc., Clifton, New Jersey) was employed. Approximately 0.1 ml of the undiluted lotion was used for each test.

Selection of Animals and Application of Odors. Preliminary observations showed that only higher ranking individuals would react uninhibitedly

towards odor-smeared companions. The odors under test were therefore always applied to the lowest-ranking individuals of the groups since this option promised to be the most sensitive in revealing any effects of the treatments.

All odor preparations were applied by means of a cottonwool swab and were rubbed thoroughly into the fur of the hindquarters, flanks and head of the selected rabbits.

Experimental Procedures

Tests were conducted during the first 2 hr of daylight, a period of the day when rabbits are normally active. The odor under test was applied to the third-ranking individual without removing it from the shelter box and with minimum possible disturbance to the other two occupants. The behavior of the group was observed from a watchtower commanding a clear view of the pens. Observations continued for 30 min from the time any member of the group emerged from the shelter box. If no rabbit emerged within 1 hr after smearing, observations were discontinued.

The incidences and durations of the following forms of behavior for each rabbit were recorded: agonistic behavior (chasing, biting), following other rabbits, sniffing at other rabbits, mounting, grooming, and exploration. The data were recorded by one observer using a multichannel event recorder.

It was often apparent from the noises produced that the rabbits were active within the shelter boxes but since neither the animals involved nor the exact forms of behavior could be observed, this information has not been used.

To assess the long-term effects of the odor applications, all rabbits were examined on each of the three days following the test and their body weights, general conditions, and fresh injuries were recorded.

The effect of each odor source was tested within 20 groups of rabbits, the same groups being used for all treatments. For each treatment 10 groups were tested on two consecutive mornings. The different treatments were separated by an interval of three days and all tests were carried out within a period of 20 days.

The order in which the different odor sources were tested was as follows: the treated animal's own inguinal gland secretion, perfume, male chin gland secretion, male urine, female inguinal gland secretion, male inguinal gland secretion. Preliminary observations indicated that treatments with certain sources of odor were likely to disrupt the groups to the extent that some of them might not be suitable for further experimentation. Since it was expedient that the same groups of rabbits were used for all treatments, the odor sources thought most likely to produce serious disturbances were tested last.

Treatment of Data

For the purposes of this paper, only the incidences of attacks and injuries are taken into consideration. The more detailed data will be presented elsewhere together with the results obtained in further experiments.

Statistical comparisons between the incidences of aggression or injuries in the different treatments were carried out by means of the test statistic:

$$T = \frac{p_1 - p_2}{\sqrt{\frac{npq}{n-1}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where p_1 = proportion of tests in which aggression or injuries occurred in treatment 1; p_2 = proportion of tests in which aggression or injuries occurred in treatment 2; p = proportion of tests in which aggression or injuries occurred in treatments 1 and 2 combined; q = proportion of tests in which aggression or injuries did not occur in treatments 1 and 2 combined; n_1 = number of tests in treatment 1; n_2 = number of tests in treatment 2; n = number of tests in treatments 1 and 2 combined.

This is the analog of the t test for comparing the equality of population means, when both populations are assumed to have the same variance σ^2. In dealing with proportions, an unbiased estimator of the common variance is: $\sigma^2 = npq/n - 1$.

RESULTS AND DISCUSSION

Figure 1 shows the numbers of tests in each treatment in which there was evidence of aggressive behavior towards the smeared and unsmeared rabbits, respectively. The first- and second-ranking animals have both been included in the category of unsmeared rabbits. Observed attacks by either one or both penmates or injuries detected up to three days after the treatment were accepted as evidence of aggression.

It can be seen that the incidence of aggression was lowest when the subordinate animals were smeared with their own inguinal gland secretions. This is regarded as a control series. The results include any effects due to the handling of animals during odor application or to the unusual siting of the odor sources on the bodies of the smeared rabbits.

Aggression took place in slightly more tests involving perfume, male chin, and male urine. The frequencies recorded are too small to allow statistical comparisons between any of the categories in those tests, but the fact that the increase occurred only in aggression directed towards the smeared animals suggests that it may have been stimulated by the unfamiliar odors.

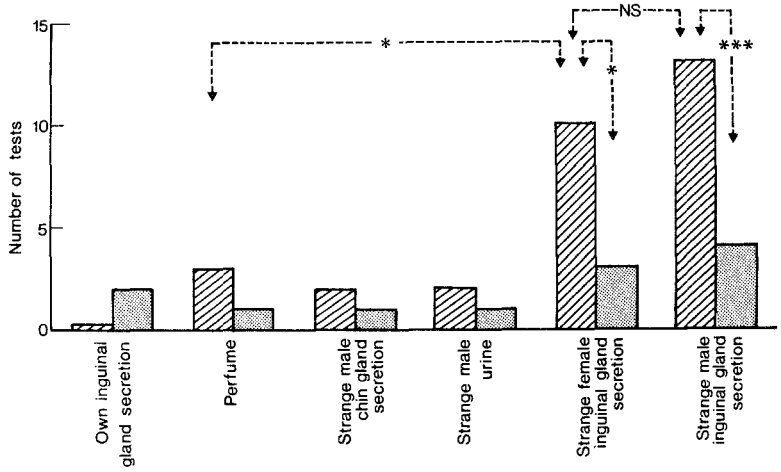

FIG. 1. The number of tests in which rabbits, *Oryctolagus cuniculus*, smeared with various odors were attacked by penmates in all-male groups. Comparable data for unsmeared members of the groups are also shown. $N = 20$ for each odor treatment. Hatched columns = smeared rabbit. Stippled columns = unsmeared rabbits. NS, not significant; $*P < 0.05$; $***P < 0.001$.

A clear increase in aggressive activity occurred when strange female and male inguinal gland secretions were used. The results of these two treatments do not differ significantly from one another. The frequencies of aggression towards smeared animals differed significantly from that directed to the unsmeared rabbits in both treatments (female inguinal, $P < 0.05$; male inguinal, $P < 0.001$). They were also significantly higher than the frequency of aggression displayed towards the animals smeared with perfume ($P < 0.05$).

The combined data for tests using strange inguinal gland secretions show a fivefold increase in the frequency of aggression towards smeared rabbits (57.5% of tests) compared with the combined results for tests in which the other sources of unfamiliar odor were used, i.e., perfume, male chin secretion, and male urine (11.7% of tests). This increase is highly significant ($P < 0.001$). A similar comparison of the combined data for unsmeared animals shows a much smaller, but still significant ($P < 0.05$) increase in the tests involving unfamiliar inguinal gland secretions. This aggression towards unsmeared penmates was undoubtedly due to the general increase in activity. Whenever the normal pattern of social interactions within a group is disturbed, such as by the intrusion of a strange animal or a severe conflict between two residents, all high-ranking rabbits and particularly the dominant animal are stimulated to reinforce their positions within the social hierarchy. They do so by intensifying their aggressive activities towards all subordinate group members and may even attack a higher-ranking individual.

It is interesting to note that there was no significant difference between the frequencies with which aggression was displayed towards animals treated with inguinal gland secretion from strange males and strange females. This is consistent with our knowledge of the territorial behavior of the wild rabbit. Owners of a territory exclude any intruder, irrespective of its sex (Mykytowycz and Hesterman, 1975).

Table 1 presents the data on attacks and injuries and also shows the frequencies with which dominant and second-ranking individuals were aggressive towards the stimulus animals. As can be seen, both dominant and second-rankers were disturbed by the presence of an animal carrying a foreign inguinal odour. Although there is a suggestion that the second-ranking animals were more frequently aggressive to penmates smeared with female inguinal secretion than the dominant males, the difference is not significant.

More animals were injured after being smeared with strange inguinal gland secretions than following treatment with other odor sources. However, the overall incidence of injuries was too low to show a firm trend.

In an earlier study inguinal gland secretion, unlike anal and chin gland secretions and to a lesser extent urine, failed to stimulate territorial confidence in rabbits (Mykytowycz et al., 1976). The results of the present experiments show conclusively that the application of the inguinal gland odor from a foreign rabbit to the subordinate member of a group markedly increases the frequency with which it is attacked by its penmates. In contrast, the odors of foreign chin gland secretions or urine are no more effective in this behavioral context than a nonspecific, unfamiliar odor. Anal gland secretion was not

TABLE 1. TESTS IN WHICH SUBORDINATE RABBITS SMEARED WITH VARIOUS ODORS WERE ATTACKED BY DOMINANT OR SECOND-RANKING PENMATES OR SUSTAINED INJURIES WITHIN THREE DAYS OF TREATMENT[a]

	Number of tests		
	Observed attacks		
	By dominant	By second-ranker	Injured
Own inguinal gland secretion	0	0	0
Perfume	2	1	1
Strange male chin gland secretion	1	1	1
Strange male urine	1	1	0
Strange female inguinal gland secretion	3	7	2
Strange male inguinal gland secretion	7	8	3

[a]Twenty tests were performed in each odor treatment.

used for comparison in these tests because of the difficulty of obtaining samples in the quantity and form suitable for this type of experimentation.

The fact that foreign inguinal gland secretions were the only odor sources which caused rabbits to be attacked by other members of their groups strongly suggests that the inguinal gland odor carries information concerning identity.

However, whether this information identifies the animal as an individual or merely as a group member cannot be determined from the type of experimentation used in these studies, as misidentification of the smeared animal by its penmates on either basis would lead to them treating it as a foreign intruder.

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A MATERIAL ISOLATED FROM HUMAN HANDS THAT ATTRACTS FEMALE MOSQUITOES[1,2]

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Abstract—The residue left on glass surfaces by human hands was found to be attractive to female *Aedes aegypti* (L.) and *Anopheles quadrimaculatus* Say mosquitoes. The material lost half of its activity in 1 hr. A solvent wash technique was developed to recover and concentrate the residuum from handled glass beads. The residuum could be recovered effectively with absolute ethanol and less effectively with several other solvents. More mosquitoes were attracted to heated than to unheated residuum, an indication of its volatility. Also, attraction of the residuum decreased with decreasing concentration or dose. Concentrated residuum collections, stored under refrigeration and tested for longevity, showed no appreciable loss of attractiveness up to 60 days of storage.

Key Words—Attractants, human, mosquito, collection, concentration, *Aedes aegypti*, *Anopheles quadrimaculatus*, Diptera, Culicidae.

INTRODUCTION

A great deal of effort has been made to identify agents produced by humans that attract mosquitoes. Carbon dioxide, water, heat, and chemical emanations have been investigated and a number of proposals made to explain their significance. Bar-Zeev et al. (1977) cited 43 publications in this regard. Acree

[1]Diptera: Culicidae.

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et al. (1968) showed that L-lactic acid isolated from acetone washes of human skin was attractive to *Aedes aegypti* (L.). Smith et al. (1970) evaluated the importance of lactic acid in host-seeking behavior of *Ae. aegypti* and reported that lactic acid was attractive only in the presence of carbon dioxide. They concluded that the presence of an attractive chemical(s) was indicated in addition to lactic acid. In tests with highly refined collecting and bioassay apparatus, Price et al. (1979) also concluded that airborne human emanations other than carbon dioxide and water attracted *Anopheles quadrimaculatus* Say and suggested that lactic acid was not the only attractant present.

In all of these studies, either skin washes or airborne effluents were used in olfactometer bioassays and chemical determinations. However, working with a dual-port olfactometer, Schreck et al. (1967) observed that contact with the acrylic plastic interior of the system with bare hands caused erratic test results. Subsequent investigations showed that surfaces touched by hands retained a residuum that was attractive to laboratory-reared *Ae. aegypti*; researchers wore disposable plastic gloves thereafter when operating the olfactometer (Acree et al., 1968). Bar-Zeev et al. (1977) decontaminated their olfactometer by flushing it with air. Price et al. (1979) showed that passage of human emanations from the hand and forearm through 1-cm-diam silicone tubing reduced attractiveness in proportion to the length of the tubing, an indication that the attractive substances adhered to the inside of the tubing. Subsequent studies were conducted to measure the magnitude of attraction of the residuum and the length of time it persisted. When polyethelene gloves were worn then turned inside out in an olfactometer, they attracted up to 57% of *Ae. aegypti*; activity declined after 1–3 hr (unpublished data).

Because mosquitoes were attracted in these tests without externally applied heat, moisture, or CO_2, we concluded that the attractiveness was the result of a chemical(s) in addition to or other than lactic acid. This is a report of tests conducted to obtain information about the nature of the attractive material and methods developed for its collection, concentration, and bioassay for ultimate identification.

METHODS AND MATERIALS

In all five experiments, subjects manipulated various numbers of 15-mm-diam glass beads with their hands for various periods of time. All bioassays were competitive (treatment vs. no treatment); attractive residuum (AR) was evaluated with a dual-port olfactometer (Schreck et al., 1967) containing ca. 125 seven-day-old female *Ae. aegypti* or *An. quadrimaculatus*.

The first experiment was conducted to determine the effect of aging on the response of mosquitoes to the AR. Four sets of 6 beads were handled for

10 min, aged for 0, 15, 30, or 45 min, placed in a clean, untouched watch glass, then tested for attractancy to *Ae. aegypti*. Since each test required ca. 15 min, treatments were designated as 0–15, 15–30, 30–45, and 45–60, min. Each test was replicated 16 times. A linear regression analysis was used to relate response to duration of aging.

The second experiment was conducted to determine if the AR could be removed from the beads with various solvent washes. Twelve beads were handled for 15 min; then six were placed in a glass funnel, washed with 15 ml of a solvent, allowed to drip dry, and competitively tested for attraction to *Ae. aegypti* in an olfactometer against acetonitrile, ether, absolute ethanol, and distilled water. Each test was replicated four times. Analysis of variance was used to compare mean responses.

The third experiment was conducted to determine whether mosquitoes were attracted to the AR recovered from handled beads and redeposited on clean beads. Two subjects each handled 12 beads (6/hand) for 6 hr. The beads were washed every 30 min during this time with 10 ml of one of the five solvents. Preliminary data showed no difference in attraction response between 10, 15, and 30 min handling time, therefore, 30 min was chosen for the remaining studies for logistical reasons in scheduling collections and tests. The original 10 ml of each solvent were used in successive rinses, and a small amount of clean solvent was added for the final rinse to bring the volume back up to 10 ml. Solvents were stored at 4° C until tested. Two ml of solvent were then pipetted over six clean beads in a watch glass, and 2 ml of a clean sample of the same solvent were pipetted onto a second set of six clean beads. Both sets were then competitively tested for attraction to *Ae. aegypti* in an olfactometer. Each test was replicated three times on different days and with different populations of mosquitoes. Analysis of variance was used to compare mean responses.

The fourth experiment was conducted to determine the dose–response relationship of solvent washes of handled beads to *Ae. aegypti* and *An. quadrimaculatus*. Twenty-four subjects handled 12 beads (6/hand) for a total of 302.5 accumulated hours of handling. The beads were washed every 30 min with 200 ml of absolute ethanol. Each wash was then divided into two 100-ml samples, and one sample was serially diluted three times. Although we did not have a quantitative measure of the actual amount of AR present, the samples were considered as 100, 50, 25, or 12.5% of the original wash and represented four different levels or doses of the AR. The samples were then bioassayed as before except that 2 ml of each sample were pipetted onto 12 instead of 6 beads. The surface area of the 12 beads plus the watch glass container was ca. 10 cm^2. Samples were bioassayed in a randomized fashion so that the dilutions were not necessarily tested in descending order. Similarly, the two mosquito

species (new populations each test day) were randomly placed in two of three olfactometers. Each replication consisted of a response test with each of the four sample doses and with both mosquito species on the same day (eight tests total). The dose-response data were analyzed statistically with a probit analysis procedure, from which a linear regression equation relating probit of response to the log of dose was obtained. A *t* test was used to determine differences between equation slopes.

Although it was not necessary to heat the beads before applying a solvent wash to attract mosquitoes, preliminary tests showed that preheating the beads to 34–37° C resulted in greater attractive response by both *Ae. aegypti* and *An. quadrimaculatus*. This increase was probably the result of a more rapid volatilization of the AR from the heated surfaces and a greater amount of the AR being available for detection. We therefore preheated the beads for the third, fourth, and fifth experiments. The temperature of the beads at the start of a test after the pipetting was 38.9° C and at the end of a 3-min test was 36.4° C.

The fifth experiment was conducted to determine the activity and longevity of concentrated washes from handled beads thru bioassay. Sets of beads (12 per subject) were handled, and at 30-min intervals each set was washed with the same 10 ml portion of absolute ethanol. Periodically, the ethanol was made up to 10 ml. Each collection was terminated at the end of 20 hr of handling time by several individuals. The concentration of each 20 hr collection in a 10-ml ethanol solution to 100 μl was achieved using compressed nitrogen as described by Price and Carlson (1981).

Samples of AR wash concentrates were stored in small glass vials at 4° C for testing after minimum storage (2–6 days) as collection, concentration, and bioassay cannot be done on the same day. Stored AR concentrate samples were tested as three groups, 2–6, 29–33, and 39–61 days, using 10-μl aliquots, each equivalent to 2 hr of collection. The 10-μl aliquot was brought up to 2 ml with ethanol and pipetted on the surfaces of glass beads for bioassay as described in experiment 4.

A linear regression test was used to analyze for the longevity of attraction of the AR. A two-tailed *t* test was devised to determine the significance of the slope obtained.

RESULTS AND DISCUSSION

Figure 1 gives results of the first experiment. The plotted data represent the mean percentage of mosquitoes attracted in 16 tests in each 15-min time interval. The vertical lines represent the standard deviation for each time. Regression analysis indicated a significant negative slope (1% level, *F* test) for

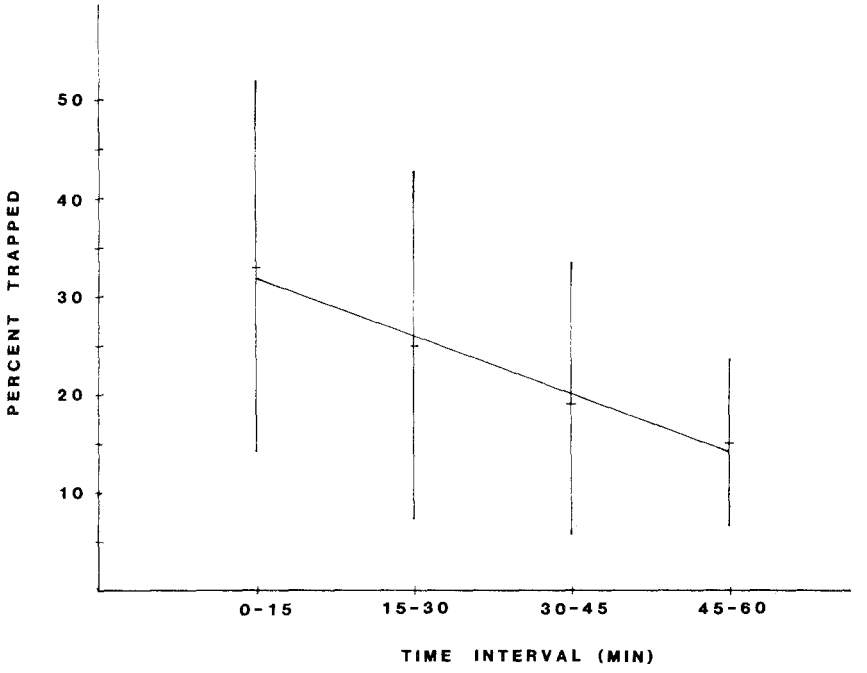

FIG. 1. Relationship between mean response level (percent trapped) and duration of aging in 15-min time intervals of the AR on handled glass beads. Vertical lines represent SD for the 16 replications/interval.

TABLE 1. ATTRACTION OF *Aedes aegypti* TO HANDLED GLASS BEADS UNWASHED OR WASHED WITH INDICATED SOLVENTS AND BIOASSAYED IN A DUAL-PORT OLFACTOMETER (4 REPLICATIONS)

	% Attraction (± SD)			
	Washed beads		Unwashed beads	
Solvent	Range	Mean	Range	Mean
Acetone	0-1.0	0.25 ± 0.35	26-71	42.25 ± 19.8
Acetonitrile	0-9.0	2.75 ± 4.1	34-48	40.5 ± 5.8
Ether	0-5.0	2.75 ± 2.1	20-59	45.0 ± 17.8
Absolute Ethanol	0-2	0.75 ± 0.80	35-61	44.5 ± 11.5
Distilled Water	0-3	1.0 ± 1.2	33-75	48.25 ± 19.9

TABLE 2. ATTRACTION OF *Aedes aegypti* TO HANDLED GLASS BEAD WASHES COLLECTED WITH INDICATED SOLVENTS, REDEPOSITED, AND BIOASSAYED IN A DUAL-PORT OLFACTOMETER (3 REPLICATIONS)

Solvent	% attraction (± SD)[a]	
	Range	Mean
Absolute ethanol	81-92	84.8 ± 6.2 a
Acetonitrile	51-58	55.4 ± 3.8 b
Acetone	44-57	51.5 ± 6.8 b
Ether	33-72	51.2 ± 19.4 b
Distilled water	0-15	5.8 ± 8.3 c

[a]Means followed by the same letter are not significantly ($P = 0.01$) different.

the regression equation, even though its value was quite low (−0.396) and the data were somewhat variable. The attractiveness of the AR was significantly reduced (by ca. 50%) within 60 min.

Table 1 gives results of the second experiment. Analysis of variance showed that all five solvents were equally effective in removing the AR from the beads. Attractiveness was significantly ($P = 0.01$) different between washed and unwashed beads, whereas attractiveness was no different among solvents used.

Table 2 gives results of the third experiment. The attractiveness of ethanol washes was significantly greater than of the other solvents, and the response to water washes was significantly ($P = 0.01$) lower than to the other

TABLE 3. DOSE-RESPONSE RELATIONSHIP OF ATTRACTION OF *Aedes aegypti* AND *Anopheles quadrimaculatus* TO 4 CONCENTRATIONS OF HUMAN HAND RESIDUUM ON GLASS BEADS IN A DUAL-PORT OLFACTOMETER (6 REPLICATIONS/TREATMENT)

% wash concentration	% attraction			
	Ae. aegypti		*An. quadrimaculatus*	
	Range	Mean	Range	Mean
100	60-95	78.5	20-67	43.8
50	12-77	44.5	3-55	20.8
25	11-55	28.0	0-42	10.5
12.5	3-11	5.7	2-25	11.3

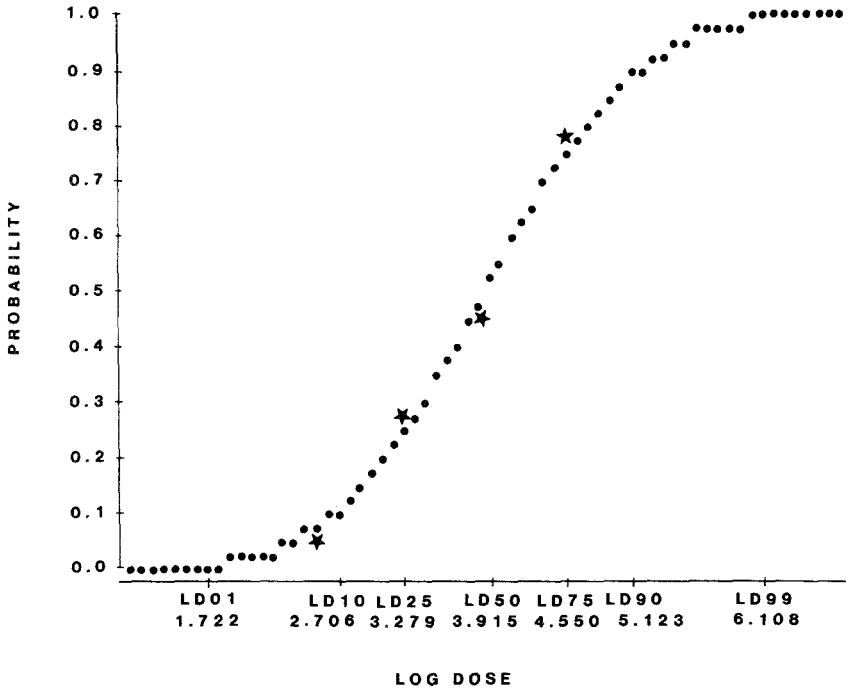

FIG. 2. Probit analysis curve showing the relationship between mean response (probability) and log dose (solvent wash dilution) for *Ae. aegypti.* Stars are actual data points.

solvents. Although the five solvents used in experiments 2 and 3 were equally efficient in removing the AR, they were not equally efficient in redepositing the AR back on clean beads in a form which was equally attractive.

Table 3 gives results of the fourth experiment (dose–response relationship of the AR). The response of *Ae. aegypti* was ca. 2X that of *An. quadrimaculatus.* The mean percentage of mosquito response was reduced by ca. 50% with each 50% reduction in concentration in all but the 12.5% sample.

Figures 2 and 3 give dose–response curves from the probit analyses. The *t* test on the regression equation slope indicated a significant ($P = 0.05$) dose–response relationship for *Ae. aegypti* but a nonsignificant dose–response relationship for *An. quadrimaculatus*; however, the overall response level for *An. quadrimaculatus* was lower than for *Ae. aegypti.* The trend of the data indicated an increasing response with increasing attractant level for both species.

Figure 4 shows that in the fifth experiment activity of the AR was not lost during concentrating procedures. The condensate was bioassayed but was not

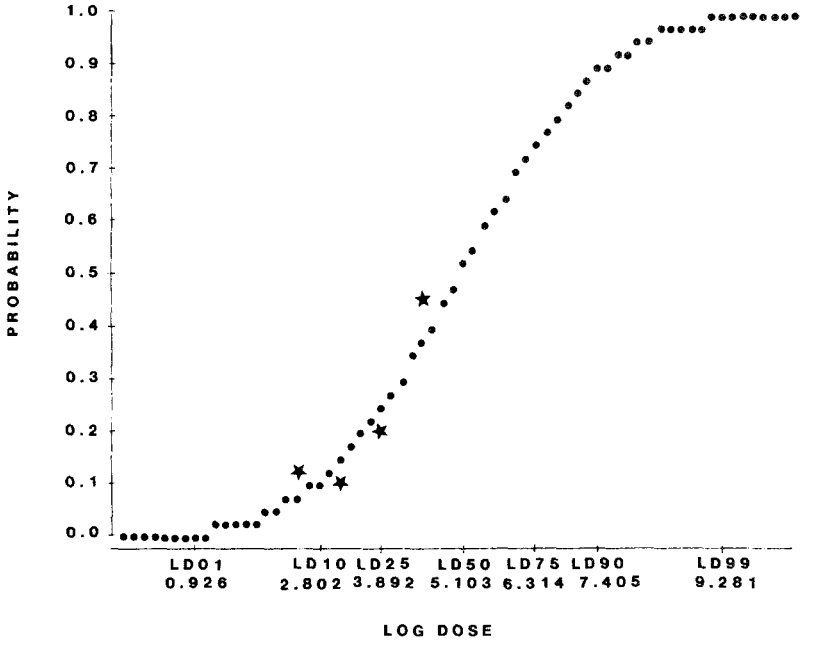

FIG. 3. Probit analysis curve showing the relationship between mean response (probability) and log dose (solvent wash dilution) for *An. quadrimaculatus*. Stars are actual data points.

active, an indication that the attractive substance was not lost in the condensing process in quantities large enough for the mosquitoes to detect.

As for longevity, overall results showed means of 44.1 ± 26.6% (SD) attraction for samples vs. 1.17 ± 1.21% (SD) for blanks, a highly significant difference. The means for each group (2–6, 29–33, and 39–61 days) were statistically indistinguishable, although means of 45, 43, and 42% attraction were indicated.

An r value of 0.99 indicated a good fit of the data, and the slope was significantly different from zero at the 99% confidence level. The indicated loss of activity was 0.05% per day, suggesting that storage of raw AR concentrate for one year should result in only 18% loss of activity, although this cannot be proven statistically due to large variability in attraction. The results indicate that AR concentrate can be safely stored for up to 60 days without appreciable loss of activity.

Future studies will be concerned with development of suitable methods of isolation and, ultimately, identification of the attractant(s) present in the residuum.

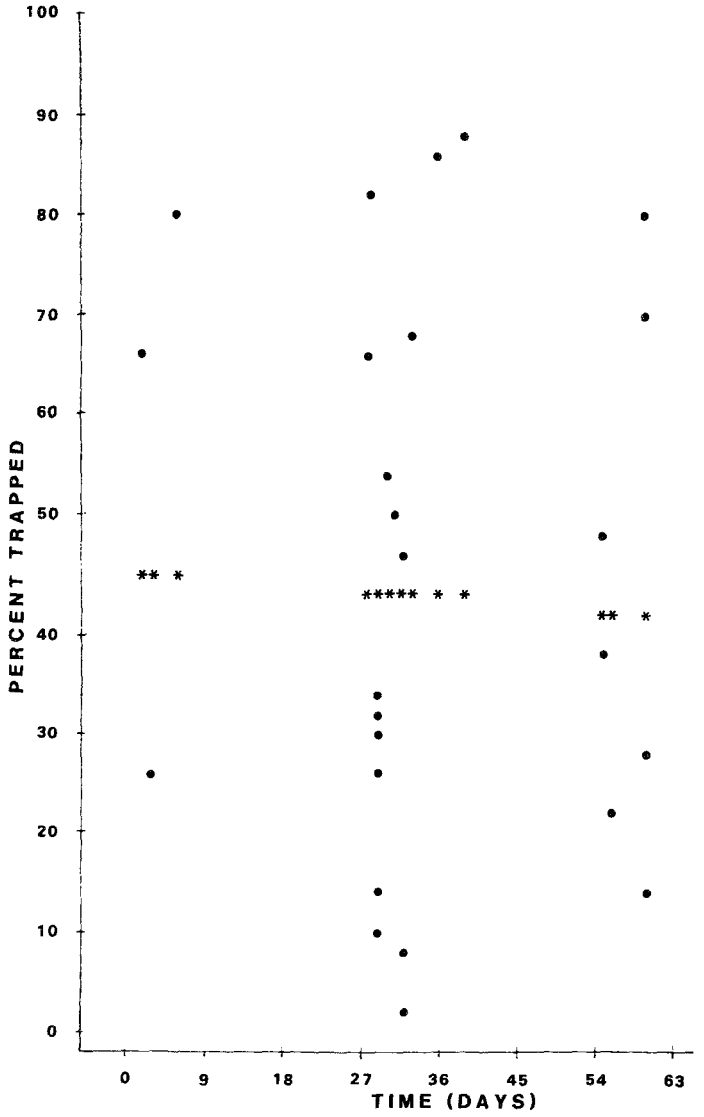

FIG. 4. Response (percent trapped) of *Ae. aegypti* to stored, concentrated AR. Dots are actual data points; asterisks show the mean percentage of mosquitoes trapped (attracted) to each group of stored AR samples.

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STUDIES ON SEX PHEROMONE OF AMERICAN COCKROACH, WITH EMPHASIS ON STRUCTURE ELUCIDATION OF PERIPLANONE-A

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Abstract—Two independently biologically active compounds, periplanone A and periplanone B can be isolated from fecal material of the American cockroach, *Periplaneta americana*. In fecal material these occur in a ratio of 1 : 10 while, in intestinal tracts only periplanone B has been found. The latter has been identified as (1*Z*,5*E*)-1,10(14)-diepoxy-4(15),5-germacradien-9-one; the identification was confirmed by synthesis. Only the CD (−) enantiomer (1*R*,2*R*,5*E*,7*S*,10*R*) exhibited activity. The lower threshold of activity of both natural and synthetic CD (−) pheromone, is 10^{-6}–10^{-7} μg. Periplanone A has been identified (apart from its stereochemical configuration) as 7-methylene-4-isopropyl-12-oxa-tricyclo[4.4.2.$0^{1,5}$]-9-dodecen-2-one).[1] The structure of this rather unstable compound could be deduced by comparing its NMR, UV, IR, and mass spectra with the NMR and mass spectra of its rearrangement product. Both structures still require confirmation by synthesis, but their spectral data are in complete agreement with the proposed structures. The presence of only periplanone B in the gut and the presence of both periplanone A and periplanone B in the feces suggests that periplanone B is a genuine sex pheromone, whereas peri-planone A might be a biologically active transformation product, which in turn can isomerize into a more stable, but inactive compound.

Key words—Sex pheromones, American cockroach, *Periplaneta americana*, periplanone-A, periplanone-B, germacrane derivatives.

[1] Name according to IUPAC, Nomenclature of Organic Chemistry, 1979 ed., page 32. For the sake of clarity we prefer to use the same numbering of C atoms as has been used for periplanone B (IUPAC, Nomenclature of Organic Chemistry, 1979 ed., page 498). This permits the assignment of the same numbers to corresponding C atoms in the various structures.

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INTRODUCTION

Several attempts have been made to identify the sex pheromone of the American cockroach, *Periplaneta americana*, but these have been unsuccessful (Jacobson et al., 1963; Chen, 1974; Chow et al., 1976; Takahashi and Kitamura, 1976; Kitamura and Takahashi, 1976). In 1974 we reported the presence of two biologically active substances (later called periplanone A and B) in extracts of feces of virgin females and also the determination of their molecular formulae (Persoons et al., 1974).

The structure elucidation of periplanone B as well as its synthesis have been described in detail (Persoons, 1977; Persoons et al., 1979; Persoons and Ritter, 1979; Still, 1979; Adams et al., 1979) and will only be briefly reviewed here. We now which to describe in detail the structure elucidation of periplanone A.

METHODS AND MATERIALS

Structural Elucidation of Periplanone B: A Short Review. For reasons of material availability (periplanone B occurs at the concentration several times higher than that of periplanone A), we first focussed our attention on the identification of periplanone B. This compound could be obtained from both fecal material and intestinal tracts, whereas periplanone A could only be extracted from fecal material. A massive cockroach rearing and extraction program in which fecal material and intestinal tracts from 75,000–100,000 virgin female cockroaches were used, led to the isolation of 200 μg of periplanone B and 20 μg of periplanone A. Interpretation of the results of spectroscopic analysis of periplanone B (GC-MS, IR, proton NMR) and of its hydrogenation product (GC-MS) led to six possible structures, all of which possessed a 10-membered alicyclic ring (Persoons et al., 1976). On the basis of biogenetic considerations, the reported activity of another germacrane derivative towards *P. americana* (Tahara et al., 1975) and certain proton NMR chemical shift criteria, a preference could be made for one out of these six tentative structures; conclusive proof was, however, lacking. Rerunning the proton NMR spectrum at 300 MHz, including decoupling experiments and computer simulations, provided very important additional information. Combination of all available analytical data led to the conclusion that, apart from uncertainties in the relative configuration at the chiral centers, the structure of periplanone B was a shown in Figure 1 (Persoons, 1977; Persoons et al., 1979).

Still (1979), in his synthesis of periplanone-B, developed a flexible route to an intermediate which established the absolute configuration at C1, C7, and C10. Three of the four possible diastereoisomers of periplanone B were

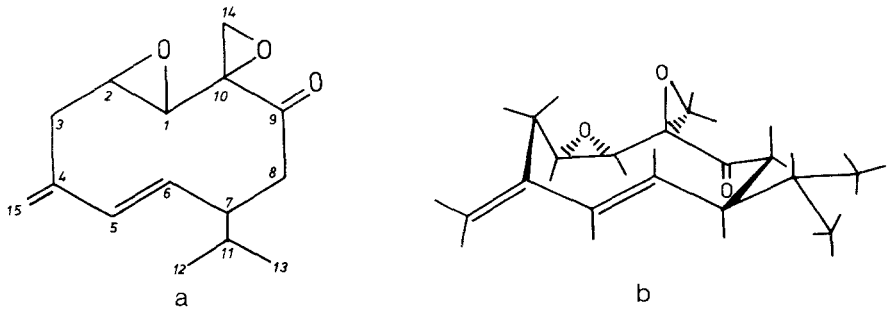

FIG. 1. Periplanone B: (1*Z*,5*E*)-1,10(14)-diepoxy-4(15),5-germacradiene-9-one: (a) planar structure; (b) sterostructure of the natural enantiomer (*1 R, 2R, 5E, 7S, 10R.*)

synthesized. One of these stereoisomers was found to be identical with the natural pheromone. Bioassays with the resolved enantiomers showed the lower threshold of activity for CD(+)-and CD(−)-periplanone B to be 10^{-2} and 10^{-6}–10^{-7} μg, respectively (Adams et al., 1979). The latter value agrees very well with that found for natural periplanone B[= CD(−)] (Persoons, 1979). The fact that the CD (+) enantiomer exhibited some activity might well be caused by contamination of this enantiomer with a small amount of the natural isomer due to incomplete separation of the MTPA esters during the HPLC procedure. On the other hand, the (+)-MTPA chloride used for the separation of the enantiomers may have been contaminated with a small amount of (−)-MTPA chloride. The absolute configuration of periplanone B (Figure 1) was established to be (1*R*,2*R*,5*E*,7*S*,10*R*) by application of the excition chirality method (Adams et al., 1979).

Isolation and Structure Elucidation of Periplanone A and its Rearrangement Product. The isolation and structure elucidation of periplanone B have been described in previous communications (Persoons, 1977; Persoons et al., 1979). Persoons (1977) described the isolation procedure for periplanone A in detail. Essentially the same procedure has been used for the isolation of the rearrangement product of periplanone A. The instrumental methods used for the structure elucidation of periplanone A and its rearrangement product have been mentioned before (Persoons, 1977; Persoons et al., 1979).

RESULTS

Collection and Purification of Crude Extracts. The active compound could only be obtained from fecal material, either by direct extraction from feces or by extraction from excrement-soiled filterpaper. Prepurification of these crude extracts on Sephadex, followed by HPLC on SiO^2 afforded the desired, but still impure, compound.

Gas chromatographic analysis via at least two different columns finally yielded the pure compound. At this stage it was found that the compound isolated was rather unstable. Storage of the pure compound in hexane at -20° C for periods of over 3 days yielded a second (biologically inactive) compound. The latter could easily be separated from periplanone A by gas chromatography, using DEGS of OV-101 as the stationary phase. As the process of rearrangement proceeded rather quickly, only small amounts of periplanone A were available at the same time. When larger amounts of it were necessary (e.g., for NMR analysis), the process of rearrangement had already started before the required amount of material had been collected. For this reason, part of the spectrometric analyses could only be carried out with mixtures of the two compounds. Because of this instability, more spectroscopic data became available for the rearrangement product than for periplanone A itself; the structure elucidation of the rearrangement product will therefore be described first.

Rearrangement Product of Periplanone A. The mass spectrum of both the rearrangement product and of periplanone A itself show the parent peak at $m/z = 232$. Because of this relationship it is likely that the molecular formula of the rearrangement product is the same as has been found for periplanone A: $C_{15}H_{20}O_2$ (Persoons et al., 1974; Persoons, 1977). This is in agreement with a total of 20 hydrogen atoms estimated from the NMR spectrum.

The proton NMR spectra of mixtures of periplanone A and its rearrangement product are depicted in Figures 2 and 3. Figure 3 shows the spectrum after the process of rearrangement was nearly complete. From this spectrum, including the results of spin-decoupling experiments and the molecular formula, the partial structures shown in Figure 4 were deduced (Table 1).

The presence of a carbonyl group could be concluded from the fact that such a group present in periplanone A (Persoons, 1977), whereas comparison of NMR spectra of periplanone A and its rearrangement product does not suggest that the process of rearrangement involves a transformation of the carbonyl group into an alcohol or ether group. Combination of the four partial structures described above and consideration of the further requirements of the NMR spectrum, leads to only one possible structure (Figure 5). On the basis of coupling constants the configuration shown in Figure 6 for this compound is expected.

Periplanone A. Although the assumed molecular ion peak ($m/z = 232$) in the mass spectrum of periplanone A was too weak to be peak-matched, the molecular formula could be derived from the elemental compositions determined for some fragment ions as $C_{15}H_{20}O_2$ (Persoons, 1977; Talman et al., 1978). The mass spectrum of hydrogenated periplanone A indicated the presence of two double bonds (parent peak at $m/z = 236$) (Persoons, 1977).

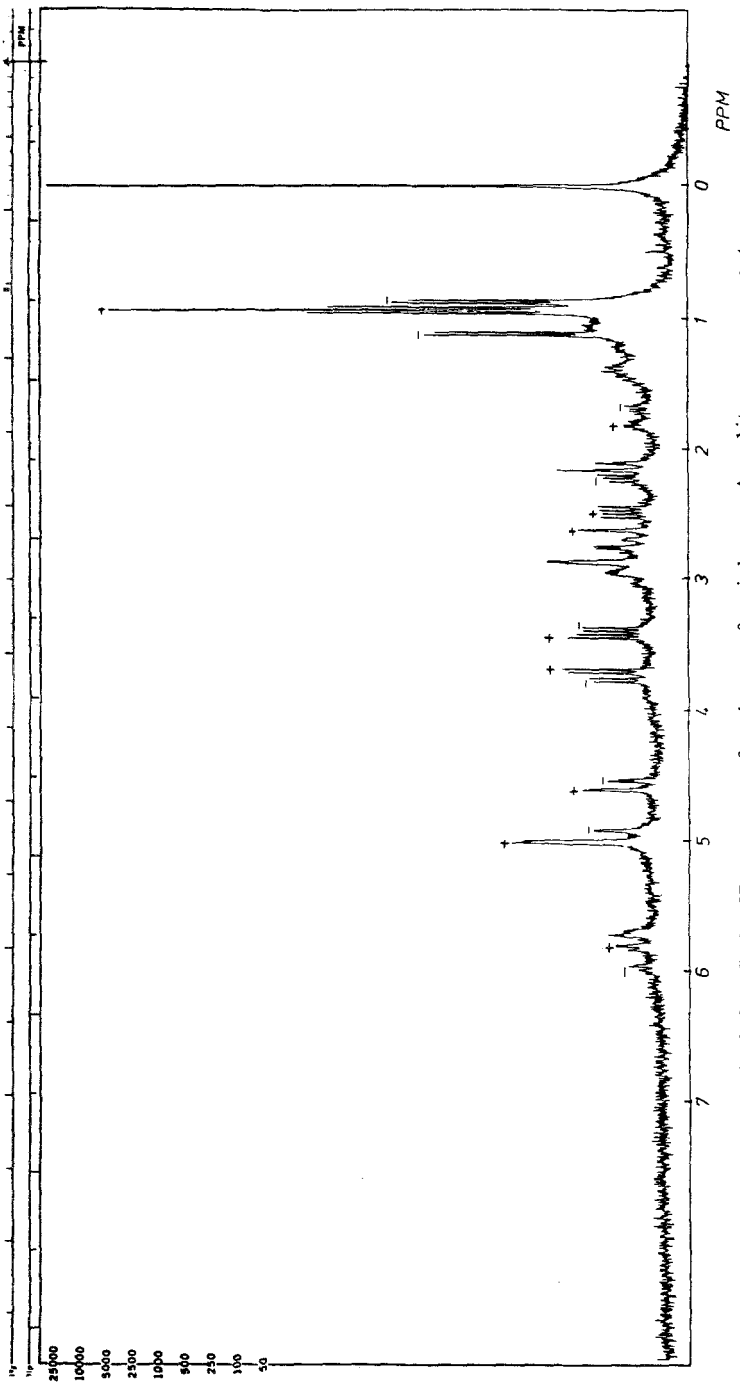

FIG. 2. 300-MHz NMR spectrum of a mixture of periplanone A and its rearrangement product; (+ signals increased as time proceeded, − signals decreased as time proceeded).

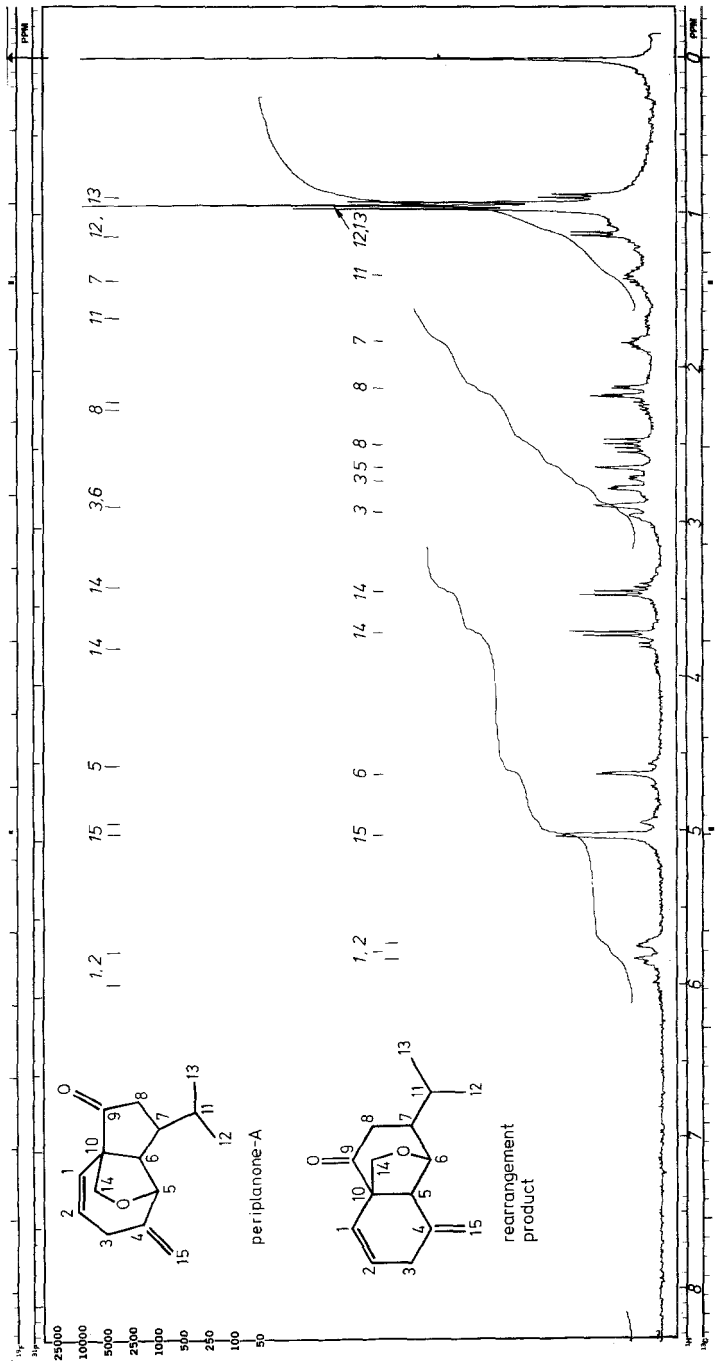

FIG. 3. 300-MHz NMR spectrum of a mixture of periplanone A (20%) and its rearrangement product (80%).

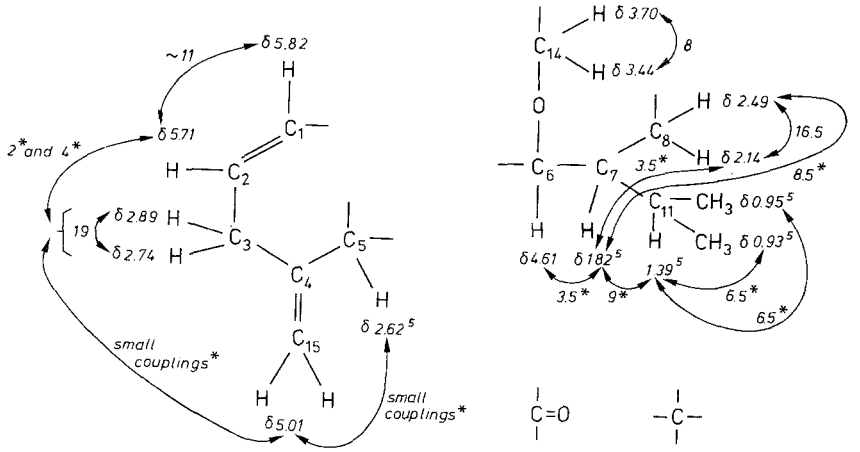

FIG. 4. Partial structures of the rearrangement product of periplanone A.

TABLE 1. 300 MHz PROTON-NMR SIGNALS OF PERIPLANONE A AND ITS REARRANGEMENT PRODUCT (IN CS_2)

Periplanone A δ (ppm)		Rearrangement product δ (ppm)	Assignment in rearrangement product
5.98	d, 11, broadened	5.82	1-H
∿5.71	d, 11, with additional splittings	5.71	2-H
∿5.01	broadened	5.01	15-H_2
4.92	broadened		
4.54	d ∿ $4\frac{1}{2}$, broadened	4.61	6-H
3.77	d ∿ 8	3.70	14-H_2
3.39	d ∿ 8	3.44	
∿2.9	probably 3 H's	2.89	3-H_2
		2.74	
		2.62^5	5-H
∿2.24	d, 16, d, $7\frac{1}{2}$	2.49	8-H_2
∿2.19	d, 16, d, $10\frac{1}{2}$	2.14	
∿1.4 ?	broad multiplet	1.82^5	7-H
∿1.66	broad multiplet[a]	1.39^5	11-H
1.12^5	3H, d, $6\frac{1}{2}$	0.95^5	12-H_3, 13-H_3
0.88	3H, d, $6\frac{1}{2}$	0.93^5	

[a]Couplings with δ 0.88 and δ 1.12^5 were confirmed by means of a spin-decoupling experiment.

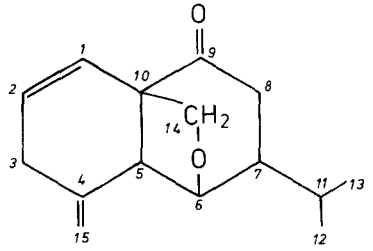

FIG. 5. Structure of the rearrangement product of periplanone A (for the numbering of C atoms, see p. 1).

The infrared spectrum (Persoons, 1977) showed the presence of the following functional groups:

$>C = 0$ (absorption band at 1710 cm^{-1}), $>C(CH_3)_2$ (bands at 1380 and 1360 cm^{-1}), $-CH_3$ (2960 and 2870 cm^{-1}), $-CH_2-$ (2925 and 2850 cm^{-1}, and $>C = CH_2$ (3085 and 875 cm^{-1}).

Both the infrared spectrum (absence of an absorption band at 1600 cm^{-1}) and the ultraviolet spectrum (no absorbance observed between 200 and 400 nm), indicated that no conjugated double bonds are present in the molecule.

The proton NMR spectra (Figures 2 and 3) showed weak signals of periplanone A (max. 45%) which partially overlap those of its rearrangement product (min. 55%). Due to the low signal-to-noise ratio, spin-decoupling experiments did not yield useful results. It was therefore necessary to assume that signals with similar chemical shifts and patterns in the spectra of periplanone A and its rearrangement product arose from identical partial structures. This assumption seemed to be justified since all data indicated that we were dealing with a compound and its rearrangement product.

Analysis of the NMR spectral data (Table 1) leads to the partial

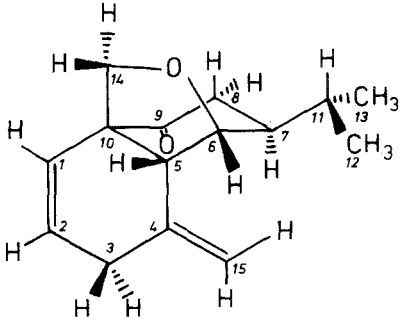

FIG. 6. Expected configuration for the rearrangement product of periplanone A.

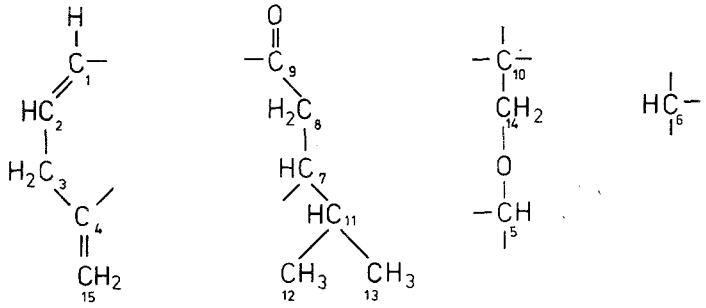

FIG. 7. Partial structures of periplanone A.

structures shown in Figure 7. Combination of these partial structures and consideration of the chemical shifts and coupling constants, leads to the structure shown in Figure 8.

From the above data two more structures could be deduced. These were, however, rejected, because, for both structures, the process of rearrangement would involve cleavage of three C–C bonds.

The structure (in Figure 8) shows, however, a great similarity with the structure of the rearrangement product of periplanone A (see Figure 5) and obeys the head-to-tail isoprene rule. Both structures can also account for the mass spectral data, including the main difference observed (Talman et al., 1978).

DISCUSSION

Periplanone B, periplanone A (proposed structure), and the rearrangement product of the latter possess closely related structures. The germacrane skeleton and the functional groups of periplanone B can, although slightly modified, be found in the other two structures. A hypothetical scheme for the transformation of periplanone B into periplanone A, which in turn isomerizes into the biologically inactive rearrangement product, is given in Figure 9.

The starting point is the assumption that the five-membered oxygen-

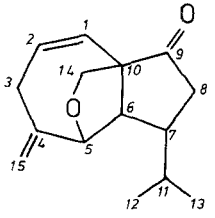

FIG. 8. Proposed structure of periplanone A. (for the numbering of C atoms, see p. 1B).

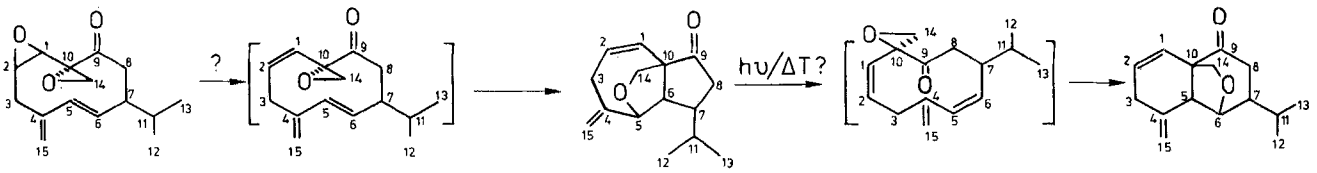

FIG. 9. Hypothetical scheme for the transformation of periplanone B into periplanone A and of periplanone A into the stable, biologically inactive product.

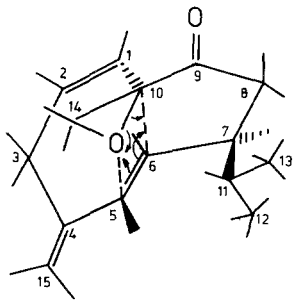

FIG. 10. Formation of periplanone A from the hypothetical 5 (*E*) isomer. (= IV in Figure 9).

containing ring in structures I and II has arisen from a transannular reaction of an *exo*-epoxide group at C_{10} with a C_5–C_6 double bond.

It can be shown with a Dreiding molecular model that both isomers (IV and V) of the hypothetic starting compound may take a conformation in which the C_{10}–0 bond of the epoxide and the Δ^5 double are nearly parallel and at bond distance.

Transannular reaction of the C_{10} epoxide with the double bond at position 5 in the *E* isomer leads to the formation of periplanone A (Figure 10), while in the case of the 5(*Z*) isomer, this will give rise to the rearrangement product (Figure 11). Intermediate IV (in Figure 9) can be assumed to originate from periplanone B by reduction of the 1 (*Z*)-epoxide group.

Cleavage of the bonds C_5—0 and C_6—C_{10} in the proposed structure of periplanone A will preferentially lead to the formation of intermediate V, rather than to that of IV, since the dihedral angle of H-C_5C_6-H in structure II is about 40°.

With the single-cell recording technique, Sasz (1980) demonstrated the presence of many olfactory hairs on the antennae that were very sensitive to

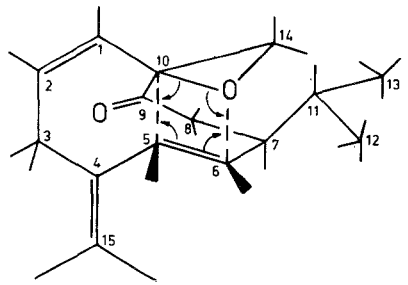

FIG. 11. Formation of the stable, but biologically inactive product from the hypothetical 5(*Z*) *isomer.* (= V in Figure 9).

periplanone A, whereas other were very sensitive to periplanone B; this may indicate that both compounds are pheromones.

It is interesting to note that compounds having the same carbon skeleton as periplanone A could be obtained by biomimetic reactions starting from epoxygermacrene D (Niwa et al., 1979). Ultimate proof of the correctness of the proposed structures of periplanone A and its transformation product will have to be provided by synthesis.

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DEFENSIVE ADAPTATIONS OF EGGS AND ADULTS OF *Gastrophysa cyanea* (COLEOPTERA: CHRYSOMELIDAE)

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Abstract—Egg clusters and adults of *Gastrophysa cyanea* are conspicuous and, like their larvae, are chemically protected. The eggs owe their bright yellow color primarily to β-carotene and, in addition, contain substantial quantities of oleic acid. At natural concentrations oleic acid effectively deters many species of ants from feeding. The use of fatty acids as deterrents against ants is discussed as a possible widespread phenomenon among insects. During defensive confrontations, adults of *G. cyanea* exhibit avoidance behavior and may also feign death. In addition, the adults may autohemmorhage or secrete a fluid from elytral or pronotal pores in response to traumatic stimuli. The secretions are effective against ants and contain a mixture of hydrocarbons as well as terpenoid components. The pattern of ontogenetic modification in the defensive chemical repertoire of *G. cyanea* is discussed.

Key Words—Defensive secretions, oleic acid, hydrocarbons, terpenoids, eggs, deterrents, ants, predation, Chrysomelidae, Coleoptera, *Gastrophysa cyanea*, reflex bleeding, elytral glands.

INTRODUCTION

Studies on the defensive chemistry of insects often emphasize a single stage in the life cycle, usually either the larva or adult. Although useful, this approach can provide little insight into the changes in chemical defense that transpire as an insect undergoes metamorphic development.

Information on the ontogeny of predator deterrents in leaf beetles

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(Chrysomelidae) is particularly sparse. Many chrysomelid species deposit bright orange or yellow egg clusters on the host plant (Klausnitzer and Forster, 1971). However, except for reports of cardinolides in the ova of *Chrysolina polita* and *C. coerulans* (Pasteels and Daloze, 1977; Daloze and Pasteels, 1979), the defensive chemistry of chrysomelid eggs has been almost completely ignored. Whereas the larvae of some leaf beetle species possess eversible tergal glands and the chemistry of these secretions has received considerable attention (Matsuda and Sugawara, 1980, and references therein), the defensive attributes of the other developmental stages of these species have only been examined in a few isolated cases. For example, some pupae retain the larval cuticle as a holdfast and gain protection from the secretory reservoirs it holds (Bromley, 1947; Hinton, 1951; Wallace and Blum, 1969). Furthermore, many adult chrysomelids are equipped with defensive glands, but only in *C. polita* have both adult and most juvenile stages been chemically assessed (Daloze and Pasteels, 1979).

Previous investigations on defenses in the chrysomelid *Gastrophysa cyanea* have been restricted to the larvae, demonstrating that eversible glands produce potent deterrents against small arthropodan predators such as ants. The secretions contain two major components: chrysomelidial, a cyclopentenoid monoterpene, and another iridoid compound, gastrolactone (Blum et al., 1978; Jones et al., 1980).

We had reason to suspect that other stages of the life cycle of *G. cyanea* are also endowed with chemical defenses. The adult beetles are conspicuous on the foliage of their host plant, *Rumex crispus* (Force, 1966), their elytra and pronotum varying in hue from metallic green to a lustrous steel-blue. The eggs are also easily noticeable, being deposited in gleaming yellow clusters on the underside of host plant leaves. The pupae are yellow as well, but they pupate unobtrusively beneath the soil. The bright coloration of these stages could constitute aposematic displays, advertising the presence of noxious or toxic compounds. We tested the possibility that eggs and adults are chemically protected, using ants which frequent the beetle's host plant as potential predators and analyzing these stages for the presence of defensive allomones.

METHODS AND MATERIALS

Interactions between *G. cyanea* and ants were observed in dense stands of *Rumex* sp. from April to June, 1980 in various localities of Georgia and Florida. The ants were often attracted to the host plant in significant numbers by honeydew-excreting aphids.

Feeding deterrence of egg or adult extracts was assessed by comparing the numbers of ants feeding on 3-mm lengths of mealworms (*Tenebrio molitor*) treated with extracts or solvent controls. The mealworm baits were

pinned along ant foraging trails on the host plant and the number of feeding ants was recorded after 5 min. Similar tests were performed in the laboratory with a minimum of five ant colony replicates per experiment.

Extracts of *G. cyanea* eggs and adult secretions were prepared in methylene chloride and analyzed for volatiles on a LKB-2091 gas chromatograph–mass spectrometer. A 2-m × 2-mm, 3% OV—1 column was used with the column temperature programed from 30° to 200° at 8° C/min.

Extracts were qualitatively tested for cardenolides by spotting on filter paper, spraying with saturated tetranitrobiphenyl in benzene and developing with 5% methanolic KOH. Cardenolides are indicated by the appearance of purple spots (Mauli et al., 1957).

RESULTS

Egg clusters were usually located in groups on the host plant, with most clusters containing 30–50 eggs (Figure 1). The majority were deposited along leaf midribs on the lower portion of the plant. The delicate eggs appeared to be coated with a thin oily material with which they adhered loosely to one another and to the substrate.

Ants encountering egg masses typically inspected them only briefly, then backed away to groom their antennae. The eggs were not fed upon, punctured,

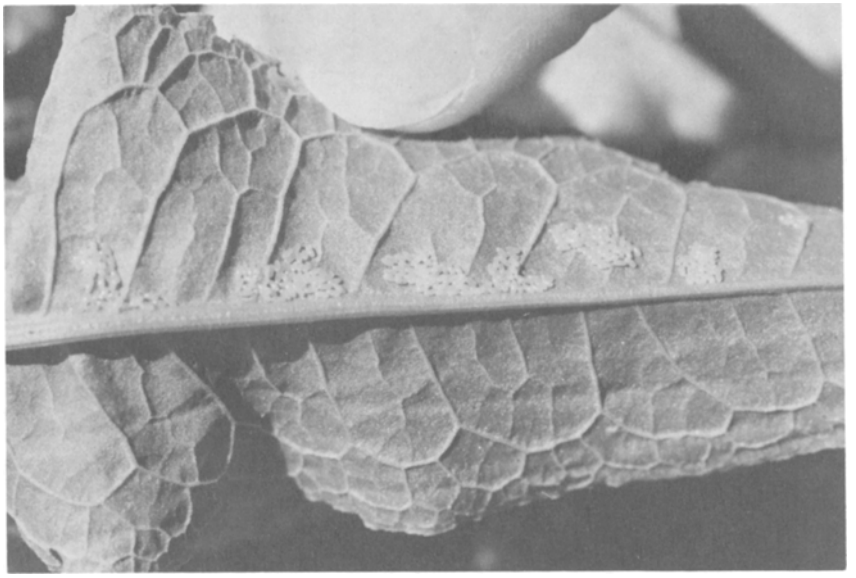

FIG. 1. Egg clusters of *G. cyanea* on the lower surface of a host plant leaf.

or even manipulated by workers of *Crematogaster pilosa*, *Campanotus pennsylvanicus*, and *Solenopsis invicta*.

One thousand eggs were harvested by carefully sliding a spatula underneath them and were then extracted whole for 20 min in methylene chloride. The extract was filtered, brought to 2 ml with additional solvent, and tested in the field for feeding deterrence against foraging workers of *C. pilosa*. In five replicates, only 1.8 ± 1.3 (mean ± SD) workers fed at mealworms treated with 5 egg equivalents of extract but 5.2 ± 1.6 ants fed at control mealworms receiving solvent alone ($P < 0.02$; Mann-Whitney test). The behavior of ants at treated baits was similar to that shown to eggs: avoidance and grooming after contact were common. However, some extract-treated baits were eventually consumed well after the 5-min test period.

Although no volatile components were detected by GC-MS analysis of fresh methylene chloride extracts, treatment with ethereal diazomethane revealed the presence of one major component in the eggs. The spectrum of this component contained the following important ions: m/z = 296m$^+$, 265, 264, 222, 221, 180, 87, and 74; this was identical to the published spectrum for methyl oleate (Stenhagen et al., 1974). The average oleic acid content was estimated 40 μg/egg.

The feeding deterrency of oleic acid (Sigma Chemical Co., 99% purity) was assayed in five laboratory colonies of *Crematogaster lineolata*. In each nest, two mealworm baits treated with one of four concentrations of oleic acid

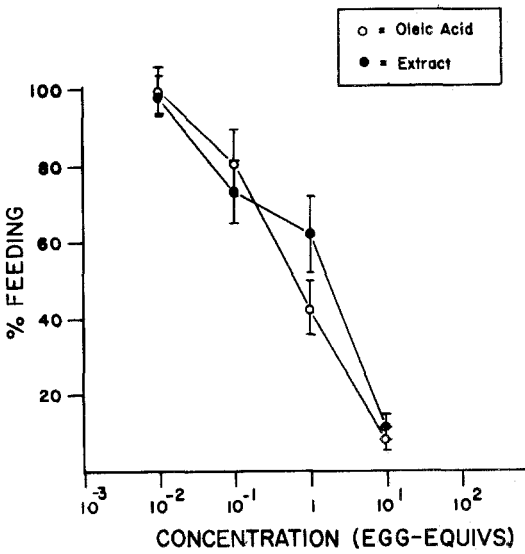

FIG. 2. Effects of oleic acid and egg extracts on feeding by *C. lineolata*. Response expressed as percent of controls (mean ± 1 standard error: $N = 6$).

(in ethanol) or one of four concentrations of ethanolic extract were compared against ethanol-treated controls. The pairing of concentrations of the two treatments was randomized. As shown in Figure 2, oleic acid in natural concentration reduced feeding as effectively as one egg equivalent of the extract. The fatty acid also retarded feeding in laboratory colonies of *Solenopsis invicta*, *Monomorium phaoronis*, *M. minimum*, *Crematogaster pilosa*, *C. ashmeadi*, and *Iridomyrmex humilis*. Workers of the latter species quickly consumed controls but covered oleic acid-treated baits with soil and other debris.

To the human palate the eggs taste somewhat bitter and evoke a numbing sensation on the tongue, but oleic acid does not produce these reactions. Cardenolides are noted for their bitterness, but tests for their presence in the methylene chloride extracts of 500 eggs were negative. Neither chrysomelidial nor gastrolactone were detected in the extracts.

To determine the chemical basis of their yellow coloration, approximately 500 *G. cyanea* eggs were crushed and extracted with petroleum ether until colorless. After filtration the solution was reduced in volume and banded on silica gel (Eastman chromagram sheet) for thin-layer chromatography using toluene as a solvent. A yellow band noted on the chromatogram was removed and eluted with hexane for visible spectrophotometric analysis according to Davies (1976). Absorption maxima were observed at 478, 445, and 423 nm, indicating a predominance of β-carotene.

Protection of Adults. Due to their bright coloration, aggregating tendencies, and penchant for basking on exposed regions of the host plant, the adult beetles could easily be perceived from a distance. The beetles tolerated the close approach of humans but capturing them with forceps proved challenging. When disturbed, many of the beetles folded their appendages close to their body and tumbled from the plant to the litter below. Since they often landed with their dull ventral surface upward and remained motionless for extended periods, the adults were difficult to relocate.

Beetles often eluded ants by running for short distances but since they do not move rapidly, they were sometimes overtaken. As the ants crawled over them, the adults assumed a characteristic "headstand" posture, pressing their head and antennae against the substrate while tilting the abdomen upward (Figure 3). This stance could also be elicited by tapping the dorsal surface of a captive beetle with forceps, but tactile stimulation of other areas was less effective. Harassment of headstanding beetles provoked the release of yellow fluid from the buccal region or, more frequently, a clear liquid accumulated on the pronotal and elytral surfaces. Neither of these fluids was noticeably odoriferous.

Staining with the Wrights–nicotine–oxalic acid procedure (Yeager, 1938) revealed blood cells in the yellow buccal fluid. Since the fluid emerged from

other than ant-inflicted wounds, autohemmorhaging (reflex bleeding) is indicated. In a few instances, hemolymph appeared at the tibiofemoral joints. Most ants whose antennae or mouthparts contacted the blood quickly relinquished their hold, staggered about, and later groomed intensively.

The clearer dorsal fluid issued from a series of pores along the margins of the pronotum and elytra. The defensive nature of the secretion was graphically demonstrated when fire ants (*Solenopsis invicta*), blundering into it, dropped from the beetle and convulsed or curled up as if paralyzed. Some ants held their antennae to the rear as they grappled with the beetle and later groomed themselves for prolonged periods (behaviors not normally seen when they manipulate palatable prey). The beetles improve the exposure of the secretion by rotating their dorsal surface toward and leaning into the source of tactile stimulation. Most beetles escaped without apparent injury, although a few were overrun in populous laboratory colonies of fire ants. In contrast, larvae and adults of *T. molitor* quickly succumbed to the ants.

Secretion from 500 beetles was absorbed on small filter paper squares which were extracted into methylene chloride. Mealworm pieces treated with 1 beetle equivalent of the extract were examined only briefly by most fire ant workers, and grooming of antennae and mouthparts was commonly elicited. After 5 min, a mean of 5.6 ± 1.6 workers (mean $\pm$ SD; $N = 7$) contacted the treated baits whereas 11.3 ± 2.2 ants fed at controls ($P < 0.001$; Mann-

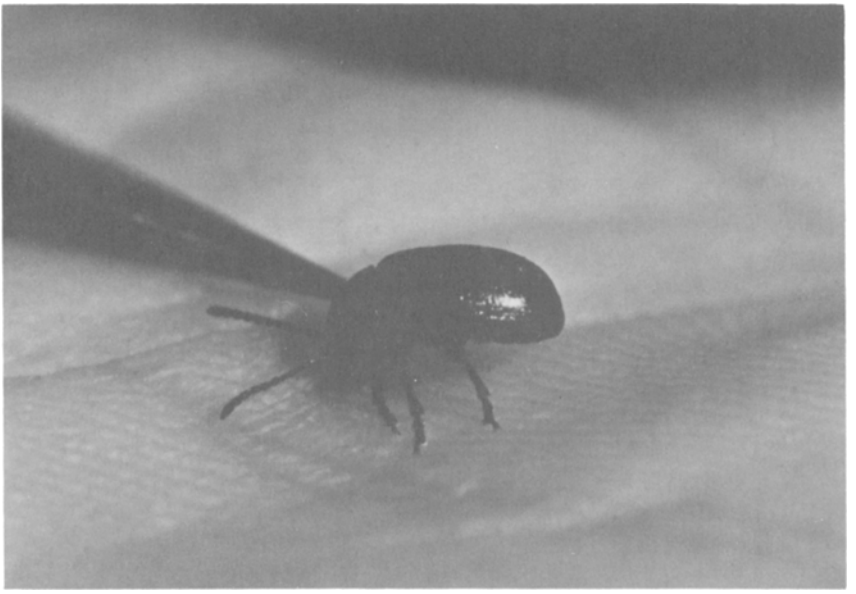

FIG. 3. Headstand posture elicited by prodding the elytra.

Whitney test). Tests for the presence of cardenolides in 10 beetle equivalents of secretion were negative. GC-MS analysis of diazomethane-treated methylene chloride extracts showed a mixture of straight-chain hydrocarbons, readily identifiable by their characteristic mass spectra (Stenhagen et al., 1974), including (with relative proportions in parentheses): tetradecane (1), pentadecane (2), hexadecane (4), heptadecane (7), and octadecane (8). An additional unidentified major component (relative proportion = 5) was detected with characteristic ions at m/z 182, 150, 95, 94, 88, and 79. This mass spectrum suggests a methyl ester of a terpenoid α-methyl carboxylic acid. Oleic acid was not detected, nor were the two larval iridoids.

Preliminary evidence suggests that adult *G. cyanea* are unpalatable to some birds. A captive yellow-throated warbler readily consumed small crickets and mealworms but quickly released unharmed a *G. cyanea* adult it had grasped in its beak. When offered additional beetles the bird ignored them, but it would accept other insects. On the other hand, the beetles certainly do not enjoy universal protection from predation. In two instances in the field, adults were found being devoured by the predatory pentatomid, *Stiretrus anchorago*.

DISCUSSION

Rigorous examples of chemically protected eggs are curiously sparse, a finding which prompted Orians and Jansen (1974) to point out the need for systematic studies of the subject. In the majority of investigations of insect defensive chemistry, the eggs are simply ignored. Even when they are chemically analyzed, eggs are usually tested only for compounds previously identified from larvae or adults, and bioassays against appropriate predators or parasites are generally omitted. With these limitations, it is likely that the chemical attributes of insect eggs have been underrated.

Deterrents or toxins might be anticipated in insect eggs like those of *Gastrophysa cyanea* that are conspicuous and deposited in clusters (Marsh and Rothschild, 1974; Orians and Janzen, 1974; Stamp, 1980; Hinton, 1981), especially when they are frequently exposed to predators like ants with their notoriously efficient foraging and mass-recruiting systems.

Our study demonstrates that the eggs of *G. cyanea* derive protection from oleic acid, and both the sheen of the eggs and ease of extraction suggest that this fatty acid is located near the eggs' exterior. Independent lines of evidence reveal that oleic acid and similar lipids are particularly suitable deterrents against ants. Objects coated with oleic acid are rejected from the nests of *Pogonomyrmex badius* (Wilson et al., 1958), *Myrmecia vindex* (Haskins, 1970), and *Solenopsis invicta* (Wilson et al., 1958; Blum, 1970; Howard and Tschinkel, 1976), and workers of *Monomorium pharaonis* are repelled by

fatty acids from the cornicles of aphids (P.L. Phelan, personal communication). Egg masses of the mosquito *Culex pipiens* bear droplets containing glycerides of several fatty acids (Iltis and Zweig, 1962) which interfere with predation by ants (Hinton, 1968). Although such lipids are not toxic (like compounds such as alkaloids or cardenolides), their deterrency against ants appears widespread. In the currency of biochemical pathways, they must certainly be less costly to produce than the more elaborate toxins.

Circumstantial evidence argues that the eggs of *G. cyanea* are also protected by an additional compound (or compounds). The egg's vivid yellow color is undoubtedly noticeable to many predatory arthropods and birds, against whom oleic acid may be ineffective. The bitter flavor of *G. cyanea* eggs is suggestive and, as mentioned earlier, a precedent for toxins in chrysomelid eggs has been established (Pasteels and Daloze, 1977; Daloze and Pasteels, 1979).

Carotenoid pigmentation, predominantly responsible for the yellow color of *G. cyanea* eggs, also occurs in other chrysomelids. Such pigments may function as aposematic signals, or perhaps they enhance a predator's reception of noxious scents (Rothschild, 1978, and references therein).

The diverse responses of *G. cyanea* adults to disturbances include dodging, "feigning death," buccal reflex bleeding, and secreting allomones from pronotal and elytral glands. Such reactions have been reported for other chrysomelids (reviewed by Deroe and Pasteels, 1977). The tendency of some harassed *G. cyanea* to elevate the abdomen is reminiscent of the headstand displays of certain tenebrionid beetles. In tenebrionids, the postures may serve to improve application of defensive secretion, and they may constitute warning signals to vertebrate predators (Eisner and Meinwald, 1966; Tschinkel, 1975). Perhaps similar functions apply to *G. cyanea*.

It is not yet clear whether the tendency of adults of *G. cyanea* to reflex bleed or secrete changes as the beetle ages. In the Colorado potato beetle, *Leptinotarsa decemlineata*, reflex bleeding is common in teneral adults but the elytral and pronotal glands are more frequently utilized when the cuticle toughens (Deroe and Pasteels, 1977).

The pronotal and elytral exudates of *G. cyanea* contain hydrocarbon and terpenoid components which could account for their deterrence. Aliphatic hydrocarbons also occur in the defensive glands of other insects, and it has been suggested that they enhance the spreading and penetration of defensive secretions into the enemy's cuticle (Gilby and Waterhouse, 1965). Unlike several members of the tribe Chrysolinini (Pasteels and Daloze, 1977; Pasteels et al., 1979; Daloze and Pasteels, 1979), the secretions of adult *G. cyanea* apparently lack cardenolides.

As the life cycle of *G. cyanea* progresses, some compounds are added to the defensive repertoire and others are deleted. The pattern develops from

eggs with oleic acid, to two iridoid compounds in larval secretions, to hydrocarbons and a different terpenoid molecule in the exudates of adults. This evidence does not preclude the possibility that some chemical deterrents persist throughout the insect's life. Indeed, in *Chrysolina polita*, although the complexity of cardenolide mixtures appears to increase with age, certain cardenolides can be found in all developmental stages (Daloze and Pasteels, 1979). However, our findings demonstrate that even when its life stages share a similar diet and/or habitat, an insect's catalog of defensive substances can undergo substantial ontogenetic modification. A multitude of factors must contribute to this temporal variation but noteworthy are potential differences among the stages in: (1) biochemical capabilities (Orians and Janzen, 1974), (2) predator/parasite pressure (both quantitative and qualitative), and (3) availability of alternative methods of predator/parasite avoidance (e.g., mobility). Ultimately, it may be demonstrated that a shifting chemical defense reduces the possibility that predators that have breached one defense will be able to exploit all stages of the animal's life history.

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BIOASSAY OF COMPOUNDS DERIVED FROM THE HONEYBEE STING[1]

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Abstract—Nine compounds identified from honeybee, *Apis mellifera* L., sting extracts and one compound identified from the honeybee mandibular gland were evaluated in a standardized laboratory test for their effectiveness in eliciting an alarm response from caged honeybees. Two, *n*-decyl acetate and benzyl alcohol, were judged ineffective as alarm pheromones. The remaining eight—2-nonanol, isopentyl acetate, *n*-butyl acetate, *n*-hexyl acetate, benzyl acetate, isopentyl alcohol, and *n*-octyl acetate from the sting and 2-heptanone from the mandibular gland—produced responses of similar frequency and strength.

Key words—Honeybee, *Apis mellifera* L., Hymenoptera, Apidae, alarm pheromone, isopentyl acetate, sting, mandibular gland.

INTRODUCTION

In 1962, Boch et al. identified isopentyl acetate (IPA) as an active component of the sting alarm pheromone of the honeybee, *Apis mellifera* L. When presented on cotton balls at the entrance of a colony, this compound alerted and agitated the guard bees but did not incite them to sting as did an equivalent number of odoriferous stings dissected from live bees and presented on cotton balls at the entrance. Boch et al. then suggested that IPA was only one of several active components of the sting pheromone. Free and Simpson (1968) also reported that targets treated with IPA provoked less stinging than targets treated with stings.

[1]In cooperation with Louisiana Agricultural Experiment Station. Mention of a proprietory product does not constitute an endorsement by the USDA.

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In 1965, Shearer and Boch identified 2-heptanone (2HPT) as an alarm pheromone produced in the mandibular glands of the honeybee. This compound also caused the guard bees to become alerted and agitated, issue from the hive, and attack a treated cork. Results of a comparison of 2HPT, IPA, and whole sting extracts also indicated that other sting-derived compounds contribute to the release of alarm behavior (Boch et al., 1970).

In 1978, Blum et al. analyzed extracts of honeybee stings and identified eight previously undetected compounds. We report here results of tests conducted to compare the activity as a chemical releaser of alarm behavior in the honeybee of each of these newly identified compounds and the two previously known compounds (IPA and 2HPT).

METHODS AND MATERIALS

The method of Collins and Rothenbuhler (1978) was used for bioassays. Caged brood from two individual queens (colonies) was emerged in an incubator during a 24-hr period and the young bees placed in glass-fronted wooden cages described by Kulincevic and Rothenbuhler (1973).

Newly emerged bees were used because they have not yet begun to produce alarm pheromone (Boch and Shearer, 1966) which could interfere with the assay. Although bees of this age are not normally involved in colony defense, in cages they respond with the same, but less intensive, behavior (Collins, 1980).

During the tests cages were arranged several inches apart on shelves in a 35° C walk-in incubator. Tests consisted of separate presentation to the bees of each component diluted in paraffin oil 1:9 (v/v). A 0.03-ml sample of this solution was presented under the wire floor of the cage on a small slice of No. 2 cork. The reaction by the bees involved both a flickering of the wings and increased locomotion in the cage. All tests were performed by one observer under double-blind condition; cage numbers were hidden until after testing and the cages were rearranged randomly after each complete sequence of tests.

The characters were measured as follows: (1) initial activity level—the number of bees moving on the floor, sides, and top of the cage prior to presentation of the stimulus; (2) seconds to react—the time until a group reaction was seen; (3) initial intensity of this reaction—graded as a weak, medium, strong, or very strong response based on the number and vigor of responding bees; (4) duration of the reaction; and (5) number of bees engaged in Nasonov fanning behavior at the end of the test. Following testing, a sixth character was calculated—frequency of no reaction—the number of times in which there was no reaction to the test material. Analysis of the data measured in seconds was by least-squares analysis of covariance, with seconds to react and duration being adjusted for initial activity level, and by a least significant

difference test. Intensity of the reaction, frequency of no reaction, and the number of bees fanning were analyzed using chi-square analysis. Spearman's rank correlation procedure was used to test correlations between measures.

The compounds tested were isopentyl acetate (IPA), benzyl acetate (BZA), 2-nonanol (2NL), benzyl alcohol (BZA1), *n*-hexyl acetate (nHA), *n*-butyl acetate (nBA), and isopentyl alcohol (IPA1), all obtained from Aldrich Chemical Co., Milwaukee, Wisconsin; *n*-octyl acetate (nOA) obtained from Matheson, Coleman and Bell, Norwood, Ohio; *n*-decyl acetate (nDA) obtained from Alfred Bader Library of Rare Chemicals, a division of Aldrich Chemical Co.: and 2-heptanone (2HPT) obtained from ICN Pharmaceuticals, Plainview, New York.

RESULTS

Table 1 shows the ranking of intensity, with a rank of 1 given to the compound having the most strong and very strong responses and a rank of 10 to the compound having the greatest number of weak or no reactions. The compounds ranked as the first six were not very different in intensity. IPA1 and nOA, ranked 7 and 8, showed a shift from strong to weaker or no responses.

Table 2 presents a summary of the measured responses to each of the 10

TABLE 1. INTENSITY OF RESPONSE BY CAGED HONEYBEES TO 10 COMPOUNDS TESTED AS ALARM PHEROMONES

		Intensity					
Rank[a]	Chemical	No response	Weak	Medium	Strong	Very strong	Total responses[b]
1	2-Nonanol	2	1	29	32	8	72
2	Isopentyl acetate	1	4	61	68	10	144
3	2-Heptanone	2	2	22	42	4	72
4	*n*-Hexyl acetate	1	3	33	31	4	72
5	*n*-Butyl acetate	5	5	31	27	4	72
6	Benzyl acetate	5	7	34	25	1	72
7	Isopentyl alcohol	14	10	35	13	0	72
8	*n*-Octyl acetate	17	12	28	15	0	72
9	*n*-Decyl acetate	35	5	28	4	0	72
10	Benzyl alcohol	37	12	19	4	0	72

[a]Rank was determined by relative number of observations in each category with 1 being the group with the greatest number of strong responses and 10 the group with the greatest number of weak or no responses.

[b]Eight cages with 30 bees each were tested 3 times a day for 3 days.

TABLE 2. MEASURES OF RESPONSE OF CAGED HONEYBEES TO 9 COMPOUNDS ASSOCIATED WITH HONEYBEE STING AND 1 FROM HONEYBEE MANDIBULAR GLAND

	% of total sting pheromone extract	No. nonreactors/total	$\bar{X}$ seconds to react	Intensity (rank)[a]	$\bar{X}$ duration of reactions (s)	No. of bees fanning
2-Nonanol (2NL)	8.6 mandibular gland	2/72	3.7 a	1	72.5 a[c]	203
2-Heptanone (2HPT)		2/72	4.1 a	3	52.8 bcd	91
Isopentyl acetate (IPA)	27.2	1/144	4.3 ab	2	51.0 bcd	86
n-Butyl acetate (nBA)	1.2	5/72	4.9 ab	5	45.7 bcd	21
n-Hexyl acetate (nHA)	3.4	1/72	5.0 ab	4	57.6 d	225
Benzyl acetate (BZA)	13.3	5/72	5.3 ab	6	76.3 a	432
Isopentyl alcohol (IPAl)	12.3	14/72	5.3 ab	7	43.8 bcd	8
n-Octyl acetate (nOA)	14.3	17/72	6.2 abc	8	45.2 bcd	42
n-Decyl acetate (nDA)[b]	1.2	35/72	6.9 bc	9	39.2 d	4
Benzyl alcohol (BZAl)[b]	3.4	37/72	8.6 c	10	43.5 cd	60

[a]Rank determined by the relative intensity of responses in repeated tests. Rank 1 had the most vigorous responses, rank 10 the weakest or no responses.
[b]These chemicals probably do not function as alarm pheromones based on the frequency of no reaction.
[c]Means within a column followed by the same letter(s) are not significantly different ($P < 0.01$).

compounds. The percentages of the total string pheromone are taken from the samples used for identification by Blum et al. (1978).

The two compounds, nDA and BZA1, which caused no response more than half the time, were judged to be ineffective in stimulating alarm behavior. When the bees did react to these compounds, the responses were slow, weak, and brief. For the remaining eight compounds, mean seconds to react were not significantly different, although they ranged from 3.7 to 6.2 sec. These results indicate that acetates with alcoholic moieties in the range C_4 (*n*-butyl) to C_8 (*n*-octyl) are of relatively equivalent activity. Furthermore, some secondary alcohols (e.g., 2-nonanol) appear to be as active as esters in releasing alarm behavior in worker bees.

The duration of the reaction was more variable than the speed of the reaction. Significantly longer mean responses were seen for BZA and 2NL, as well as larger numbers of fanning bees, as compared to the other compounds.

The correlations between these characters are presented in Table 3. The percentage of the total pheromone extract was not significantly related to any of the measures of response. The frequency of no reaction, the speed of the reaction, and the intensity of the reaction were all highly correlated ($P < 0.01$) with each other. The duration of the reaction was slightly less ($P < 0.05$) but still significantly correlated to those three measures. The only significant correlation for number of bees fanning was with the duration of the reaction.

Not only were there differences in the overall response to each of the compounds, but there were also colony differences. Table 4 shows a difference

TABLE 3. SPEARMAN'S RANK CORRELATION COEFFICIENTS FOR PROPORTION OF TOTAL PHEROMONE EXTRACT OF 9 COMPOUNDS ASSOCIATED WITH HONEYBEE STING AND 5 MEASURES OF RESPONSE BY CAGED HONEYBEES TO THESE COMPOUNDS

	Frequency of no reaction	Speed of reaction	Intensity of reaction	Duration of reaction	No. of bees fanning
Percent of pheromone extract	−0.176	0.069	−0.073	0.194	0.194
Frequency of no reaction		0.852**[a]	0.936**	−0.736*	−0.603
Speed of reaction			0.973**	−0.676*	−0.439
Intensity of reaction				−0.745*	−0.515
Duration of reaction					0.903**

[a] ** Significant at $P < 0.01$; * significant at $P < 0.05$.

TABLE 4. SIGNIFICANT COLONY DIFFERENCES IN RESPONSE OF CAGED HONEYBEES TO *n*-OCTYL ACETATE[a]

	Colony number	
	1	2
No. nonreactors/total	4/36	13/36*[b]
$\bar{X}$ seconds to react ± SD	4.5 ± 4.0	8.2 ± 6.8*
Intensity		
Weak	2	10**
Medium	17	11
Strong	13	2
Very strong	0	0
$\bar{X}$ duration of reaction (s) ± SD	55.0 ± 19.7	25.6 ± 21.5**
No. of bees fanning	23	19 NS

[a]Four cages of 30 bees each from each colony tested 3 times a day for 3 days.
[b]* Significant at $P < 0.05$; ** significant at $P < 0.01$; NS-not significant.

in response by the two colonies to nOA. Colony 2 showed a greater frequency of no response and responded more slowly, for a shorter period of time, and with less intensity, when it did show a response, than did colony 1.

In a preliminary experiment of the same design where a slightly different group of compounds was tested, three colonies were used. The responses of these colonies to three of the compounds in the test are shown in Table 5. Colony 7 responded equally to all three compounds. Colony 8 responded more slowly to IPA than the other two colonies. Colony 9 responded more slowly to IPA and BZA. Clearly, these results imply genetic differences in response to alarm pheromones.

TABLE 5. COLONY DIFFERENCES IN SPEED OF RESPONSE BY CAGED HONEYBEES TO 3 ALARM PHEROMONES[a]

	Chemical		
Colony No.[b]	IPA	IPAl	BZA
7	5.08	5.63	5.65
8	4.20	7.58[c]	5.56
9	7.54[c]	4.93	11.17[c]

[a]Values are mean seconds to react.
[b]Four cages of 30 bees each per colony were tested 3 times a day for 3 days.
[c]Significantly different from other values in column and row at $P < 0.05$.

DISCUSSION

No striking differences were observed in the effects of nine compounds identified from extracts of the honeybee sting and bioassayed for their possible function as alarm pheromones. Rather, the response to these compounds varied along a continuous scale. An observation was made, based on the frequency of nonresponse, that two of the compounds (nDA and BZA) were not functioning as alarm pheromones under the cage test conditions. Among the remaining seven and 2HPT, response varied gradually. Boch et al. (1970) reported that 2HPT was significantly less effective as an alarm pheromone than was IPA. However, results of our tests seem to indicate that, at least under the test conditions outlined, 2HPT is as effective an alarm releaser as several of the most active sting-derived compounds.

Boch and Shearer (1971) also compared six of the compounds tested here to IPA. Responses were ranked from 5 (equivalent to IPA) to 0 (not effective) based on the concentration required to attract a similar number of guard bees to a cork at the colony entrance. 2HPT and nBA were scored as 4, nHA as 2, IPA1 as 1, and nOA and BZA as 0. None of these were significantly different in our laboratory cage test. The difference in results may be explained by the fact that the cage test measures only alarm, whereas Boch and Shearer's test required attraction to the source and a higher level of activity. In addition, the bees used in the two experiments may have different reactivities such as the colonies mentioned earlier.

Why is this array of compounds produced by the honeybee if they all produce a similar reaction? Several investigators comparing IPA and whole stings (Boch et al., 1962; Free and Simpson, 1968) reported that, whereas IPA-marked targets elicited no stinging response, previously stung targets were stung again frequently. Some of the other chemicals tested probably mark objects, causing further stinging, or simply incite stinging by alerted bees, a behavior not measured by the cage test. It is also possible that some of the sting-derived compounds provide longer lasting alarm signals than IPA (i.e., 2NL and BZA). Further research is underway to examine these hypotheses and to determine whether additive or synergistic effects may occur when these compounds are used in combination.

It is not surprising that there are colony differences in the response to these pheromones. With such a complex array of possible alarm pheromones, it is not unexpected to find that some bees may respond more effectively to certain components than to others. Two inbred lines examined by Collins (1979) showed strikingly different responses to the same chemical, IPA, in cage tests as well as quite different extremes of defensive and stinging behavior in the field. It is possible that, in addition to the difference in response, a more careful analysis of sting extracts from genetically different colonies may show variation in the type and quantity of alarm pheromones present.

The lack of correlation between the relative amount of a compound in the extract and the resulting behavioral response is consistent with the results obtained by Boch and Rothenbuhler (1974), who found no correlations in behavior resulting from manipulation of field colonies and quantitative measures of IPA production. However, correlations between defensive behavior of field colonies and 2HPT was reported by Brazilian investigators (Kerr et al., 1974).

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FIELD RESPONSES OF REDHEADED PINE SAWFLY MALES TO A SYNTHETIC PHEROMONE AND VIRGIN FEMALES IN FLORIDA[1,2]

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Abstract—Field tests using the sawfly pheromone [3,7-dimethylpentadecan-2-ol acetate with (−)-*erythro* configuration (2*S*,3*S*)] were conducted near Gainesville, Florida, during 1978–1981 to determine the attraction of *Neodiprion lecontei* males to baited traps with respect to time of year and time of day. Greatest numbers of males were caught during May, July, and September in traps placed within a pine stand from July 1978 to July 1979. Males were only caught between 1400 and 2000 hr on 10 dates in June and 10 dates in September–October 1980, and 10 dates in June 1981, with greatest catches from 1600–1800 hr. Catches in a synthetic-baited trap and in virgin female-baited traps were similar with respect to time of day.

Key Words—*Neodiprion lecontei*, Hymenoptera, Diprionidae, 3,7-dimethylpentadecan-2-ol acetate, trapping, pheromone, time of year, time of day.

INTRODUCTION

The redheaded pine sawfly, *Neodiprion lecontei* (Fitch), is one of the most important native forest insects defoliating young pines in eastern North America (Benjamin, 1955). For several decades after World War II, spraying larval populations with insecticides was one of the most widely used

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techniques for controlling this pest. More recently, the use of nuclear polyhedral viruses alone or in conjunction with insecticides has shown considerable promise against *N. lecontei* (Wilkinson, 1969; Hopewell, 1977; DeGroot et al., 1979; Morris, 1980).

One useful tool that might also be used in the management of *N. lecontei* populations is a synthetic pheromone that would attract males. The existence of a sex attractant in *N. lecontei* females was first identified by Benjamin (1955). The free alcohol, 3,7-dimethylpentadecan-2-ol, was identified in female pine sawflies of the genera *Neodiprion* Rohwer and *Diprion* Schrank, and the acetate ester of this alcohol was found to be a major component of the *N. lecontei* sex attractant (Jewett et al., 1976). It was later determined that only one isomer (A-II) with (−)-*erythro* configuration (2*S*,3*S*) was attractive to *N. lecontei* males (Matsumura et al., 1979).

Mertins et al. (1975) developed a mathematical model to assess the feasibility of population suppression of the introduced pine sawfly, *Diprion similis* (Hartig), using pheromone-baited traps. According to this model, four generations of intensive trapping of males would theoretically be sufficient to eliminate *D. similis* from an isolated area. With this model in mind, we decided to obtain information on *N. lecontei* male responses to baited traps that could be used in designing later tests aimed at suppression of *N. lecontei* populations by the male-trapping technique. Here we report on the numbers of *N. lecontei* males caught in traps baited with synthetic A-II pheromone, with respect to time of year and time of day. Also reported are time-of-day responses of males to virgin females. All tests were conducted in areas where *N. lecontei* larval (feeding) populations were scarce or apparently absent.

METHODS AND MATERIALS

Time of Year. One Pherocon II® sticky trap was mounted at 1.5 m on the northeast side of five typical slash pines, *Pinus elliottii* Engelm. var. *elliottii*, distributed in an X pattern within a 50-tree block of 12-year-old trees located at Gainesville, Florida. One hundred μg of synthetic A-II pheromone [(−)-*erythro* (2*S*,3*S*) isomer of 3,7-dimethylpentadecan-2-ol acetate] in solvent was applied to a cotton dental wick ca. 2.5 cm long, which was then held in a clip at the upper center of one side of the inner sticky surface of each trap. The five traps were baited continuously from July 11, 1978, through July 30, 1979, with fresh baits placed out at ca. 2-month intervals. *N. lecontei* males were collected into xylene 3 times a week and identified by their relatively large size plus the presence of orange-brown pigment on the frons, clypeus, labrum, and mandible bases.[6]

[6]Identification and information were supplied by H.N. Greenbaum.

Time of Day. Two traps, each baited as above with 30 μg of A-II, were placed at 1 m elevation on PVC pipes located at the Forest Insect Laboratory in Gainesville and ca. 150 m north of the laboratory on the south side of Lake Alice. Several loblolly (*Pinus taeda* L.) and slash pines were growing within 50–100 m of the traps. Traps were checked at 2-hr intervals from 0600 to 2000 hr EDST on 10 dates during June and 10 dates during September–October 1980. Traps were not checked at 2-hr intervals from 2200 to 0400 hr, but were checked the next morning at 0600 hr to determine if overnight catches occurred. To determine if the male responses to the A-II pheromone were similar to responses to virgin females regarding time of day, four virgin female-baited sticky board traps (Coppel et al., 1960) were placed on longleaf (*P. palustris* Mill.) pine branches in a block of pine plantings in Gainesville, Florida, on June 1, 1981. On June 4, two additional traps were set out in similar manner. One Pherocon II trap baited with 30 μg of A-II also was placed on a longleaf pine branch in this area. To prevent possible cross-attraction of the pheromone, traps were placed at least 10 m apart. Traps were periodically checked as above and monitored for a 10-day period. Dead females were replaced with live females daily.

RESULTS

There were three peak periods of male catches during the growing season, two small peaks, one each in May and July, and a larger peak during September (Figure 1). One or more males were trapped during each month of this test. Males were trapped between 1400 and 2000 hr only, with the peak

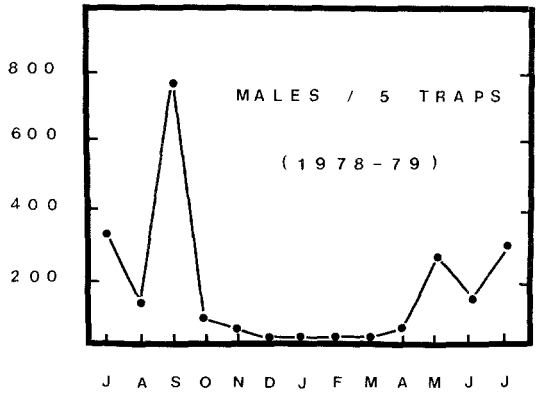

FIG. 1. Monthly catches of *Neodiprion lecontei* males in five traps, each baited with 100 μg of A-II pheromone [(−)-*erythro* (2*S*,3*S*) isomer of 3,7-dimethylpentadecan-2-ol acetate] and placed within a pine stand at 1.5 m elevation from July 11, 1978, through July 30, 1979, at Gainesville, Florida.

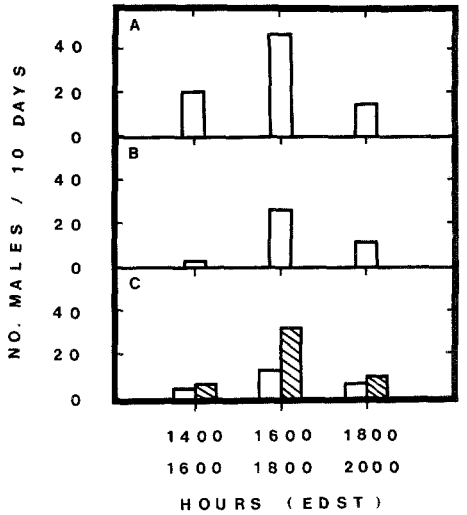

FIG. 2. Total numbers of *Neodiprion lecontei* male adults captured per 2-hr interval for a 10-day period. (A) Male adult catches in two traps baited with 30 μg of A-II pheromone [(−)-*erythro* (2*S*,3*S*) isomer of 3,7-dimethylpentadecan-2-ol acetate] at 1 m elevation during June 1980 in Gainesville, Florida. (B) Male adult catches in two traps baited with 30 μg A-II pheromone at 1 m elevation during September–October 1980 in Gainesville, Florida. (C) Male adult catches in 4–6, virgin female-baited traps (cross-hatching) and one A-II pheromone trap placed on longleaf pine branches during June 1981 in Gainesville, Florida. All traps (A–C) were continuously exposed in the field, but no males were caught outside of the periods shown.

period of catches from 1600 to 1800 hr (Figure 2). No males were caught overnight on any date. The catch peak periods were not different between the virgin female and A-II-baited traps. The number of female-baited traps attracting males varied from 0 to 3 daily (average 2.4 active traps/day), with mean catches of 1.67 males/active trap/day. This is similar to 1.8 males/A-II trap/day.

DISCUSSION

Figure 1 suggests that there are at least three generations of *N. lecontei* per growing season in north Florida, in agreement with Benjamin (1955). *Neodiprion insularis* (Cresson), a very closely related species found on Caribbean pine [*P. caribeae* (Morelet)] in Cuba, is reported to have four generations yearly (Hochmut, 1972). Field experience with N. *lecontei* in Florida over a 20-year period indicates that the fall larval generation is always greatest in numbers and the most destructive. This would appear to coincide

with the surprisingly large number (775) of males caught during September (Figure 1), despite the fact that larval (feeding) populations were rarely found during the entire 13-month period of this test.

Suppression of *N. lecontei* populations might theoretically be accomplished within two growing seasons, assuming that the male-trapping model given for *D. similis* by Mertins et al. (1975) applies to *N. lecontei* and that diapause is not a problem. Males were caught during late afternoon (1400–2000 hr EDST) in all three tests over a one-year period and at two locations. Male responses to the synthetic and virgin female-baited traps were similar, suggesting that in north Florida males respond to the female pheromone only during the afternoon. Further studies are necessary on male responses to A-II pheromone at different dosages, seasons, altitudes, distances, and in relation to parallel development of larval (feeding) populations of *N. lecontei*, before attempting suppression tests.

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RESPONSES OF THE OLFACTORY RECEPTORS OF *Scolytus scolytus* (F.) (COLEOPTERA: SCOLYTIDAE) TO THE STEREOISOMERS OF 4-METHYL-3-HEPTANOL

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Abstract—Electroantennogram (EAG) and single-cell recording techniques have been used to demonstrate the presence of separate receptors for (−)-threo- and (−)-erythro-4-methyl-3-heptanol on the antenna of *S. scolytus*. The majority of single-cell recordings showed spikes of two different amplitudes. The cell giving spikes of larger amplitude responded to the (−)-threo stereoisomer while the cell with the small-amplitude spikes responded to (−)-erythro-4-methyl-3-heptanol. It is suggested that in most recordings the two cells are associated with a single sensillum basiconicum.

Key Words—*Scolytus scolytus*, Coleoptera, Scolytidae, pheromone, 4-methyl-3-heptanol stereoisomer, olfaction, electrophysiology.

INTRODUCTION

Research during the past six years has established that bark beetle pheromones are stereospecifically biosynthesized and that the insects may respond differently to the enantiomers of the pheromonal components (Silverstein, 1979, and references therein). Thus, the aggregation pheromone of *Gnathotrichus sulcatus*, 6-methylhept-5-en-2-ol (sulcatol), is produced as a 65:35 mixture of the *S*-(+) and *R*-(−) enantiomers (Byrne et al., 1974), while that of *G. retusus* has recently been identified as *S*-(+)-sulcatol (Borden et al., 1980). In the field *G. sulcatus* responds only when both enantiomers are present (Borden et al., 1976), whereas attraction of *G. retusus* to *S*-(+)-sulcatol is inhibited by the presence of the *R*-(−) enantiomer (Borden et al., 1980).

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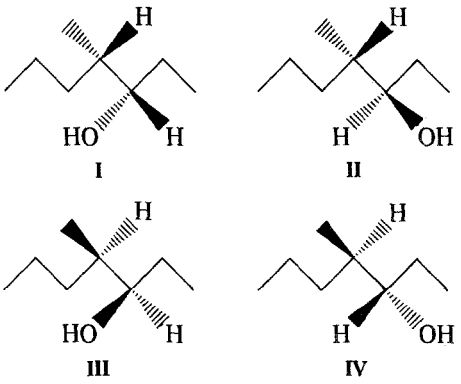
This ability to discriminate between enantiomers implies that the receptors for these compounds are chiral (Lensky and Blum, 1974), and receptor specificity for enantiomers has been demonstrated in several electrophysiological studies of bark beetles. In *Ips pini* and *I. paraconfusus*, single-cell recordings indicated the presence of cells specialized for individual components of the aggregation pheromone (Mustaparta, 1979). Thus the attractant ipsdienol, and the inhibitor ipsenol which is not produced by *I. pini*, stimulate separate groups of cells. Furthermore, two types of ipsdienol cells have been distinguished which differ, by about an order of magnitude, in their response to the enantiomers. In *I. pini* the majority of these cells responded preferentially to (−)-ipsdienol while the situation is reversed in *I. paraconfusus* (Mustaparta et al., 1980).

The large European elm bark beetle, *Scolytus scolytus*, aggregates in response to a male produced secondary attractant (Borden and King, 1977). The key components of the pheromone mixture are (−)-threo- (I) and (−)-erythro-4-methyl-3-heptanol (II). Males produce both (−)-threo and (−)-erythro stereoisomers while females produce smaller amounts of the (−)-erythro stereoisomer (Blight et al., 1979a).



Field trials of the 4-methyl-3-heptanol isomers indicated that only the (−)-threo and (−)-erythro stereoisomers attract more beetles than the control; the (+)-threo (III) and (+)-erythro (IV) stereoisomers were inactive rather than inhibitory (Blight et al., 1979b).

Initial results from an electrophysiological study using coupled gas chromatography/electroantennogram (GC-EAG) techniques indicated that, although the antennae of both male and female *S. scolytus* respond to all four isomers of 4-methyl-3-heptanol, significantly greater responses were elicited by the naturally occurring I and II than by III and IV (Blight et al., 1979a). We report here a more detailed electrophysiological study, using both EAG and

single-cell recording techniques, of the olfactory perception of the 4-methyl-3-heptanol stereoisomers by *S. scolytus*.

METHODS AND MATERIALS

Chemicals. 4-Methyl-3-heptanol (Aldrich, >99%; (±)-threo: (±)-erythro = 1 : 1) was separated by spinning band distillation using a Perkin-Elmer model 151, 200 plate, adiabatic, annular Teflon still, into (±)-threo- and (±)-erythro-4-methyl-3-heptanol.

The preparation of the stereoisomers of 4-methyl-3-heptanol and the analytical methods used to determine their purity have been previously described (Blight et al., 1979a). All samples were subjected to a final purification by preparative gas chromatography. The chemical purities of all the 4-methyl-3-heptanol samples are shown in Table 1.

Preparation. The insects used were obtained from laboratory cultures originating from several sites in the south of England and from Aargan, Switzerland. The EAG study was carried out with English beetles alone, while single-cell recordings were made from insects obtained from both the English and Swiss laboratory cultures. Newly emerged adults were immobilized by cooling for a few minutes at 0° C and were prepared using the techniques described by Angst and Lanier (1979).

EAG Recordings. EAGs were recorded with Ag–AgCl glass electrodes filled with saline solution (composition as in Maddrell, 1969, but without the glucose). The recording electrode was positioned in the distal end of the antennal club and the indifferent electrode was positioned either in the scape or mouthparts.

The signals generated by the antenna were passed through a high-

TABLE 1. CHEMICAL PURITIES OF THE 4-METHYL-3-HEPTANOL SAMPLES

Compound	Chemical Purity[a]	Impurities[a]
(±)-threo-4-methyl-3-heptanol	99.1%	0.9% (±)-erythro
(±)-erythro-4-methyl-3-heptanol	98.2%	1.8% (±)-threo
(+)-threo-4-methyl-3-heptanol	>99.9%	<0.1% (−)-erythro
(−)-threo-4-methyl-3-heptanol	99.7%	0.3% (+)-erythro
(+)-erythro-4-methyl-3-heptanol	99.5%	0.4% (−)-threo
		0.1% (−)-erythro
(−)-erythro-4-methyl-3-heptanol	99.5%	0.3% (+)-threo
		0.2% (+)-erythro

[a]Estimated by gas chromatography and high pressure liquid chromatography (See Blight et al, 1979a).

impedance preamplifier ($>10^{10}$ Ω) and an automatic DC offset, to a Devices 3121 biological oscilloscope. The output from the oscilloscope was monitored on a chart recorder (pen response 380 mm/sec).

Single-Cell Recordings. Recordings from olfactory cells associated with individual sensilla were made using tungsten microelectrodes (Boeckh, 1962). The indifferent electrode was positioned in the insect's mouthparts and the recording electrode was brought into contact with the surface of the antennal club until impulses were recorded. Signals were amplified with an AC-coupled amplifier, displayed on an oscilloscope, and stored on magnetic tape. Permanent copies of the responses of the olfactory cells were made on a Siemens Mingograph 34T ink jet recorder.

Stimulation. Several delivery systems were used. In most cases the stimulus was delivered into a purified and humidified airstream (800 ml/min) which flowed continuously over the preparation.

EAG Studies. The stimulus delivery system used for the pure stereoisomers of 4-methyl-3-heptanol utilized a disposable Pasteur pipet cartridge. The sample, in 10 μl purified pentane, was applied to a 20 × 4-mm filter paper strip, the solvent was allowed to evaporate, and the filter paper was inserted into a Pasteur pipet. The vapor from the cartridge was injected into the airstream passing over the antenna by means of a second airstream. The latter was controlled with a solenoid valve which was operated by an electronic timer. The stimulus duration was 1 sec, and the volume of air delivered through the pipet was 5 ml. Fresh cartridges were prepared for each stimulation.

A second delivery system was used for the (±)-threo- and (±)-erythro-4-methyl-3-heptanol samples and employed a 10-ml glass syringe. Samples were again applied in 10 μl pentane to 20-mm filter paper disks. After the solvent had evaporated, the filter paper was placed in the syringe which was then sealed and stored at −20° C. Paired syringes were prepared for each stimulus concentration. Five ml of the vapor from the syringe was injected manually into the airstream passing over the antenna. The stimulus duration was approximately 1 sec.

Samples were presented twice to each antennal preparation. A stimulus with pure pentane served as the control and the amplitude of the response obtained was subtracted from the sample EAGs. Responses were normalized with respect to the response obtained from a filter paper loading of 1.6×10^{-7} g (±)-threo-4-methyl-3-heptanol. In order to allow complete recovery of the sensory cells, stimuli were presented at intervals of 2–20 min; the exact interval was determined by the concentration of the previous stimulus.

Single Cell Studies. Two stimulus delivery systems were again used, one of which is to be described elsewhere (Angst et al., in preparation). The second delivery system utilized a glass cartridge (volume 1.7 ml) prepared in the same

manner as the Pasteur pipets used in the EAG study. Paired cartridges were prepared for each stimulus concentration and were stored at −20° C until required. The vapor from the cartridge was injected, by means of the solenoid valve and electronic timer system (see above), into the airstream (800 ml/min) which passed continuously over the antenna. The stimulus duration was 2 sec, and the volume of air delivered through the cartridge was 5 ml. The presentation of the samples and the intervals between stimulations were the same as for the EAG study. Impulse frequency was determined as the number of spikes elicited during the first 1 sec after stimulus initiation.

Estimation of Stimulus Concentration for the EAG Study. The concentration of the stimulus arriving at the antenna was established by gas chromatographic analysis. Vapor samples from the delivery systems and liquid calibration samples were both analyzed, under the same conditions, on a 38-m × 0.5-mm ID Carbowax 20M WCOT column. The latter was fitted into a Carlo Erba series 2150 gas chromatograph equipped with a flame ionization detector and an LDC 304-50 computing integrator. Samples were injected at ambient temperature, and after 2 min the oven temperature was raised rapidly to 80° C. This procedure maintained peak shape for the vapor samples.

A calibration curve of peak area vs. concentration was constructed. Five ml of the vapor from the delivery system was then injected, and the concentration was calculated from the calibration curve. The stimulus concentration was estimated in terms of the number of molecules per milliliter of air which stimulated the antenna.

RESULTS

The stimulus calibration curves for the delivery systems used in the EAG study are shown in Figure 1. Values below the detection limit of the gas chromatographic system were obtained by extrapolation.

EAG Responses. The EAG responses of *S. scolytus* to the stereoisomers of 4-methyl-3-heptanol were similar in shape and amplitude to those given by other Scolytidae to their pheromonal components (e.g., Angst and Lanier, 1979). The response of individual preparations remained relatively constant over a period of several hours (Figure 2).

The mean responses of male and female antennal preparations to the stereoisomers of 4-methyl-3-heptanol over the concentration range 1.8×10^{6}–1.8×10^{13} molecules/ml of air (corresponding to filter paper loadings of 10^{-12}–10^{-5} g) are shown in Figure 3. Although all four isomers elicited EAG responses from both sexes, the responses to the (−)-threo and (−)-erythro stereoisomers were greater than those to (+)-threo- and (+)-erythro-4-methyl-

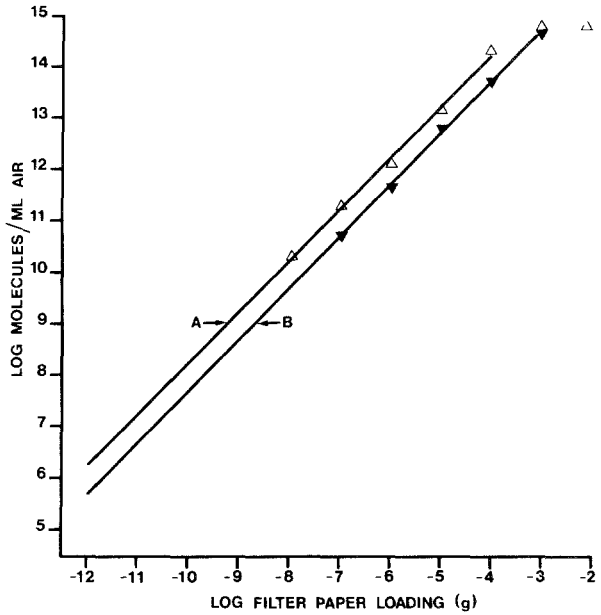


FIG. 1. Calibration of the EAG stimulus delivery systems showing the number of odor molecules/ml of air stimulating the antenna for different filter paper loadings of 4-methyl-3-heptanol. (A) Using the Pasteur pipet delivery system (extrapolated from 10^{-9} g filter paper loading). (B) Using the glass syringe system (extrapolated from 10^{-8} g filter paper loading).

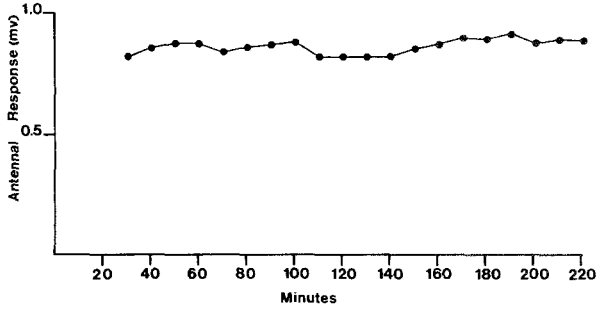


FIG. 2. Response of an *S. scolytus* male antenna to repeated stimulation with the (±)-threo-4-methyl-3-heptanol standard. The first stimulation was 30 min after implantation of the electrodes. Thereafter stimuli were presented at 10-min intervals. The mean response of the preparation was 0.85 ± 0.01 mV.

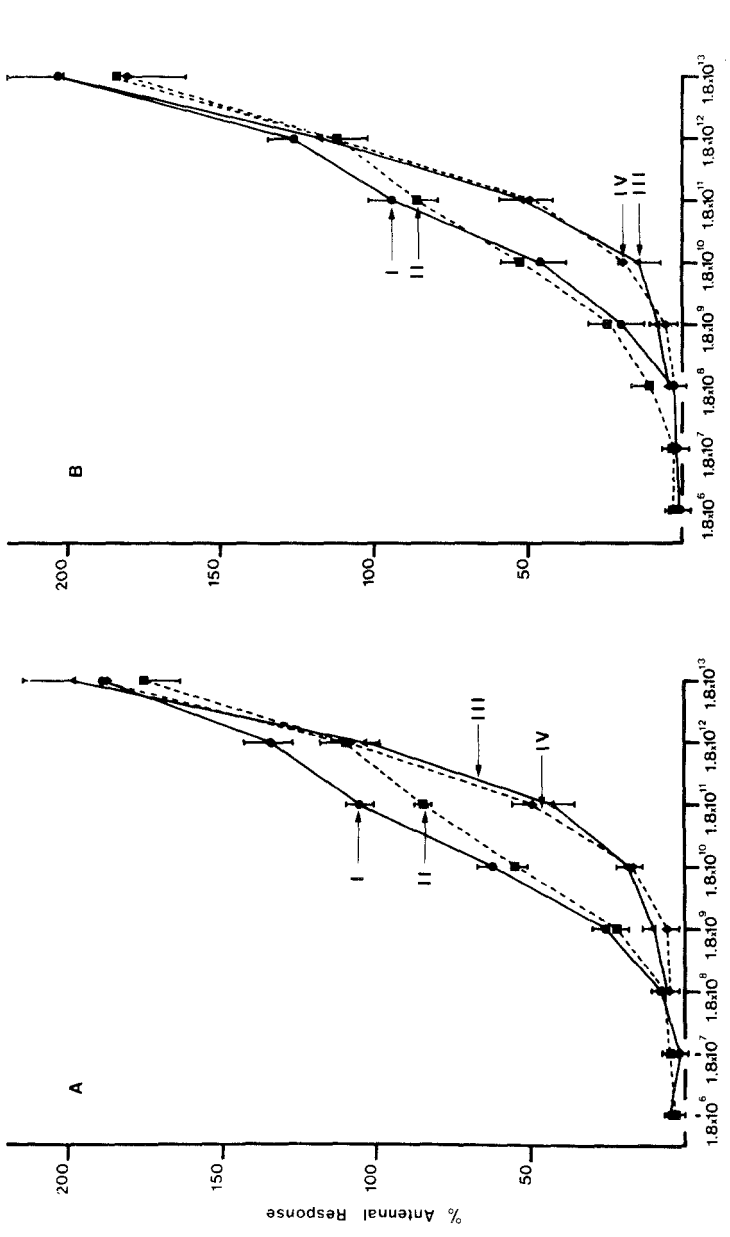


FIG. 3. EAG responses of (A) males and (B) females to (−)-threo- (I), (−)-erythro- (II), (+)-threo- (III), and (+)-erythro-4-methyl-3-heptanol (IV). Each point is the mean response ± SE normalized with respect to the (±)-threo-4-methyl-3-heptanol standard. Where standard errors overlap only half the SE bar is shown. For I and II, eleven antennal preparations of each sex were tested; for III and IV, seven preparations of each sex were tested.

3-heptanol. Moreover the threshold concentrations for both sexes were at least an order of magnitude lower for the naturally occurring (−) stereoisomers than for the (+) isomers.

There were no differences between the amplitudes of the EAG responses given by female antennae to the (+)-threo and (+)-erythro isomers or between the responses to the (−)-threo and (−)-erythro stereoisomers. Similarly, no differences were apparent between the responses of males to (+)-threo- and (+)-erythro-4-methyl-3-heptanol. However, there were differences in the amplitudes of the responses of male antennae to the (−)-threo and (−)-erythro isomers over part of the concentration range tested (Figure 3).

Figure 4 shows the EAG responses of males and females to (±)-threo- and (±)-erythro-4-methyl-3-heptanol over the concentration range 4.8×10^5–5.7×10^{14} molecules/ml of air (corresponding to filter paper loadings of 10^{-12}–10^{-3} g). With these substances the stimuli were presented using the syringe delivery system (see Methods and Materials). Threshold response concentrations of male and female preparations were the same for both isomers. Although no differences were found between the amplitudes of the EAG responses of females to either isomer, there were differences in the responses of male antennae to (±)-threo- and (±)-erythro-4-methyl-3-heptanol.

Single-Cell Responses. Single-cell recordings were obtained from both male and female preparations. Forty of the cells recorded were specialized to one of the naturally occurring (−)- stereoisomers of 4-methyl-3-heptanol to which they responded strongly at low stimulus concentrations. In addition these cells responded only weakly, or not at all, to high concentrations of all other compounds tested. These included the mixed isomers of multistriatin and the terpenes (−)-β-pinene and (−)-α-cubebene. The spontaneous activity of the cells was low (<5 impulses/sec) and the responses remained constant for several hours. The majority of recordings were obtained from cells situated in fields 1 and 2 (Figure 5), and usually spikes of two different amplitudes were observed. The cell giving spikes of the larger amplitude was specialized to the (−)-threo stereoisomer, while that giving the smaller amplitude spikes responded preferentially to (−)-erythro-4-methyl-3-heptanol (Figure 6). Both cells responded weakly to the (+) stereoisomers. The dose–response curves for such a pair of cells are shown in Figure 7.

In addition, two recordings showed the presence of one cell only, belonging to one of the cell types shown in Figure 7. A cell recorded from a male *S. scolytus* preparation was specialized to the (−)-threo stereoisomer while another cell recorded from a female preparation was specialized to (−)-erythro-4-methyl-3-heptanol.

No differences were apparent between the response of cells recorded from English and Swiss populations of *S. scolytus*, and no major differences were

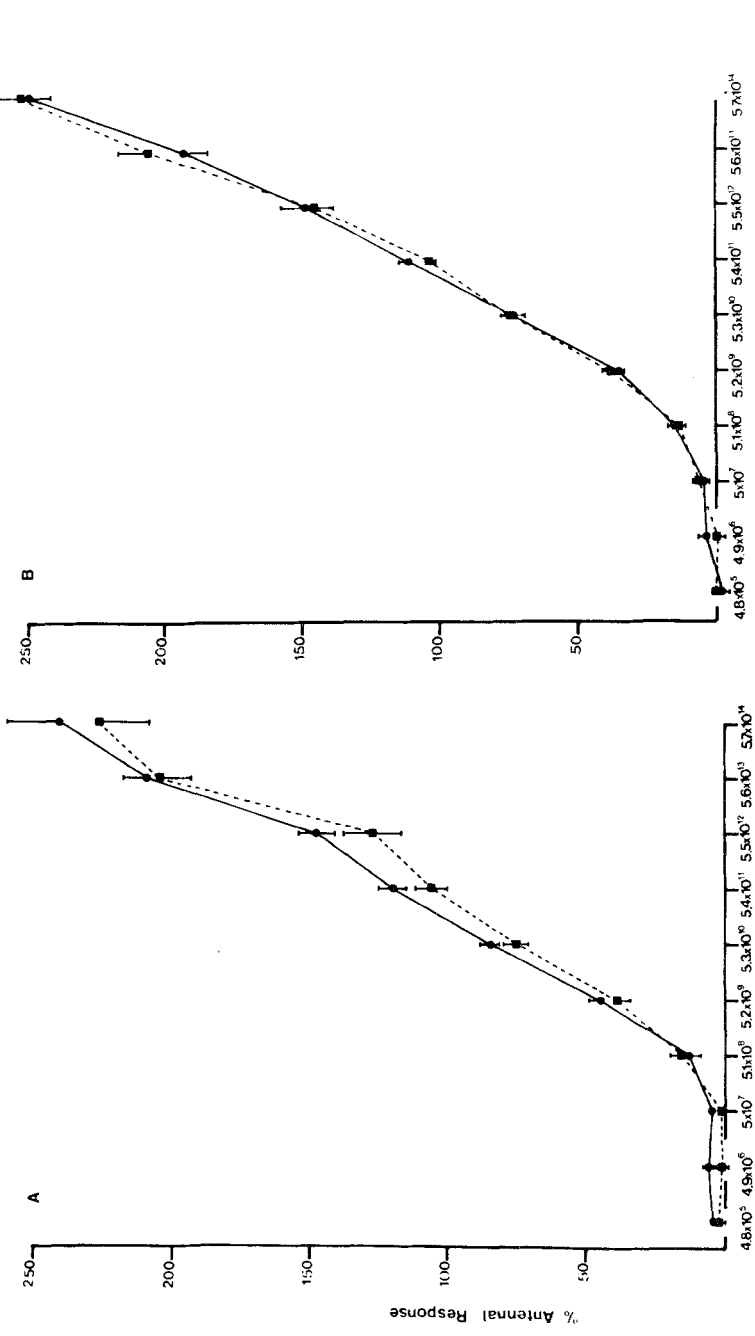


FIG. 4. EAG response of (A) males and (B) females to (±)-threo-(●) and (±)-erythro-4-methyl-3-heptanol (■). Each point is the mean response (15 preparations) ± SE normalized with respect to the (±)-threo-4-methyl-3-heptanol standard. Where standard errors overlap only half the SE bar is shown.

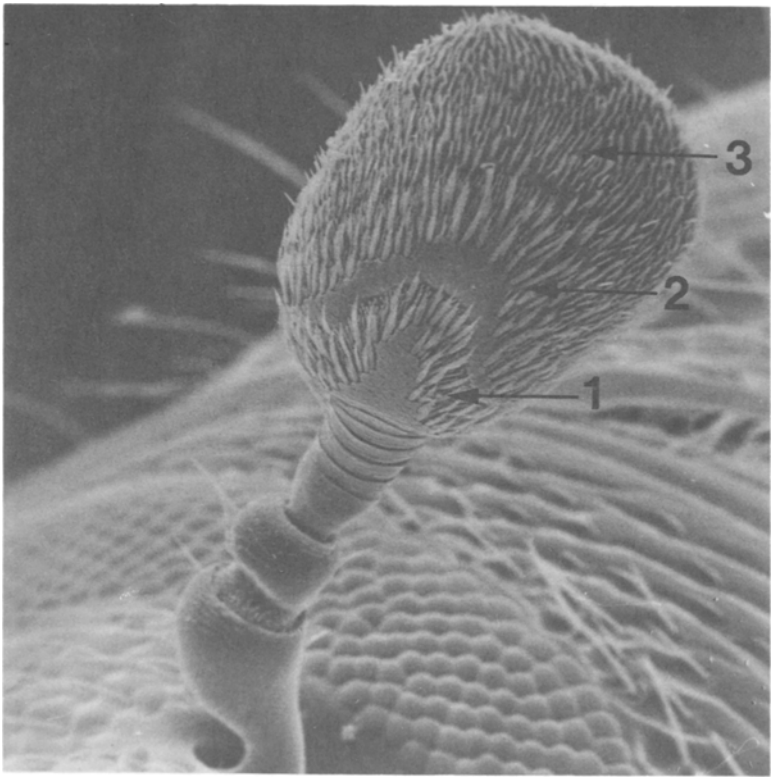


FIG. 5. Scanning electron micrograph of the antennal club of an *S. scolytus* male beetle showing the three sensory fields.

observed between cells recorded from males and females. However, small variations in the responses to the (+) stereoisomers were apparent between individual cells.

Threshold response concentrations for the (−) stereoisomers were similar for each pair of cells, although occasionally differences were observed in the threshold concentration where more than one recording was made from the same preparation. Thus a pair of cells recorded from field 1 were found to have threshold concentrations two orders of magnitude greater than another pair recorded from field 3 of the same preparation. However, despite this difference, the overall responses of both pairs of cells to the test compounds were essentially similar. The cells with high and low threshold concentrations appeared to belong to the same cell types.

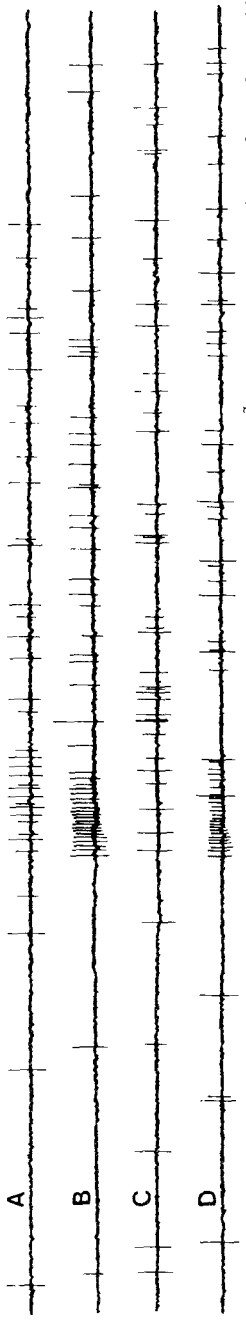


FIG. 6. Responses of two olfactory cells from an *S. scolytus* male to stimulation with 10^{-7} g (filter paper loading) of (+)-threo- (A), (−)-threo- (B), (+)-erythro- (C), and (−)-erythro-4-methyl-3-heptanol (D).

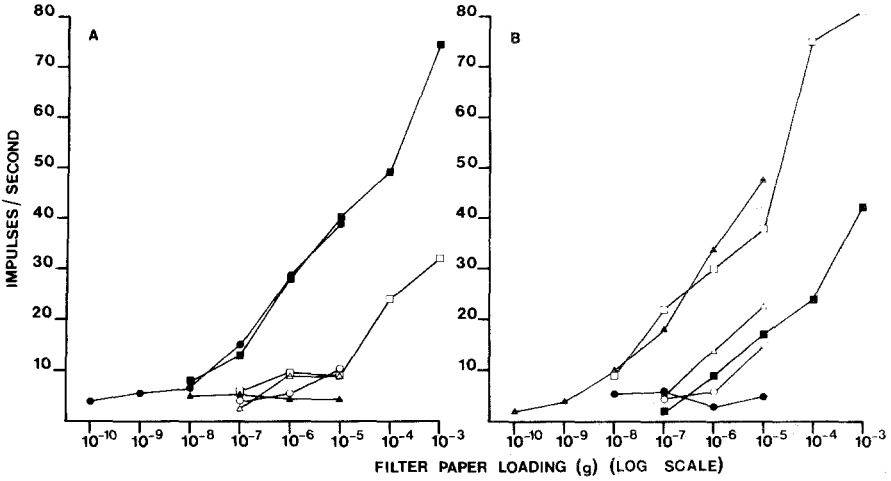


FIG. 7. Dose–response curves of a typical pair of olfactory cells, from an *S. scolytus* male preparation, specialized to (−)-threo- and (−)-erythro-4-methyl-3-heptanol. The responses of the cell giving the large-amplitude spikes (A) and the cell giving smaller-amplitude spikes (B) are the means of two stimulations and are shown as the number of spikes elicited during the first 1 sec. after the beginning of stimulation with (+)-threo-[○], (−)-threo-[●], (+)-erythro-[△], (−)-erythro-[▲], (±)-threo-[■], and (±)-erythro-4-methyl-3-heptanol [□].

DISCUSSION

The antennal discrimination observed in the EAG study in favor of (−)-threo- and (−)-erythro-4-methyl-3-heptanol reflects the known production and field activity of these compounds and is in agreement with the previous electrophysiological study (Blight et al., 1979a,b). The results obtained with the coupled GC-EAG technique showed differences in the response of males to stimulation with 2.5×10^{-9} g of each of the (−) stereoisomers. It was suggested (Blight et al., 1979a) that the significantly greater response elicited by the (−)-threo stereoisomer might be an experimental artifact associated with a deterioration in column efficiency, but the results reported in this paper show that this was not the case.

It is difficult to estimate the stimulus concentration delivered to the antenna in the coupled GC-EAG system. However, since the peak widths of compounds eluting at short retention time from the WCOT columns were only a few seconds, the maximum stimulus concentration (resulting from the injection of 2.5×10^{-9} g of stereoisomers) was probably of the order of 10^{11}–10^{12} molecules/ml of air. It is over this concentration range that the differences in amplitude of male response to (−)-threo and (−)-erythro

stereoisomers and to (±)-threo- and (±)-erythro-4-methyl-3-heptanol are most apparent in the present EAG study. This contrasts with the situation in females where no amplitude differences were apparent in the EAG responses elicited by (±)-threo and (±)-erythro samples. This is also in agreement with the results obtained with the (−) stereoisomers using the coupled GC-EAG technique (Blight et al., 1979a).

These observations suggest that the differences in the response to the (−) stereoisomers reflect their interaction with separate receptors on the antenna. The present single-cell study has confirmed this hypothesis and demonstrated the presence of separate olfactory cells specialized to either (−)-threo- or (−)-erythro-4-methyl-3-heptanol.

The majority of single-cell recordings have shown the presence of two cells, of which the one with the larger amplitude responded preferentially to the (−)-threo stereoisomer while that with the smaller amplitude showed receptor specificity for (−)-erythro-4-methyl-3-heptanol. In general, the number of impulses resulting from stimulation of each pair of cells with the same concentration of the appropriate (−) stereoisomer was similar, suggesting the presence of equivalent numbers of receptors on each cell, since impulse frequency is thought to be directly related to the number of stimulus acceptor interactions (Kaissling and Priesner, 1970).

The amplitude of the EAG response is also considered to be related to the number of receptors responding (Boeckh et al., 1965). Thus the occurrence of pairs of methyl heptanol-specific cells, each with approximately the same number of receptors, cannot account for the observed EAG amplitude differences between males and females to the two (−) stereoisomers. The fact that two recordings have shown the presence of individual cells with receptor specificity for one of the (−) stereoisomers may be pertinent. However, the number of cells recorded is too small to be representative of the distribution on the antenna as a whole and a larger sample is required to demonstrate differences in the proportions of methyl heptanol-specific cells.

The density of sensilla in fields 1 and 2 precludes the implantation of an electrode into a predetermined sensillum (see Figure 5). However, only two types of sensilla are found in appreciable numbers in these fields: sensilla basiconica and sensilla chaetica (Henderson and Wadhams, 1981). Of these, only sensilla basiconica have been implicated in the olfactory perception of pheromones in bark beetles (Payne et al., 1973; Dickens and Payne, 1978). Transmission electron microscopy has shown that the sensilla basiconica of *D. frontalis* are innervated by one or two bipolar cells (Dickens and Payne, 1978). The sensilla basiconica and sensilla trichodea of *S. multistriatus* are innervated by two cells (Borg and Norris, 1971), and it is probable that a similar situation exists in the closely related *S. scolytus*. Since the majority of recordings of methyl heptanol-specific cells have been obtained from fields 1

and 2 and show the presence of two cells, we suggest that they are associated with a single sensillum basiconicum.

Variations were observed in the threshold concentration (expressed as filter paper loading) between pairs of cells recorded from fields 1 and 3 on the same antennal preparation. This may reflect the shielding of the sensilla basiconica in fields 1 and 2 by the overlying sensilla chaetica. Since the EAG response curves for the (−) stereoisomers provide an overall estimate of the sensitivity of the olfactory cells to these compounds, no attempt has been made to determine the stimulus concentrations, in molecules per milliliter of air, for the single-cell studies.

The differences in the slopes of the EAG response of both male and female *S. scolytus* to the (+) and (−) stereoisomers of 4-methyl-3-heptanol are not fully understood. The plot of response against log dose for the (−) stereoisomers in the EAG study (Figure 3) is a smooth curve which gives a linear relationship between log dose and log response. The corresponding plots for the (−)-threo and (−)-erythro stereoisomers appear to be a summation of this same smooth curve with sigmoid curves having the upper saturation response limits of ca. 60% and 50%, respectively, at a stimulus concentration of approximately 10^{11} molecules/ml of air. It is tempting to suggest that the sigmoid components arise from the specific (−)-threo and (−)-erythro receptors while the smooth curve is associated with the stimulation of cells specialized to other pheromone components but which are unable to discriminate between the 4-methyl-3-heptanol stereoisomers. However, this hypothesis is so far unsupported by single-cell recordings. Alternatively the EAG response curve for the (+) stereoisomers may reflect their ability to interact, albeit weakly, with both (−)-threo and (−)-erythro receptors whereas both specialist cells usually show little if any response to the "wrong" (−) stereoisomer at the highest stimulus concentration tested.

It has recently been shown that *S. multistriatus* also possesses olfactory cells specialized to (−)-threo-4-methyl-3-heptanol, although no cells specific to the (−)-erythro stereoisomer are present on the antenna (Angst et al., in preparation). Since female *S. multistriatus* produce only the (−)-threo stereoisomer (Lanier et al., 1976) it appears that the 4-methyl-3-heptanol receptor systems of *S. scolytus* and *S. multistriatus* are tuned to the stereoisomeric composition of their individual aggregation pheromones. However, the receptor system of *S. scolytus* is more flexible, enabling it not only to discriminate between (−)-threo- and (−)-erythro-4-methyl-3-heptanol, but also to distinguish between the different proportions of the (−)stereoisomers. It is interesting to note that air entrainment extracts of male *S. scolytus* boring into *U. procera* have shown variations in the proportions of the (−)-threo and (−)-erythro stereoisomers produced (Blight et al., 1978).

Further work is in progress to elucidate the role of the naturally occurring stereoisomers of 4-methyl-3-heptanol in the chemically mediated behavior of *S. scolytus*.

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DISRUPTION OF MALE SPRUCE BUDWORM ORIENTATION TO CALLING FEMALES IN A WIND TUNNEL BY SYNTHETIC PHEROMONE

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Abstract—Male spruce budworm [*Choristoneura fumiferana* (Clem.)] moths were held for 3 hr in a wind tunnel and subjected to various concentrations of background synthetic pheromone. They were then exposed to calling females and their response was recorded. The background pheromone was presented either as discrete turbulent plumes or as a uniform permeation throughout the tunnel. The numbers of males wing-fanning and flying in response to the calling females decreased as the concentration of background pheromone increased. Of the males which flew, a higher proportion progressed upwind in the discrete plumes than in the uniform permeation, an indication that structure in the pheromone cloud is necessary for upwind progression. In both discrete plumes and uniform permeation fewer males were able to locate the females (i.e., disruption was greater) as the concentration of synthetic pheromone increased, but for the same total release rates, disruption was greater when the synthetic pheromone was released in discrete plumes rather than in a uniform permeation. This implies that disruption which involves luring males to sources of synthetic pheromone is more effective than masking female plumes by uniform permeation and suggests that it is more efficient to release pheromone from a few potent sources than from numerous low-potency sources.

Key Words—*Choristoneura fumiferana* (Clem.), Lepidoptera, Tortricidae, sex pheromone, mating disruption, sex attraction.

INTRODUCTION

Numerous attempts have been made to disrupt the mating behavior of Lepidoptera, but as yet the processes involved in disruption are poorly

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understood [see Richerson (1977) and Cardé (1981) for summaries of the possible mechanisms]. Furthermore, most reports include few data on the amounts of chemical released into the air space. Usually only the quantities of formulation are cited, and even these usually refer to quantities of formulation applied, rather than to the deposit achieved. Exceptions are the quantification of atmospheric concentrations of chemical by USDA workers in Beltsville, Maryland (Plimmer et al., 1979; Caro et al., 1977, 1980; Bierl-Leonhardt et al., 1979), and of spruce budworm pheromone by the New Brunswick Research and Productivity Council in Canada (Wiesner et al., 1980). However, even in these studies no correlation has yet been established between atmospheric concentration of chemicals and degree of mating disruption.

This paper reports on experiments carried out in a wind tunnel to determine the effects of different atmospheric concentrations of the synthetic pheromone of the spruce budworm [*Choristoneura fumiferana* (Clem.)] on one component of the mating process, the ability of male spruce budworm to locate calling females over a distance of more than 1 m.

METHODS AND MATERIALS

Insects. The insects were first or second laboratory generation, reared on artificial diet [McMorran (1965), as modified by Harvey (1974)]. They were separated by sex as pupae. Emerged male moths were collected each day between 1000 and 1600 hr and kept under a 17 : 7 light–dark cycle, with scotophase from 2030 to 0330 hr. Female moths were collected daily and transferred to a 17 : 7 light–dark cycle, with the scotophase advanced 3.5 hr (1600–2300), so that the females would start calling at 1300 hr (Sanders and Lucuik, 1972). All rearings and the adult moths were kept at 21° C and ca. 70% relative humidity. The adult holding cages were sprayed with water daily to provide drinking water.

Pheromone sources. The sex pheromone of the spruce budworm is an approximately 96: 4 blend of (*E*,*Z*)-11-tetradecenal (Sanders and Weatherston, 1976). The two isomers, supplied by ChemSampCo., Cleveland Ohio, were analyzed for purity and further purified at the Forest Pest Management Institute (Canadian Forestry Service) in Sault Ste. Marie, Ontario. Each isomer was >98% pure and contained none of the congeneric acetate or alcohol.

A 96 : 4 blend of the two isomers was incorporated into a solid PVC formulation (Fitzgerald et al., 1973; Daterman, 1974; Sanders, 1981) to give a range of concentrations from 3% pheromone by weight down to 0.0003% in factors of 10. The PVC was cut into cylindrical pellets, 5 mm long by 4 mm in diameter. Experiments were conducted with pellets which had been exposed at room temperature for 20–50 days. Estimates of release rate by weight loss

(Sanders, 1981) indicated that a pellet of this size, containing 3% of the synthetic attractant, was releasing pheromone at about 1 μg/hr and that each tenfold decrease in concentration gave a tenfold decrease in release rate. A pellet containing 0.03% would then release ca. 10 ng/hr, which is close to the rate of release by a virgin female spruce budworm (Silk et al., 1980; Sanders, 1981). Fluorescent dyes were incorporated into each formulation to avoid confusion. When not in use, the pellets were stored at $-11°$C.

Wind tunnel. The wind tunnel consisted of a working space, 90 × 90 cm in cross-section by 195 cm long, with an impeller fan blowing air in one end and a large hood evacuating air out of the building at the other. The upwind end of the working space was covered by two removable screens, 30 cm apart, each consisting of a double layer of cheesecloth dyed dark grey to reduce contrast with the tunnel floor and sides. These two screens created a mixing chamber, similar to that described by Kennedy et al. (1981). The upwind screen had taped onto its upwind side a 9 × 9 grid of 2.5-cm-wide masking tape. This procedure created turbulence, and tests with cigarette smoke showed that the air in the space between the two screens was thoroughly mixed as a result. The downwind screen, which was left untaped, then acted as a smoothing screen, and air passing through it flowed smoothly through the observation area. When a single source of pheromone was pinned on the downwind side of the downwind screen, the resulting plume was very narrow with little turbulent structure, and the success rate of moths in arriving at the source was low. Therefore, each pheromone source on this screen was backed by a 2.5 × 2.5 cm square of black tape. This created a well structured plume with time-averaged diameters of 12 cm at 50 cm downwind from the release point, 25 cm at 100 cm, and 30 cm at 195 cm, the exit end of the tunnel. The tunnel was illuminated from above by two 40-watt fluorescent lamps, giving a light intensity of 75 lux at the tunnel floor. Air flow was maintained at 33 cm/sec throughout.

Experimental Design. Experiments were designed to determine the success rate of males released at the downwind end of the tunnel in locating two calling females at the upwind end in the presence of synthetic pheromone.

The synthetic pheromone was presented in two ways. The first method created a uniform permeation of attractant throughout the tunnel. Eighty-one pheromone sources were pinned individually at the intersections of the 9 × 9 tape grid on the upwind side of the upwind screen. The pheromone-laden air was then thoroughly mixed by turbulence in the space between the upwind and downwind screens and passed into the observation area as a structureless fog of pheromone. The second method created discrete plumes of pheromone in the tunnel working space. Nine pheromone sources were pinned to 2.5-cm-square pieces of tape on the downwind side of the downwind screen in a 3 × 3 grid. By using pheromone sources of the appropriate concentration, it was

possible to approximate the same total release rate via the two systems. For instance, 81 sources of 0.003% concentration released approximately the same amount as $9 \times 0.03\%$. An unquantified amount of pheromone was presumably adsorbed by the screens in the uniform permeation treatment. However, this adsorption was minimized by setting up the treatment the previous afternoon to allow the screens to become saturated with the pheromone. To avoid problems of contamination, different upwind and downwind screens were used for each method. Also, in any one series of experiments, the lowest concentration was tested first and increasingly higher concentrations were tested on subsequent days. At the end of a series, the glass surfaces of the tunnel were cleaned with hexane and then washed with soap and water. All cages for releasing the insects in the tunnel were washed each time after they were used. When the tunnel was not in use all fans were left running, the cages were placed in the tunnel, and a 15-watt UV lamp was placed in the tunnel to break down any residual pheromone.

Experimental Procedure. The synthetic pheromone sources were placed in position the previous afternoon to reduce adsorption losses during the experiment, as already described. The following morning two 2-day-old female moths were housed individually in circular screen cages, each 3 cm in diameter by 2.5 cm long. Two females were used to guard against the possibility that one would be defective and would not call normally. These females were taped side by side to a wire stand placed at the upwind end of the tunnel adjacent to the screen (Figure 1). They were then left untouched

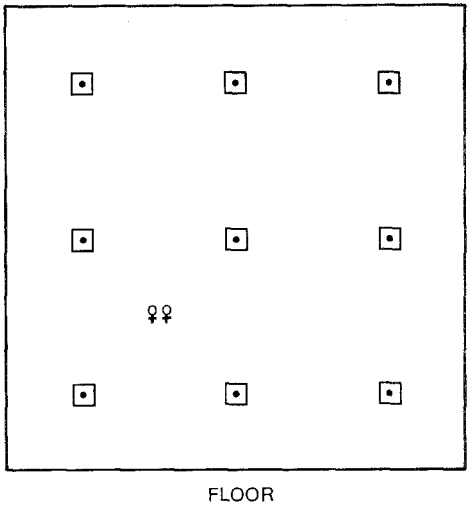

FIG. 1. Diagram of screen at upwind end of tunnel showing location of the nine discrete pheromone sources and of the two females.

throughout the experiment so that there would be no interference with their calling.

Fifteen males were assayed against each pair of females. The males were each 1 day old and were placed individually in cylindrical screen cages, ca. 3.5 cm in diameter and 7 cm long, on the morning of the assay. These cages were placed in the wind tunnel at 1130 hr on wire shelves on the opposite side of the tunnel to the female so that the females were subjected to the prevailing pheromone treatment but not to the female pheromone plume. The experiments began at 1430 hr, by which time the males had been exposed to the synthetic pheromone for 3 hr, simulating conditions in a field disruption trial where males are subjected to pheromone throughout their adult lives. Assays were carried out by moving the cages, one at a time, into the center of the female pheromone plume at the downwind end of the tunnel. After exposure of 0.25 min, the lid of the cage was removed to permit the male to escape. Males which had not flown at the end of 1 min were forced into flight by a gentle jarring of the cage.

The following activities were recorded: wing fanning; and the times that males reached a point 50 cm upwind from release, a point 150 cm upwind, the upwind screen, and the female cages or pheromone source.

All times were recorded to the nearest 0.01 min with a watch subdivided into units of 0.01 min. Observations on each male were terminated as soon as the male landed on the cages housing the females, on the upwind screen or on the sides, roof, floor, or downwind screen and remained there for 0.1 min. The data on males responding to the females by wing fanning or flying and on males reaching the females or the synthetic pheromone were analyzed for independence by χ^2. The flight times were subjected to analysis of variance. The significance level for differences was 0.05 in all analyses.

RESULTS

The response of the males to the different treatments is shown in Table 1. The experimental protocol clearly promoted a high level of responsiveness in the males. Of the controls, 95% took flight voluntarily, and of these, 93% arrived successfully at the calling females. Exposure for 3 hr to the lower concentrations of background pheromone, $9 \times 0.003\%$, $81 \times 0.0003\%$, and $81 \times 0.003\%$, did not significantly reduce the number of males responding to the calling females by wing fanning and flying. However, as the background concentration increased, the incidence of wing fanning and voluntary flight decreased. Of those males taking flight voluntarily, a constant percentage made its way upwind to 150 cm in the presence of the discrete plumes, regardless of concentration of pheromone. However, fewer males progressed

TABLE 1. PERCENTAGES OF MALE SPRUCE BUDWORM RESPONDING TO CALLING FEMALES IN A WIND TUNNEL IN PRESENCE OF VARIOUS CONCENTRATIONS OF SYNTHETIC ATTRACTANT RELEASED AS DISCRETE PLUMES OR AS UNIFORM PERMEATION

Treatment	Pheromone sources[a]	Average concentration of pheromone in tunnel (pg/m^3)	Male response							
						% Voluntary fliers arriving at				
			N	% wing fanning[b]	% flying voluntarily[b]	N	150 cm[b]	♀♀[b]	Synthetic source[b]	Upwind screen[b]
Control			45	93a	95a	43	93a	93a	0b	0b
Discrete plumes	9 × 0.003%	10	60	95a	97a	58	95a	86a	5b	2b
	9 × 0.03%	100	105	83b	93a	97	96a	49b	41a	2b
	9 × 0.3%	1,000	85	29d	77c	58	97a	19c	76a	0b
	9 × 3%	10,000	60	22d	63c	38	92a	8c	71a	0b
Uniform permeation	81 × 0.0003%	10	60	90a	93a	56	93a	88a	–	5b
	81 × 0.003%	100	105	90a	99a	104	91a	88a	–	3b
	81 × 0.03%	1,000	105	62c	87b	92	80b	50b	–	24a
	81 × 0.3%	10,000	60	7e	61c	37	46c	0c	–	41a

[a]Percentages refer to concentrations of pheromone by weight in lures.
[b]Numbers in each column followed by same letter are significantly different at $P = 0.05$.

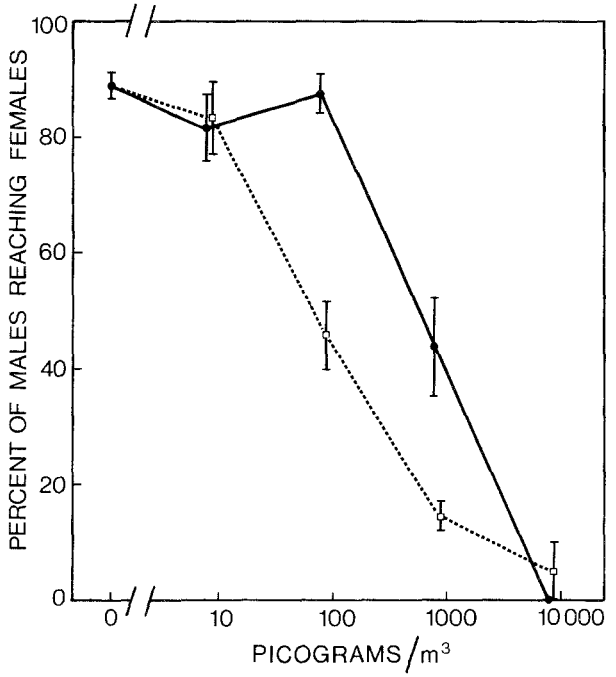

FIG. 2. Percentages of male spruce budworm successfully locating two calling females in the presence of different amounts of synthetic pheromone released either from nine discrete sources (squares and dotted line) or as a uniform permeation (circles and solid line).

upwind to 150 cm in the higher background concentrations of uniform permeation.

The reduction in number of males unsuccessfully locating the calling females provides a measure of disruption of male orientation. This is shown in Table 1 and graphically in Figure 2. The tabulated data are shown as percentages of the total numbers of insects for analysis by χ^2, but the data in Figure 2 are shown as the average percentage of each replicate of 15 insects ($\pm$ 1 SE, to give an indication of the reproducibility of the data).

As the background concentration of pheromone was increased by increasing the concentration of either the discrete plumes or the uniform permeation, fewer males were able to locate the calling females. In the case of the discrete plumes, this evidence of confusion became particularly marked when the individual pheromone sources were releasing pheromone equal to or greater than 10 ng/hr, which approximates the release rate of a virgin female. In the case of the uniform permeation where there were no discrete plumes competing with the females, disoriented males flew directly to the screen, ignoring the females. However, evidence of disruption in the uniform

permeation first occurred at a higher total pheromone release rate than with the discrete plumes, and this suggests that different mechanisms may be operating.

A higher proportion of the males had to be forced into flight as the concentrations increased (i.e., those not flying voluntarily in Table 1), but once they were in flight their behavior did not differ significantly from that of the voluntary fliers. A total of 91 males had to be forced to fly, and of these, 45 (49%) progressed upwind to the end of the tunnel. Of the 45, 11 reached the females, but of those 11 only 1 was successful in the presence of the highest pheromone concentration; hence, even when the forced fliers were included with the voluntary fliers, the resulting percentages reaching the females did not differ significantly.

The times taken for the males to fly 100 cm under the different concentrations of the discrete plumes and of uniform permeation are shown in Table 2. Clearly, in both instances, flight times increased as the concentration of the background pheromone increased. However, a complication arises here which affects the interpretation of the results. At the lower concentrations of background pheromone ($81 \times 0.0003\%$, $81 \times 0.003\%$, and $9 \times 0.003\%$) most of the males arrived at the calling females, and this implies that they had followed the female plume, whereas at the higher concentrations ($81 \times 0.03\%$ and $9 \times 3\%$) the males missed the females, the implication being that they were not following the female plumes. In the middle range of background concentrations ($81 \times 0.003\%$, $9 \times 0.03\%$) numbers were more evenly distributed between those locating the females and those missing the females. Comparison between the two categories (Table 3) shows that differences were not significant in the uniform permeation, but that flight times were significantly longer for males arriving at synthetic sources than for those arriving at the females against the background of discrete plumes. As a result, flight times in Table 2 in the presence of $9 \times 0.03\%$ discrete sources were not significantly different from times in the absence of background pheromone or in the presence of $9 \times 0.003\%$ because most went to the females. However, in the presence of $9 \times 0.3\%$ and $9 \times 3\%$ discrete sources, times were significantly longer because most males went to the synthetic pheromone sources. In view of these differences the analysis should have been carried out separately for those males arriving at females and those missing the females. However, this was impractical. First of all, it is only in the middle ranges that the two categories occur together. Secondly, it proved impossible to determine definitively whether a male was progressing upwind throughout his flight under the influence of the female or of the synthetic pheromone. Under uniform permeation, male behavior ranged from appearing to be following the plume but losing it close to the source to flying upwind well outside the boundaries of the female plume. In the case of the discrete plumes, most

TABLE 2. FLIGHT TIME (SEC X 10^{-2}) OF MALE SPRUCE BUDWORM OVER 1 M IN A WIND TUNNEL IN PRESENCE OF TWO CALLING FEMALES AND FOUR DIFFERENT ATMOSPHERIC CONCENTRATIONS OF SYNTHETIC PHEROMONE RELEASED IN NINE DISCRETE PLUMES OR AS UNIFORM PERMEATION

	Average atmospheric concentration of synthetic pheromone (pg/m^3)				
	0 (controls)	10	100	1000	10,000
Discrete plumes					
Pheromone sources	0	9 X 0.003	9 X 0.03	9 X 0.3	9 X 3
N	39	55	94	63	43
Time	8.44 ± 0.60a	12.14 ± 1.79ab	14.23 ± 1.35b	22.54 ± 1.83c	21.77 ± 2.71c
Uniform permeation					
Pheromone sources	0	81 X 0.0003	81 X 0.003	81 X 0.03	81 X 0.3
N	39	54	94	69	24
Time	8.44 ± 0.60a	10.67 ± 0.66b	11.29 ± 0.99b	11.20 ± 0.60bc	15.04 ± 1.74c

[a]Numbers in each row followed by the same letter not significantly different at $P = 0.05$.

TABLE 3. FLIGHT TIME (SEC X 10^{-2}) OF MALE SPRUCE BUDWORM OVER 1 M IN A WIND TUNNEL IN PRESENCE OF TWO CALLING FEMALES AND TWO DIFFERENT CONCENTRATIONS OF SYNTHETIC PHEROMONE RELEASED IN NINE DISCRETE PLUMES OR AS UNIFORM PERMEATION OF ATMOSPHERE, SUBDIVIDED INTO MALES ARRIVING AT CALLING FEMALES AND THOSE UNSUCCESSFUL (ARRIVING AT DISCRETE SOURCES OR END OF TUNNEL).

Pheromone treatment	Pheromone sources	Average concentration of synthetic pheromone (pg/m^3)	Males arriving at females	Males missing females
Discrete plumes	9 X 0.03%	100	10.81 ± 0.96a (N = 48)	17.80 ± 2.49b (N = 46)
Uniform permeation	81 X 0.03%	1000	11.36 ± 0.78a (N = 45)	10.92 ± 0.95a (N = 24)

[a]Numbers in same row followed by same letter not significantly different at $P = 0.05$.

males, at the higher concentrations, followed a plume from a pheromone source rather than the female plume, often switching from one plume to another as they progressed upwind. However, other males clearly followed the female plume initially, but veered off to a synthetic plume nearer to the source where the plume boundaries were sufficiently narrow to become distinct from one another.

DISCUSSION

There is a good correlation among the incidence of wing fanning, voluntary flight, and success in locating females. All three decrease with increasing concentration of background pheromone, and this suggests either that the males cannot perceive the female-produced pheromone against the background of synthetic pheromone or that the males become adapted after 3 hr of exposure at a rate proportional to the pheromone concentration. If the two females are each emitting pheromone at 10 ng/hr (Silk et al., 1981) then, with a windspeed of 33 cm/sec and a plume 30 cm in diameter, the concentration of pheromone at the downwind end will be 250 pg/m^3. This concentration is well below the background produced by the 9 × 0.3% or 81 × 0.03% treatments (Table 1) and the reduction in wing fanning, voluntary flight, and location of females in these two treatments is therefore to be expected.

At the next highest concentration there was considerable disruption by the discrete plumes, 9 × 0.03%, but very little by the uniform permeation

created by the 81 × 0.003% treatment, even though the 9 × 0.03% and the 81 × 0.003% treatments were producing similar time-averaged concentrations. This can be explained by the fact that turbulence in the discrete plumes will produce much higher concentrations locally in bursts (Murlis and Jones, 1981) and the female-produced plume will therefore contain much higher concentrations than the time-averaged 250 pg/m^3, enabling the male moths to detect the plume against the uniform background of 100 pg/m^3 created by the 81 × 0.003% treatment. The 9 × 0.03% pheromone sources, on the other hand, each create a turbulent plume very similar in concentration to the female-produced plume. Therefore, a significant number of males would be expected to follow the plume of synthetic pheromone instead of the female-produced plumes, as did in fact occur (Table 1).

The contrast between plume and background will become more pronounced as the male flies upwind, since the pheromone plume becomes narrower, causing the concentration to increase well above the concentration of the background uniform permeation. At 150 cm up the tunnel (ca. 50 cm from the source) the plume is 12 cm in diameter, giving a time-averaged concentration for a female-produced plume of 1500 pg/m^3. Consequently, a male crossing the female plume at the 150 cm mark may be able to differentiate the plume and so home in on the female even against a background of 1000 pg/m^3; this would explain the high proportion of males locating the female at the 81 × 0.03% treatment. At the 81 × 0.3% treatment, however, where the background concentration is in the order of 10,000 pg/m^3, the female plume would be well masked even at very short range, a supposition which is supported by the fact that no males were able to locate the females under that treatment. It is also possible at this high concentration of uniform permeation that the males have become adapted. This possibility is supported by the fact (Table 1) that fewer males progressed upwind in the higher concentrations of uniform permeation than at comparable concentrations distributed among discrete turbulent plumes. The assumption is that, in turbulence, the variations in concentration prevent adaptation from occurring.

Cardé and Hagaman (1979) found that gypsy moth males flew more slowly at higher pheromone concentrations, but the significance of this was somewhat confounded by the fact that the moths also slowed down at the lowest concentrations tested. The results here demonstrate a consistent trend over a wide range of concentrations. They also establish that such reduction in flight speed is in response to an increase in absolute concentration, not to an increase in the contrast between the plume and its surroundings. In clean air an increase in the concentration of the plume will increase the contrast, whereas in these experiments the increase in background decreased the contrast, but still produced a decrease in flight speed.

Decrease in speed in response to increased pheromone concentration will in itself tend to produce a lower success rate among males in a field situation since it exposes them for a longer period to the vagaries of sudden gusts of wind. On the other hand, the tighter zig-zagging which this behavior produces (Kennedy et al., 1980, 1981) makes it less likely that the males will fly out of the plume and not be able to relocate it. Consequently, the end result is debatable.

It is therefore concluded that disruption of male orientation can be achieved in two ways. First, the background concentration may be raised to such a high level that the males cannot perceive the female plume (diffuse permeation). Results here suggest that concentrations in excess of 1000 pg/m^3 will be necessary to achieve disruption by this method. Second, discrete sources of pheromone can be used, each producing a plume at least as concentrated as that of a virgin female. The effectiveness of this method will depend upon the potency of sources used, but the evidence here suggests that a smaller total amount of pheromone would be required to achieve the same level of disruption as with uniform permeation.

Schmidt et al. (1979), using very high release rates (300 and 1800 mg pheromone/hectare/hr), recorded reduction in the number of spruce budworm matings in small field cages, but the atmospheric concentrations were not measured. Carles et al. (1979) did measure atmospheric concentrations of pheromone in laboratory experiments on mating reduction of grape vine moths (*Lobesia botrana*). At 18 $\mu g/l$ (1.8 $\mu g/m^3$), a massive dose compared with those in the present study, they achieved 52% mating reduction, but in their experiments six pairs of moths were confined in a 4000-ml glass cylinder, which would make chance encounters highly probable and long-range orientation by the males irrelevant.

Field studies on the effects of different concentrations and of the density of individual pheromone sources on mating disruption were carried out by Shorey and his coworkers on *Trichoplusia ni* and *Pectinophora gossypiella* (Farkas et al., 1974; Shorey and Gaston, 1974; Gaston and Shorey, 1974). Their results suggested that the total amount of synthetic material released was more important than the number and spacing of the pheromone sources. However, their pheromone sources all released pheromone far faster than a virgin female, so there was no comparison between uniform permeation and discrete sources as described here. Similarly, most formulations currently being tested for mating disruption are acting as discrete sources, each releasing pheromone faster than a female.

Microcapsules are an exception: each capsule releases small amounts, but large numbers of capsules collectively produce a uniform permeation. If discrete sources, each out-competing a virgin female, are more effective than the same amount of pheromone released as uniform permeation, then microcapsules, although convenient because they are easily applied, would be less efficient than discrete sources such as hollow fibers or plastic flakes.

A number of field experiments have compared the effectiveness of microcapsules, plastic laminates, and hollow fibers (e.g., Taschenbert et al., 1976; Richerson, 1977; Schwalbe et al., 1979; Gentry et al., 1980), but interpretations of the results in the context of the present discussion are impossible since no comparative data on release rates are provided in any of these studies.

If, as postulated here, the use of discrete sources depends on attracting the maximum number of males to the wrong source, then it is essential that the optimum blend of the pheromone components be used to maximize attractancy. Also, since it is possible that males, once attracted to a synthetic source, may subsequently leave it and find a female, the effectiveness of this technique would be greatly enhanced if males arriving at pheromone sources were removed from the population by trapping or by killing them with the addition of an insecticide or sterilant to each pheromone source.

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MATE-SEEKING BEHAVIOR AND REDUCED MATING BY *Ephestia cautella* (WALKER) IN A SEX PHEROMONE-PERMEATED ATMOSPHERE[1]

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Abstract—The mating rate of *Ephestia cautella* (Walker) was inversely related to the level of pheromone permeating the air. However, permeation did not prevent mating by altering the number of females calling or the frequency, duration, or pattern of male flight. Instead, permeation probably prevented mating by reducing the distance from which a male can respond to a female. The pheromone level necessary to achieve a particular reduction in mating increases with moth density.

Key Words—Almond moth, *Ephestia cautella*, Lepidoptera, Pyralidae, stored products, pest management, behavior, density, flight.

INTRODUCTION

Control of insect pests generally means either outright killing them, although this may be delayed until metamorphosis with juvenile hormones, or denying them some essential resource such as mate, food, oviposition site, or shelter. Several methods of denying pests functional mates have been widely tested. These include the use of sterile insects (LaChance, 1974), mass trapping (Hüber et al., 1979), and permeation of the atmosphere with sex pheromone to block premating communication (Mitchell, 1975). Sower and Whitmer (1977) demonstrated that permeation of the air with pheromone substantially

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

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reduced mating and thus population growth rates of the almond moth, *Ephestia cautella* (Walker), and a closely related species *Plodia interpunctella* (Hübner). For both species, the mating rate was inversely related to population density. Further, Sower et al. (1975) showed that mating rate of *P. interpunctella* is inversely related to the concentration of pheromone permeating the air.

We compare here the behavior of *E. cautella* in permeated and nonpermeated atmospheres; demonstrate that permeation does not prevent mating by altering the number of females calling or the frequency, duration, or pattern of male flight; and suggest that permeation actually prevents mating by reducing the communication distance. The influence of pheromone release rate and moth density upon behavior and mating rate are also examined.

METHODS AND MATERIALS

The *E. cautella* used in this study were reared on laboratory diet at 27° C and 60% relative humidity under a 14-hr light–10-hr dark photoperiod with scotophase beginning at 1400 hr EST. Unmated adults of known age were obtained at ca. 0900 hr by collecting each adult in a separate vial as it eclosed. Males were released into the room the day prior to the test and were 28 hr old at the beginning of the test period. Females were used the same day and were 4 hr old.

Most of the observations were made at temperatures within the 20–30° C range, except those with 100 μg/hr pheromone release rate which were made at temperatures between 30 and 35° C. Statistical analyses show that temperature did not affect results within the 20–30° C range. Each of the tests was done in one of three 5.9 × 5.9 × 2.4-m rooms. The lighting, the marking of ceiling with a 5 × 5 grid of 1.4-m^2 blocks, and the release and observation of moths were as previously described (Hagstrum and Davis, 1980). Briefly, with four pairs of moths the locations of all moths were mapped, and the duration of all flights or calling bouts, the time at which each flight or calling bout began, and the time of each mating were recorded during a 100-min observation period. Most matings occurred on the ceiling. With 40 pairs, only the time of each mating was recorded. At the lowest moth density, some flights were followed by noting the time at which a male entered each block so that we knew the sequence in which blocks were entered and could determine the time spent flying in each block. The presence of other moths and in particular whether a female was calling or not when a male entered a block was also noted.

The sex pheromone of the almond moth, (*Z,E*)-9,12-tetradecadien-1-ol acetate (Brady et al., 1971; Kuwahara et al., 1971), contained <5% of the *Z,Z*

isomer as an impurity and was evaporated from Conrel® fibers placed at 4 or 40 locations. When 4 hollow fibers were used, they were attached to the ceiling 3/5ths of the way between the center of the room and the midpoint of each wall. Males did not spend more time in blocks adjacent to these 4 fibers than in other blocks. When 40 fibers were used, one fiber was attached in the center of each of 24 ceiling blocks (fiber was not placed in the block in the center of the room because a 15-W light bulb was already located there), and the remainder were distributed evenly along the walls 3/4ths of the way between the floor and the ceiling. At either fiber density males did not land and approach artificial sources as often as they would approach a calling female, i.e., observed on only five occasions. Release rates of 1, 10, 100, and 1000 μg/hr, as estimated by meniscus recession, were obtained with 1 fiber at each of 4 locations and 1, 10, or 100 fibers at each of 40 locations, respectively. Because release rate decreased as the meniscus receded, increasing the air space in the fiber above the pheromone, all fibers were cut off below the pheromone level so that a new meniscus would form near the open end of the fibers. Fibers were placed in the room 24 hr prior to observations. Controls and tests with 1, 10, 100, and 1000 μg/hr release rates were replicated 12, 8, 10, 4, and 4 times, respectively, using four pairs of *E. cautella* in the room and 6, 4, 12, and 4 times with 40 pairs (no 1000 μg/hr tests with 40 pairs). An analysis of variance was used to analyze data and only differences significant at the 1% level are considered statistically significant.

RESULTS

The percentage of flights ending in mating was inversely related to the sex pheromone release rate, but permeation did not prevent mating by reducing the numbers of females that were calling during male flights or the frequency or durations of male flights (Table 1). Also, the pattern of male flight was not consistently altered in such a way that males mated less often because they entered blocks in which females were calling less often. Instead, permeation reduced the percentage of these entries that resulted in mating from 13% in control to 4% at the 1 μg/hr release rate. Over all treatments, males entered blocks with calling females in proportion to their frequency in the room, i.e., 5.9% of entries (SD = 5.0, N = 23) were into blocks with calling females when females were calling in 1 or 2 blocks and 15.2% of entries (SD = 8.9, N = 29) were into blocks with calling females when females were calling in 3 or 4 blocks.

Males, flying mainly in the upper half of the room, entered blocks along the wall more than expected and those in the center of the room less than expected, but this pattern was similar for all treatments (Figure 1). The time spent in each block was also similar for all treatments except for the corner

TABLE 1. EFFECT OF PHEROMONE LEVEL UPON FLIGHT PATTERN AND MATING RATE OF MALE *Ephestia cautella* AT A DENSITY OF 4 PAIRS IN 5.9 X 5.9 X 2.4-m ROOMS

Type of observation	Mean response in each treatment[a]				
	Control	1 μg/hr	10 μg/hr	100 μg/hr	1000 μg/hr
Percentage of flights ending in mating[b]	66.7 a	22.8 b	6.9 c	0 c	0 c
Females calling during each male flight[b]	2.5 b	2.6 b	2.6 b	3.6 a	2.6 b
Flights per male[b]	1.2 b	1.3 b	1.5 b	2.3 a	1.6 ab
Duration of flight (min)[b]	14.3 b	22.8 ab	25.0 a	19.8 ab	23.6 ab
Percentage of blocks entered in which female calling[d]	12.2 a	12.3 a	9.0 a	14.9 a	4.6 a
Percentage traversing 3 blocks[c]					
at 180°	21.8 b	26.7 b	26.0 b	45.8 a	29.7 ab
at 360°	50.5 a	39.5 ab	39.1 ab	24.1 b	39.9 ab
at 90°	27.7 a	33.8 a	34.9 a	30.1 a	30.4 a

[a]Means in each row followed by the same letter are not significantly different at the 1% level with Duncan's multiple-range test.
[b]All 280 observed flights included.
[c]Only 90 flights for which the time in each block (N = 7889 entries) were recorded are included.
[d]Only 52 flights which were of sufficient duration for at least 25 times in block to be recorded are included.

blocks where the time for the control (7.6 sec) was less than that for other treatments (9–12 sec). Further, the frequency of flight between three blocks at 180, 360, and 90° angles to one another were generally similar for all treatments (Table 1). In addition to the similarity in pattern of flight with respect to location of blocks in the room and their location relative to one another, the numbers of blocks entered for the 1st time during each of the successive 2-min intervals of a flight (10.8, 6.4, 3.7, 2.2, and 1.1) were also generally similar for all treatments, suggesting a similar searching efficiency. Only during the 2nd of these 2-min intervals did the control differ from the other treatments (5.3 vs. 7.5).

Within blocks other than those in which females were calling, males usually spent a similar period of time in all treatments (Table 2). Thus, in addition to not altering the pattern of flight between blocks, permeation did not directly affect the time that a male spent within a block. However, just prior to mating, males generally did spend much more time in blocks in which females were calling, i.e., 49.8 sec in control and 25.5 sec at 1 μg/hr rate, than

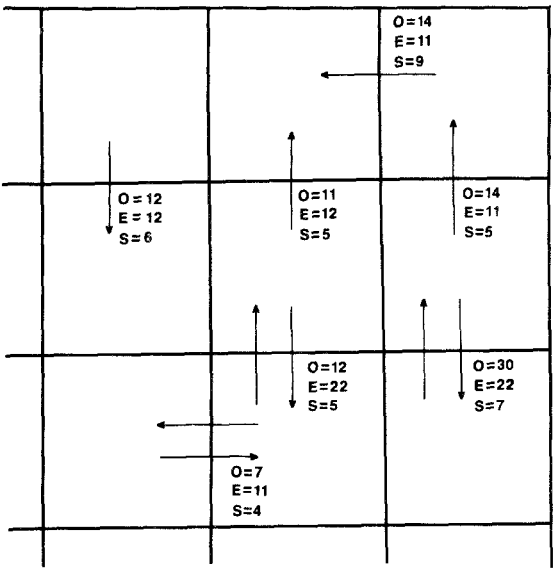

FIG. 1. Frequency of each of possible categories of between-block movements illustrated in 9 of the 25 1.4-m^2 blocks in one corner of the room. For example, the observed (O) and expected (E) percentage of block-to-block movements that occurred between an adjacent and a corner block were 14 and 11, respectively. The number of sec (S) spent in this adjacent block prior to moving into corner block was 5.

TABLE 2. EFFECT OF INTRASPECIFIC INTERACTIONS BETWEEN *Ephestia cautella* ON MEAN TIME (SEC) A MALE SPENT IN A BLOCK EACH TIME ONE WAS ENTERED

	Mean time in block (sec) in each treatment[a]				
Type of block	Control	1 μg/hr	10 μg/hr	100 μg/hr	1000 μg/hr
Block with calling female, and entry results in mating	49.8 a A	25.5 a A			
Block with calling female, but entry does not result in mating	8.3 b A	9.9 b A	4.5 b B	4.6 b B	6.0 a B
Block with female not calling	7.8 b A	8.0 bc A	4.2 b A	5.0 b A	5.7 a A
Block with male	5.1 b A	6.0 c A	8.5 a A	11.4 a A	7.4 a A
Block without male or female	5.8 b AB	6.7 c A	5.9 b AB	5.0 b B	5.9 a AB

[a]Means in each column followed by the same lower case letter or means in each row followed by the same upper case letter are not significantly different at the 1% level with Duncan's multiple-range test.

TABLE 3. EFFECT OF DENSITY AND PHEROMONE RELEASE RATE ON MATING RATE OF *Ephestia cautella*

Pheromone release rate (μg/hr)	Mating/100 sec				Ratio of mating rates for 40 vs. 4 pairs
	4 Pairs[a]		40 Pairs[a]		
	N	$\bar{X}$	N	$\bar{X}$	
0	12	0.051 a	6	2.08 a	41
1	8	0.025 b	4	0.96 b	38
10	10	0.0067 c	12	0.47 b	71
100	4	0 c	4	0.046 b	

[a]Means in each column followed by the same letter are not significantly different at the 1% level with Duncan's multiple-range test.

in other blocks (Table 2). In the control and 1 μg/hr rate, even males that did not mate spent more time in blocks in which females were calling than in other blocks, but this response disappeared at pheromone release rates above 1 μg/hr. Inexplicably, males also spent more time in blocks containing males than in other blocks at the 10 or 100 μg/hr release rates.

The mating rate was influenced by both the pheromone release rate and the pair density (Table 3). Each 10-fold increase in release rate roughly halved the mating rate and, at a particular release rate, a 10-fold increase in density resulted in a roughly 40- to 70-fold increase in mating rate. Both release rate and density significantly affected mating rate in a two-way analysis of variance, and the interaction term was also significant, indicating that increasing the release rate was less effective in reducing mating with 40 pairs than with 4 pairs.

DISCUSSION

The duration and pattern of flight resulted in *E. cautella* males flying throughout most of the room (23 of 25 blocks) within the first 10 min of a flight. Permeation did not reduce the area covered. In a room larger than the one used for the present study, the rate at which males entered new blocks probably would have declined more slowly and males would have flown over a much larger area. However, reentering blocks did contribute to the chances of a male mating in the present study since males often entered the blocks in which females were calling several times before responding and eventually mating. This perhaps indicates that a male actually searched only part of a block each time he entered and that the communication distance in our tests was less than half the width of the 1.2 × 1.2 m block or <60 cm on the average.

Also, males entered blocks in which females were calling in proportion to their frequency rather than at a higher rate as would be expected if males were attracted into blocks by female-released pheromone. The first observed response of a male to a calling female was a localization of male flight within the block in which the female was calling. This localized flight generally continued for quite a long time (49.8 and 25.5 sec) before a male finally located the female and mated. Permeation could therefore reduce the chances of mating by raising the threshold level of pheromone to which a male responds with a localization of flight in the vicinity of a calling female.

Given that the concentration of female-released pheromone decreases with distance away from female (Mankin et al., 1980) and that males no longer responsive to a certain level of pheromone can still respond to a higher level (Shorey et al., 1967; Bartell and Lawrence, 1976; Kennedy et al., 1980), the reduced mating in a sex pheromone-permeated atmosphere may be explained by a reduction in communication distance. As the level of pheromone permeating the air increases, increasing the threshold level to which a male responds, the active space around the female in which the concentration of pheromone was above the male response threshold would shrink, reducing the communication distance (Figure 2). Permeation does not directly affect the behavior of a male searching for a pheromone plume, so a male would fly the

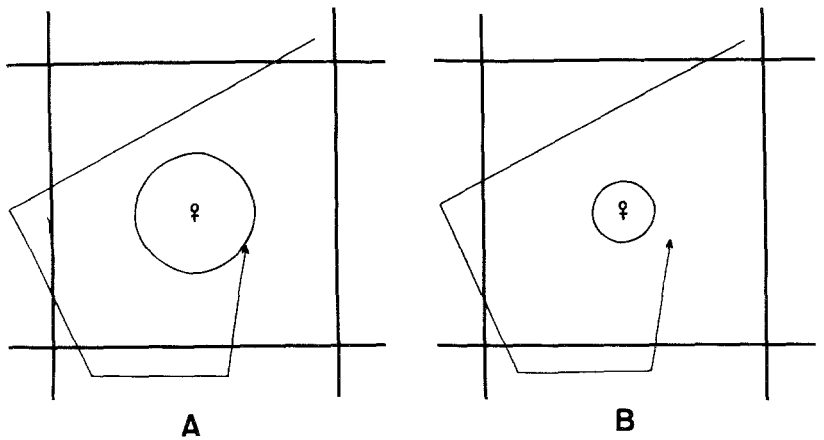

FIG. 2. Diagrammatic representation of an explanation for reduced mating by *E. cautella* in a sex pheromone-permeated atmosphere. A single 1.4-m^2 block (bold line) from a nonpermeated (A) and a sex pheromone-permeated (B) room are shown with a calling female in the center. The circle represents the active space in which the female-released pheromone is above the male response threshold and the narrow line the male flight path which is the same in both rooms since behavior was not directly affected by permeation. Clearly the male locates the female in nonpermeated but not in permeated air.

same pattern as in a nonpermeated atmosphere, but would mate only in the nonpermeated atmosphere. Both *Choristoneura fumiferana* (Clemens) and *Lymantria dispar* (L.) have been observed to search for females in restricted areas, i.e., close to foliage and tree trunk, respectively (Sanders and Lucuik, 1972; Richerson, 1977) and like *E. cautella*, these species may be able to locate mates in a sex pheromone-permeated atmosphere by persistent flight.

Cardé (1981) lists three principle mechanisms for disruption of communication when the atmosphere is permeated with synthetic sex pheromone: (1) elevation of pheromone response threshold; (2) attraction to the artificial sources of pheromone; and (3) camouflaging of the boundaries of the pheromone plume. He indicates that these mechanisms are not mutually exclusive. In the present study, the boundaries of the pheromone plume seem to be camouflaged by raising the male response threshold, and attraction of males to artificial sources is unimportant. We have observed that males were, however, attracted more often when pheromone release rate was reduced by diluting 10-fold with tetradecen-1-ol acetate (unpublished data), and attractiveness might also be greater if the full pheromone blend were used (Brady, 1973; Read and Haines, 1979). Thus, the efficiency of permeation in reducing mating might be enhanced by combining attraction to artificial sources with the elevation of response threshold.

In practice, mating rates would probably be higher than those observed here, because males would probably seek mates over a period longer than our 100-min test period on each of several nights. Our observations with *E. cautella* suggest that the most effective response to these higher mating rates would be finding a time when the population density is lower rather than increasing the pheromone release rate, since a 10-fold increase in release rate roughly halved the mating rate, while at a particular release rate a 10-fold reduction in density reduced mating rate by roughly 40- to 70-fold.

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ALLELOCHEMICS PRODUCED BY THE HYDROPHYTE *Myriophyllum spicatum* AFFECTING MOSQUITOES AND MIDGES

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Abstract—An extract of the hydrophyte *Myriophyllum spicatum* L. was found to be toxic to first- and fourth-instar larvae of the mosquitoes, *Culex quinquefasciatus* Say, *Culex tarsalis* Coquillett, *Culiseta incidens* (Thomson), *Aedes aegypti* L., and chironomid midges in the laboratory. When first-stage larvae were exposed to the extract, the lowest concentration (6.4 mg extract/100 ml H_2O) produced 86, 60, and 48% mortality in *C. incidens*, *C. quinquefasciatus*, and *A. aegypti*, respectively. Higher concentrations caused 100% mortality in both first and fourth instars (63.75 mg/100 ml H_2O or more). In general, the biocidal activity of the extract was found to be similar when first- and fourth-stage larvae were exposed. Some mortality in the successive pupal and adult stages was observed when fourth-stage larvae were exposed to the extract, but mortality occurred only in the various larval instars when first instars were exposed. Among the chironomids, *Tanytarsus* spp. was more tolerant to the extract than *Chironomus* spp. In addition to the biocidal activity against immature mosquitoes, the extract was found to show a unique activity acting as an attractant to both sexes of adult *C. quinquefasciatus* and *A. aegypti*. The response elicited in *A. aegypti* appeared to be somewhat higher than that in *C. quinquefasciatus*. In general, the percentage of males responding to water treated with the extract was higher than that of the females. The extract at higher concentrations seemed to show some repellency at the outset to both species, but induced positive responses later on.

Key Words—Eurasian milfoil, *Myriophyllum spicatum*, toxicity, attractant, mosquitoes, chironomid midges, allelochemics *Chironomus*, Diptera, Chironomidae, Culicidae, *Culex tarsalis*, *Culex quinquefasciatus*, *Aedes aegypti*, *Culiseta incidens*.

INTRODUCTION

As an essential and fundamental entity of freshwater ecosystems, aquatic vegetation serves as the primary source of food for a wide variety of

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heterotrophic organisms. However, certain hydrophytes are known to produce and/or release chemical substances that are antagonistic to the development of aquatic invertebrates. The nature of antagonism between the aquatic flora and invertebrates could be physical or chemical interferences. Among aquatic invertebrates, the phenomenon of antagonism between aquatic plants and mosquitoes has been reported to involve physical (Smith, 1910; Twinn, 1931; Furlow and Hays, 1972) and chemical characteristics (Matheson and Hinman, 1929; Angerilli and Beirne, 1974; Lalonde et al., 1980).

The green algae, *Chorella ellipsoidea* Gerneck and *Rhizoclonium hieroglyphicum* Kütz, have been shown to be inhibitory to the development of mosquitoes (Dhillon and Mulla, 1981; Dhillon et al., 1982). Among the aquatic angiosperms, *Myriophyllum* spp. have been found to produce substances that were either repellent and/or lethal to the water flea, *Daphnia rosea* (Pennak, 1973), and mosquitoes (Bachmann, 1921).

During the course of our studies on nuisance chironomid midges in Spring Valley Lake, in southern California, we noted a profuse growth of the Eurasian milfoil, *Myriophyllum spicatum* L., and observed that this weed appeared to regulate the population density of chironomid midges. As a consequence of the above observations, a study was designed to isolate the toxins of *M. spicatum* and to evaluate their biological activity against mosquitoes and midges.

METHODS AND MATERIALS

Foliage of *M. spicatum* was collected from Spring Valley Lake, California, washed several times with tap water, and air-dried in a greenhouse. The dried material was chopped into small pieces and mechanically pulverized in a mill (Willey Mill, A.H. Thomas Co., Philadelphia, Pennsylvania). A portion of the pulverized material was used for extraction purposes, and the rest was stored in a freezer.

Extraction. The ground-up plant material (200 g) was gently refluxed for 2 hr with a mixture of benzene and methanol (3:1, 1 liter). After cooling, the mixture was filtered and the residue was washed with a mixture of benzene and methanol (3:1, 200 ml × 2). The filtrate and the washings were combined, and the solvents were removed in a rotary evaporator. The residue obtained was dissolved in petroleum ether (100 ml) and chilled in a refrigerator for 4 hr after which the mixture was filtered and washed with the same solvent (40 ml × 2). The combined filtrate and washings were evaporated in a rotary evaporator to give an extract. The procedure was repeated until a total of 1 kg of *Myriophyllum* was extracted. All the extracts from 1 kg of the dried material were combined, dissolved in acetone, and assayed at various

concentrations against larvae of mosquitoes and chironomid midges under laboratory conditions. Solvents alone were run through the same procedure and served as checks.

Bioassays. The extract was tested against larvae of *Culex quinquefasciatus* Say, *Culex tarsalis* Coquillett, *Culiseta incidens* (Thomson), and *Aedes aegypti* L. mosquitoes. Twenty first- or fourth-stage larvae from a laboratory-reared colony were placed in a glass bowl (11 cm diam, 300 ml capacity) containing 100 ml of tap water. A known quantity of the extract was added to the bowl. Mosquito food (60–70 mg, laboratory chow and yeast) was provided every other day until a majority of the larvae pupated or died. Each treatment and check was maintained at room temperature (ca. 25.5° C) and replicated four times. Observations on the cumulative mortality and emergence were recorded every other day according to the procedure of Mulla et al. (1974).

Larvae of midges, *Chironomus* spp. and *Tanytarsus* spp., were collected from Silver Lakes, California. The general bioassay method for midges was followed according to Mulla and Khasawinah (1969). Ten field-collected fourth-stage larvae were placed in a paper cup (7 cm diam, 150 ml capacity) containing 100 ml of tap water and ca. 2.0 g of sand. A known amount of the extract was added to the cup. Treatments and checks were replicated four times. Mortality was assessed 24 hr after the larvae were exposed to the extract.

Attractancy Test. During the course of bioactivity studies against larvae, some of the emerged adult mosquitoes were attracted to the bioassay bowls containing *M. spicatum* extracts. Most of the attracted adults were found floating, with a small number being drowned. Tests were, therefore, carried out to ascertain the attractancy of *Myriophyllum* extract to *C. quinquefasciatus* and *A. aegypti*.

In choice tests, two glass bowls, each containing 100 ml of tap water and the desired concentration of extract, were placed in a screen cage (45 × 45 × 45 cm). The check bowls containing tap water and acetone only were also placed in the same cage simultaneously. From our stock colony, 150 one-day-old, unfed, adult mosquitoes of each sex were released in this cage. A total of three cages were set under laboratory conditions (24 ± 2° C). The photoperiod was maintained at 13:11 hr (scotophase–photophase). No food was provided to the adults prior to or after releasing in the cage. Seven concentrations of the extract were tested against *C. quinquefasciatus*, whereas only five concentrations were used against *A. aegypti*. The responding adults were removed every day from the glass bowls until no flying adult was noticed. The adult mosquitoes which were not attracted to either the treatment or the check died in the cage. These dead adults were collected from the bottom of the cage, counted, and designated as "unresponding."

During these studies, some of the adults were found to be drowned in an aqueous solution of *M. spicatum* extract at the two higher concentrations, (255.0 and 127.5 mg extract/100 ml). To determine if surface tension played any role in the drowning of mosquitoes, we measured the surface tension of the water treated with the extract at the two concentrations, and tap water with acetone, according to the procedure underlined by Mulla and Chaudhury (1968). A capillary tube (Scherer capillary tube, internal diam 1.1 mm) was placed vertically in the extract solution in a glass bowl. The rise of the solution in the tube was measured. The surface tension (δ) of the extract solution was calculated using the following formula: $\delta = h\gamma dg/2$. h = rise of the solution, γ = radius of the tube, d = density of the solution, g = gravity.

Data were analyzed on Statistician Compucorp 145E and significance of means was compared using Duncan's multiple-range test ($P = 0.01$).

RESULTS AND DISCUSSION

Field-collected *M. spicatum* upon air drying yielded 7% of dry material. Each gram of this pulverized dry material yielded approximately 25 mg of extract by the procedure employed.

Bioassay. The activity shown by the milfoil extract against first-stage larvae of three mosquito species is presented in Table 1. The three higher concentrations were proven to be very lethal to mosquito larvae, causing high mortalities in *C. quinquefasciatus* (93–100%), *C. incidens* (98–100%), and *A. aegypti* (70–100%). Results also showed that the three higher concentrations were not significantly different in their activity to *C. quinquefasciatus* and *C.*

TABLE 1. ACTIVITY OF *M. spicatum* EXTRACT AGAINST FIRST-STAGE LARVAE OF THREE SPECIES OF MOSQUITOES IN THE LABORATORY

Extract (mg/100 ml H_2O) (ppm)[a]	Cumulative mortality (%)[b]		
	C. quinquefasciatus	*C. indicens*	*A. aegypti*
127.5 (1275)	100 d	100 c	100 d
63.75 (637)	100 d	100 c	86 d
25.5 (255)	93 d	98 c	70 c
12.75 (127)	76 c	92 b	64 bc
6.4 (64)	60 b	86 b	48 b
Check (0)	5 a	5 a	8 a

[a]Numbers in parentheses represent concentration in ppm. Means followed by the same letter in the columns are not significantly different from each other (Duncan's multiple-range test, $P = 0.01$).

[b]Cumulative mortality until complete mortality or adult emergence.

incidens. On the contrary, the mortality sustained by *A. aegypti* was significantly lower at 25.5 mg/100 ml than at 127.5 and 63.75 mg/100 ml H_2O. The activity of the two lower concentrations revealed that, of the three mosquitoes tested, *C. incidens* seemed to be the most and *A. aegypti* the least susceptible to the extract. Further observations on the activity of the extract against the three mosquito species showed that the LT_{50} at the highest concentration (127.5 mg/100 ml) was two days, whereas at the lowest concentration (6.4 mg/100 ml) it was 5–6 days.

In addition to the mortality induced by the extract, it was also found to delay the rate of development of preimaginal mosquitoes. The development of surviving mosquitoes was delayed as much as 4–6 days as compared to the check populations. The development of *C. incidens* was delayed as much as 6 days, whereas it was equally delayed 4–5 days in the other mosquito species.

Table 2 shows the toxicity of *M. spicatum* extract against fourth-stage larvae of four species of mosquitoes. The two higher concentrations (255 and 127.5 mg/100 ml) tested induced complete larval mortality of all the species

TABLE 2. ACTIVITY OF *M. spicatum* EXTRACT AGAINST FOURTH-STAGE LARVAE OF FOUR SPECIES OF MOSQUITOES IN THE LABORATORY

	Cumulative mortality (%) at concentration (mg/100 ml H_2O)[a]					
Stage	255 (2550)	127.5 (1275)	63.75 (637)	25.5 (255)	12.75 (127)	Check
C. quinquefasciatus						
Larvae	100 g	100 g	90 f	33 e	5 ab	1 a
Pupae	0	0	4 a	16 d	14 cd	0
Adult	0	0	0	12 bcd	8 ab	0
C. tarsalis						
Larvae	100 e	100 e	96 dc	90 d	76 c	2 a
Pupae	0	0	4 ab	10 ab	13 b	1 a
Adult	0	0	0	0	0	0
C. incidens						
Larvae	100 e	100 e	91 e	66 d	43 c	2 a
Pupae	0	0	8 ab	5 ab	14 b	2 a
Adult	0	0	0	0	0	0
A. aegypti						
Larvae	100 e	100 e	67 d	22 b	15 b	0
Pupae	0	0	23 b	61 cd	54 c	4 a
Adult	0	0	0	4 a	8 a	0

[a]Numbers in parentheses represent concentration in ppm; cumulative mortality until complete mortality or adult emergence. Means followed by the same letter are not significantly different from each other (Duncan's multiple-range test, $P = 0.01$: data analyzed separately for each species).

within 48 hr. At the lowest concentration (12.75 mg/100 ml), the extract was quite active against some species, causing as high as 89% overall cumulative mortality (larval and pupal) of *C. tarsalis*, whereas its activity against *C. quinquefasciatus* was very low (27% cumulative mortality of all stages). It was observed that, at the three lower concentrations (63.75, 25.5, and 12.75 mg/100 ml), some mortality was produced in the successive stages, and this was especially noticeable in *A. aegypti* in which 61% and 54% pupal mortality was noted at 25.5 and 12.75 mg/100 ml concentrations, respectively. It should be pointed out that at 63.75, 25.5, and 12.75 mg/100 ml H_2O, the extract exhibited delayed activity as evidenced by the substantial mortality in successive stages when fourth-stage larvae were exposed. Mortality produced by these concentrations when first-stage larvae were used, however, occurred exclusively in the larval stage.

The activity of *M. spicatum* extract against fourth-stage midge larvae is given in Table 3. The extract appeared highly toxic to *Chironomus* spp., causing as high as 88% mortality at the lowest concentration used, whereas only 25% mortality was induced in *Tanytarsus* spp. at this concentration (63.75 mg/100 ml H_2O). *Chironomus* spp. thus seems to be three times as susceptible as *Tanytarsus* spp. to the extract at the lowest concentration. The three higher concentrations caused 100% mortality in *Chironomus* spp. and 35–45% in *Tanytarsus* spp., indicating a susceptibility index of ca. 2.5.

Comparing the activity of *M. spicatum* extract against mosquitoes and midges, it was noted that the fourth-stage larvae of *Chironomus* spp. were almost as susceptible as mosquito larvae. However, other midge species (*Tanytarsus*) showed a fourfold tolerance to the extract (at 63.75 mg/100 ml) as compared with mosquitoes. It is known that midge larvae are about three to

TABLE 3. ACTIVITY OF *M. spicatum* EXTRACT AGAINST FOURTH-STAGE LARVAE[a] OF AQUATIC MIDGES IN THE LABORATORY

Extract dosage (mg/100 ml H_2O) (ppm)[b]	% Mortality	
	Chironomus spp.	*Tanytarsus* spp.
382.4 (3825)	100 c	45 c
255.0 (2550)	100 c	35 bc
127.5 (1275)	100 c	45 c
63.75 (637)	88 b	25 b
Check (0)	10 a	5 a

[a]Larvae were collected from Silver Lakes, Helendale, California.
[b]Numbers in parentheses represent concentration in ppm. Means followed by the same letter in the columns are not significantly different from each other (Duncan's multiple-range test, $P = 0.01$).

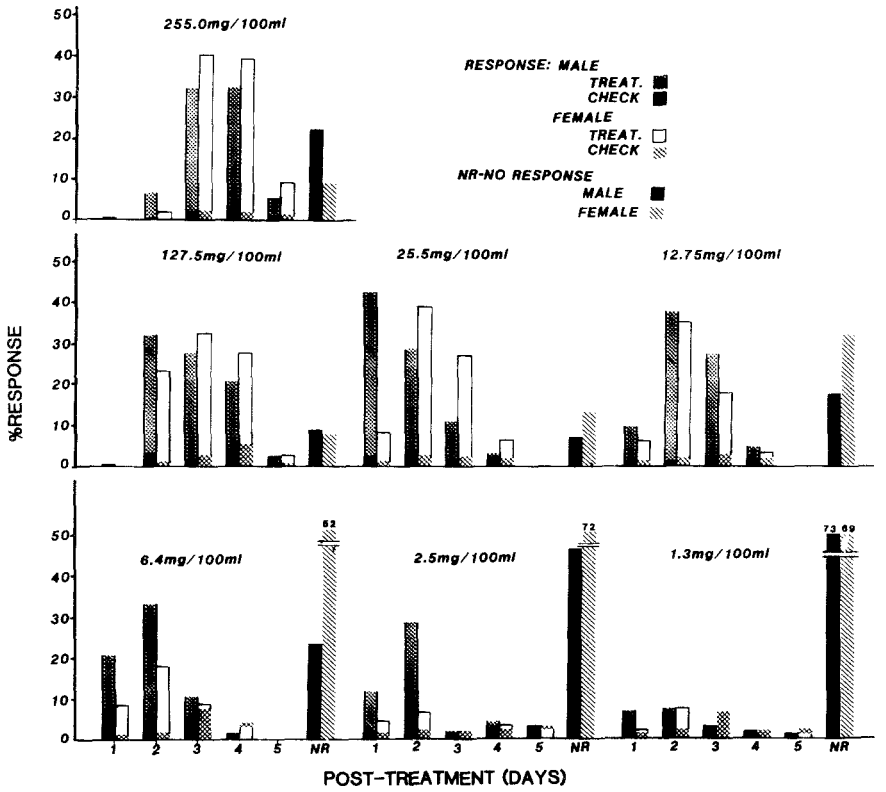

FIG. 1. Attractancy of *M. spicatum* extract listed at various concentrations to *C. quinquefasciatus* adults.

four times less susceptible than mosquitoes to synthetic larvicides (Mulla and Dhillon, unpublished data).

Attractancy Test. The *M. spicatum* extract, in addition to being active against the preimaginal stages of mosquitoes, elicited response in both adult males and females of *C. quinquefasciatus* (Figure 1). It was observed that one day after the treatment *C. quinquefasciatus* was not attracted to the two higher concentrations (255 and 127.5 mg/100 ml). The highest concentration, even two days after the treatment, did not induce any significant response in this species; however, both sexes were attracted significantly on the 3rd and 4th day after treatment to the extract at this concentration. The negative response within the first two days after treatment might have been due to the high concentration of semiochemicals which might have induced a repellent response. The majority of adults, however, responded within 1–3 days after the treatment to all lower concentrations tested (25.5, 12.75, 6.4, 2.5, 1.3

mg/100 ml). In general, the magnitude of adult response declined gradually with decreasing concentration.

The percentages of unresponding males and females of *C. quinquefasciatus* were not significantly different from one another at the three higher concentrations. However, at the next three concentrations (12.75, 6.4, and 2.5 mg/100 ml), the percentages of unresponding females were found to be significantly higher than the unresponding males. At the lowest concentration (1.3 mg/100 ml), 73% males and 69% females were observed to remain unattracted to either treatment or check. In these studies, the percent attractancy shown by all concentrations of *M. spicatum* extract except the lowest one (1.3 mg/100 ml) was always significantly higher than that of the check.

The quantitative response as shown by the adults of *A. aegypti* to the extract is presented in Figure 2. At the highest concentration (127.5 mg/100

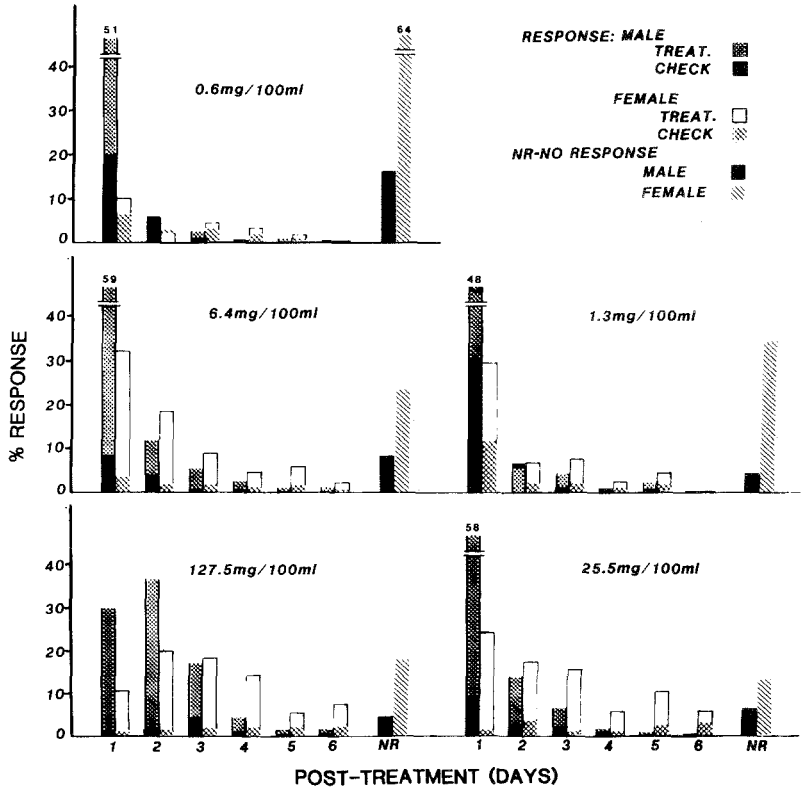

FIG. 2. Attractancy of *M. spicatum* extract tested at various concentrations to *A. aegypti* adults.

ml) both sexes showed maximum response two days after the treatment. At the five lower concentrations the maximum response occurred at one day after treatment. The response of *A. aegypti* males was significantly higher than the responding females at all concentrations one day after the treatment. Even at the lowest concentration, 58% (total response of all days) of the males and only 21% of the females were attracted to the extract. The percentage of unresponding females was significantly higher than that of unresponding males at all concentrations tested.

It can be concluded that, in general, the extract elicited higher response in the males than in the females of both test species. Furthermore, the response of adult *A. aegypti* appeared higher even at the lower concentration than those tested for *C. quinquefasciatus*.

It was noted that some of the adults were found to be drowned in the water treated with *M. spicatum* extract at the two highest concentrations. Surface tension of the water treated with the extract (71 dynes/cm) was almost the same as that of the check (tap water with acetone, 70 dynes/cm). The effect of surface tension on drowning was, therefore, ruled out.

The findings in this study have revealed that *M. spicatum*, growing profusely in stagnant water habitats, has inhibitory effects on mosquitoes and midges. The attractancy of this aquatic macrophyte to adult mosquitoes, as demonstrated in the present investigation, is indicative of the primary association between producers and consumers. For supporting its existence and survival, this hydrophyte seems to have developed defense mechanisms (antibiosis) to avoid predation by aquatic invertebrate herbivores. Hence, the toxic principles of *M. spicatum* might have evolved as a result of ecological imbalance.

Several aspects of attractant and toxic compounds of *M. spicatum* extract have yet to be investigated to determine the chemical nature and environmental implications of this hydrophyte.

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SEX PHEROMONE BIOLOGY AND BEHAVIOR OF THE COWPEA WEEVIL *Callosobruchus maculatus* (COLEOPTERA: BRUCHIDAE)[1]

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Abstract—Female cowpea weevils, *Callosobruchus maculatus* (F.), emitted a pheromone which excited males. Pheromone release began soon after emergence and continued for one week. Synchronization of pheromone release with calling behavior was demonstrated. Mating reduced pheromone release but not male response. Pheromone obtained by aeration collection was utilized for determining a quantitative dose-response relationship.

Key Words—Coleoptera, Bruchidae, *Callosobruchus maculatus*, cowpea weevil, pheromone, sex attractant.

INTRODUCTION

In the Bruchidae, Hope et al. (1967) first demonstrated the existence of a male-produced sex pheromone in the bean weevil, *Acanthocelides obtectus* (Say). Preliminary evidence for a female sex attractant in *Callosobruchus chinensis* L. which attracts males was reported by Honda and Yamamoto (1976) and in *C. maculatus* by Rup and Sharma (1978). Tanaka et al. (1981) reported that a copulation release pheromone composed of two synergistic fractions and produced by both sexes of *C. chinensis* was distinct from the female sex attractant and was released from both sexes, but affected only the male.

The bruchids are important pulse-feeding pests of cosmopolitan distribution. The cowpea weevil, *C. maculatus* (F.) is a major pest of cowpeas, *Vigna*

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unguiculata (L.), and other pulses in most parts of the tropical and subtropical world (Taylor and Agbaje, 1974).

Knowledge of pheromones of this insect will allow improved pest management strategies. Therefore we have examined the biology and behavior of *C. maculatus*, including pheromone production and release, effect of mating on release, calling behavior, gland location, and pheromone isolation, none of which were discussed in the earlier study with this species.

METHODS AND MATERIALS

The experimental insects were obtained from a field infestation in Wisconsin and reared on the host seeds, chickpeas (*Cicer arietinum* L.) in 500-ml wide-mouth jars at 27 ± 2°C, 60 ± 10% relative humidity, and a 16:8 light–dark photoperiodic regime. Peas were separated into 1.5-dr vials (2 or 3 peas/vial) prior to insect emergence. Newly emerged adults were sexed and isolated daily.

The mating behavior was observed by watching a male and female respond to each other (10 pairs) in a filter-paper-lined Petri dish following emergence (<15 hr) and during the first 2 hr of the light cycle.

To demonstrate sex pheromone release, 1 or 2 males or females (1 day old) were put into 1.5-dr vials with highly absorbent paper disks on the bottom (Burkholder and Dicke, 1966). The vials' walls were coated with Teflon® to keep the insects in contact with the disks, and the vials were capped with plastic caps having small pinholes. After 1 day, contaminated disks were bioassayed with males and females in single-choice olfactometers similar to that used by Coffelt and Burkholder (1972). The experimental disk with sample was suspended ca. 1 cm above the insect. Twenty newly emerged males or females (1–3 day old) were tested for each replicate. Each insect was exposed to the pheromone source for 1 min. A positive response was recorded when the insect became aroused and elevated and moved the antennae in a drumming motion. Untreated disks were used as controls. All biosassays were conducted under conditions similar to those in the rearing room but in a room separated from that used for culturing and handling females.

The stability and volatility of the pheromone were evaluated and tested by dividing the disks into two groups. In the first group each disk was contaminated by a newly emerged female for 4 days, using procedures similar to those in the above experiment. In the second group, pheromone samples on the disks were obtained from crude hexane extracts of aeration collections (Cross et al., 1976). Tenax® (2,6-diphenyl-*p*-phenylene oxide, Applied Sci. Labs, State College, Pennsylvania) was used as the absorbent. The test disks were stored in a 1.5-dr vial without a cover at 27 ± 2° C and 60 ± 10% relative

humidity. Untreated disks or those treated with hexane were used as controls. Pheromone activity was examined daily until males ceased to respond.

The site of pheromone production was determined by quick-freezing 1-day-old females and ablating the bodies into various sections for bioassay.

The day of peak pheromone release was determined by changing the vial and disk for the female daily and bioassaying the contaminated disk with males immediately and on subsequent days until male response ceased.

In order to observe the relationship between calling behavior and pheromone release, 40 newly emerged females were put into separate vials. Disks on the vial bottoms provided a resting substrate for the female. Vial walls coated with Teflon confined insects to the disks. Each vial was covered by a plastic cap provided with ventilation holes. Calling was observed each day after the onset of light, at which time a disk was suspended (ca. 1 cm) above the female in each vial to collect the pheromone. After the females ceased calling, the suspended disk was taken out and immediately bioassayed. Meanwhile, the disks above noncalling females were used as controls.

To examine the effect of mating on female pheromone release and male response, a newly emerged male and female were placed in a vial with a disk on the bottom. After mating, the female was transferred to a separate vial containing a clean disk. One day following contamination, biological activity of the disk was tested with unmated males and compared with the activity of disks contaminated by virgin females. Mated males were transferred to single-choice olfactometers in order to determine their response to virgin-female-contaminated disks. Unmated males were used as controls.

An insect aeration apparatus similar to that of Cross et al. (1976) was utilized to collect pheromone from female cowpea weevils. The aeration apparatus consisted of three sections: (1) a charcoal air scrubber, (2) a glass insect chamber, and (3) a pheromone collection tube. Brass screens were fixed on the top and bottom of the insect chamber to confine the adults. Pleated filter paper strips were placed on the bottom screen to provide a resting surface for insects. The collection tube was filled with Tenax (ca. 1 g) or Porapak-Q® (ca. 2.3 g ethylvinylbenzene–divinylbenzene copolymer, 60/80 mesh, Waters Associates, Inc., Framingham, Massachusetts) which had been conditioned by extraction with redistilled hexane for 20 hr in a Soxhlet apparatus. The air stream flow rate was 3 liters/min over a 7 to 8-day duration. Weevil volatiles on the absorbents were eluted with redistilled hexane and concentrated by reduced pressure distillation and bioassayed in a single-choice olfactometer to compare the collection efficiencies of Tenax and Porapak-Q. A quantitative relationship between female equivalent (FE) concentration and male response was determined with pheromone extract from Tenax at concentration series of 1.0, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 FE. Disks treated with hexane served as controls.

Bioassay data were analyzed by using the t test for independent data. Differences at the 1% level or lower were accepted as significant.

RESULTS AND DISCUSSION

After the male and female were placed together in a Petri dish, the male usually promptly searched for the female while rapidly drumming the filter-paper surface of the dish. The female periodically moved within the dish, usually followed by the male. Copulation began when the female remained passive allowing the male to mount. Copulation began within 1 min for six pairs and at 2, 3, 8, and 12 min for the other four pairs. During the first minute of copulation the male antennae stroked the female abdomen. The female remained passive in copula for an average of 5.6 ± 4.4 min; this was followed by attempts to move. The male remained passive and did not attempt to free himself until approximately 1 min after female movement. The total length of copulation was 6.8 ± 4.2 min and was 3 min longer than that reported by Paddock and Reinhard (1919).

The response of males to disks exposed to females was very strong (Table 1). However, males did not respond to males and females did not respond to either sex. There was no discernible difference in the response to disks contaminated by 1 or 2 females.

The sex pheromone of the cowpea weevil displayed relatively low volatility and high stability (Figure 1). A disk exposed to 1 female for 4 days maintained full activity for 6 days, after which the activity slowly diminished. A similar pattern of stability was observed for pheromone collected by aeration onto Tenax. While it appeared that disks spotted with hexane extract from the aeration sample were slightly less active than female-contaminated disks, the results could not be compared directly since the absolute amounts used in the tests were unknown. Under these conditions, the sex pheromone proved to be stable, a property that is very helpful for isolation and identification.

TABLE 1. EVIDENCE OF PHEROMONE RELEASE BY *Callosobruchus maculatus* FEMALES

Sex tested	% Response[a] to Disks Contaminated for 1 day by				
	1 ♂	2 ♂	1 ♀	2 ♀	Control
Male	15	15	94**	98**	10
Female	10	12	12	14	10

[a]Average of 4 replicates, N = 20 per replicate; experimental stimulus significantly different from corresponding control indicated by: ** $P < 0.01$.

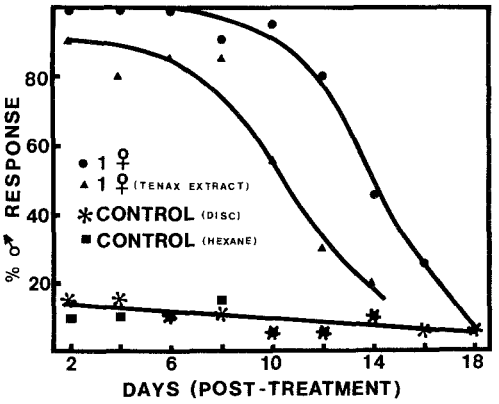

FIG. 1. Stability of *Callosobruchus maculatus* pheromone. Disks were exposed to females for 4 days or spotted with hexane extracts of Tenax from an aeration sample. The response was based on an average of two replicates, $N = 20$ per replicate. The treatments were significantly different from controls ($P < 0.01$).

Ablation of various body parts showed that the pheromone is released from the abdominal region. Female abdomens elicited a 75% response by males ($N = 40$) while female heads and thoraces produced 15% and 20% responses, respectively. Further sectioning of the abdomen revealed that all males responded to the ventral abdominal tip.

Figure 2 shows the release of pheromone by females for the first week of adult life. The level of production is high for the first 4 days, then declines until the 7th day when it no longer is detectable.

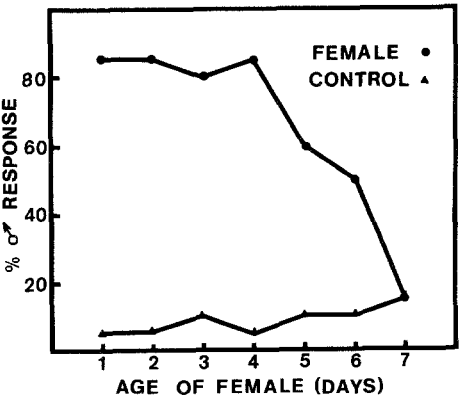

FIG. 2. Release of pheromone by young *Callosobruchus maculatus* female adults. Males were exposed to a disk contaminated by a female of the age specified. The percent response represents the average of three replicates, $N = 20$ per replicate. The treatments were significantly different from the control ($P < 0.01$).

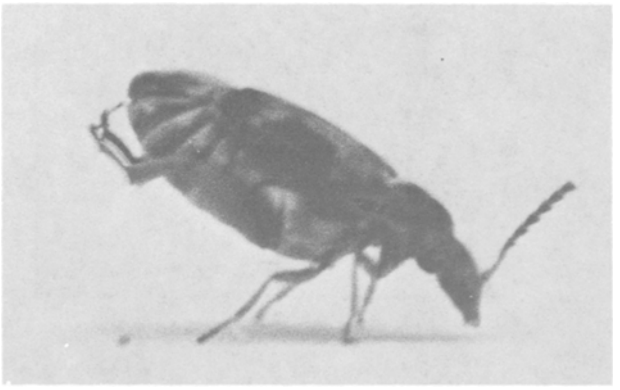

FIG. 3. Female *Callosobruchus maculatus* in calling position.

Females displayed a calling behavior associated with pheromone release. In general, calling lasted 3–5 min. However, some females maintained the posture for 15 min. While calling, the female elevates the abdomen and lowers the head by extending the middle and hind legs (Figure 3). The angle of tipping was ca. 30° from horizontal. The antennae were raised, and occasionally the abdominal tip was rubbed with the hind legs, an action which may help to disperse the pheromone. The onset of calling occurred mainly within the first 2 hr of the light period. Pheromone release was synchronized with calling behavior. Male response to disks exposed to calling females was 70% compared to 22.5% for noncalling females.

Many factors could affect pheromone release. In our experiments only the influence of mating was examined. Mating by females significantly decreased pheromone release (20% vs. 85% male response to mated and virgin females, respectively). Mating by males had a small effect on their subsequent response to females (74% vs. 94% response by mated and unmated males, respectively). Larson and Fisher (1938) reported that some males mate as many as 23 times during their lives and 6 times in a day.

Several pheromone collection methods were compared in this study. In general, aeration of a large number of females was preferable to extraction of female bodies or extraction of disks that had been in contact with females. Pheromone collection procedures by aeration are efficient since volatiles are collected continuously. Tenax was the preferred absorbent because it was easier to prepare than Porapak-Q and gave consistently higher bioassay readings, although it was not significantly different based on the t test for paired data.

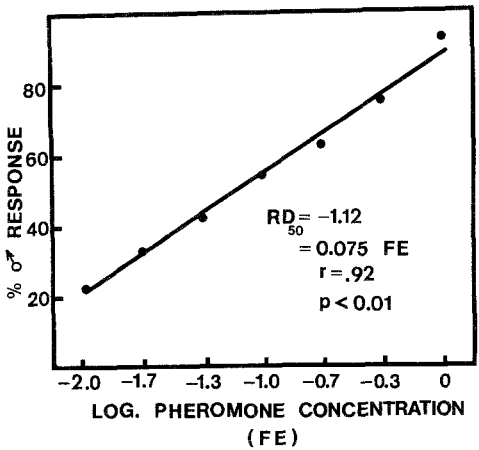

FIG. 4. Response of male *Callosobruchus maculatus* to different concentrations of pheromone extract of virgin females (FE). The response was based on an average of three replicates, $N = 20$ per replicate. The treatments were significantly different from the controls ($P < 0.01$).

The quantitative male response to the female pheromone obtained by aeration is shown in Figure 4. The concentration eliciting 50% response by males was 0.075 FE.

This is the first report of calling behavior in a bruchid. Similar calling behavior has been demonstrated in several species of dermestid beetles (Burkholder et al., 1974; Hammack et al., 1976; Barak and Burkholder, 1977; Cross et al., 1977).

Pheromone isolation and identification studies are currently in progress.

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EXOCRINE SECRETIONS OF BEES
IV. Macrocyclic Lactones and Isopentenyl Esters in Dufour's Gland Secretions of *Nomia* Bees (Hymenoptera: Halictidae)[1]

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Abstract—The volatile components of Dufour's gland extracts were analyzed in four species of Nearctic *Nomia* bees; *Nomia* (*Dieunomia*) *heteropoda*, *N.* (*Tetrazonata*) *tetrazonata*, *N.* (*Epinomia*) *nevadensis*, and *N.* (*Epinomia*) *triangulifera*. A homologous series of five saturated macrocyclic lactones ranging from C_{18} to C_{26} was identified. A series of esters (branched C_5-alkenols and fatty acids) was also identified in all species investigated. Two of these esters are new natural products reported for bees. A discussion of the significance of the Dufour's gland secretion for halictid systematics and its function in the Halictidae is also presented.

Key Words—Bees, *Nomia*, Dufour's gland, exocrine products, macrocyclic lactones, isopentenyl esters, systematics, mass spectrometry.

INTRODUCTION

The Halictidae, considered a primitive family of bees, represents a major group of Apoidea in the United States. The 500 species representing 21 genera

[1]Exocrine Secretions of Bees: I. Duffield et al. (1980), II. Fernandes et al. (1981), III. Duffield et al (1981).

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are divided into three subfamilies (Hurd, 1979). The halictids vary not only in nesting behavior but also in their social behavior, ranging from solitary to eusocial. Since behavior may be mediated by the secretions of several exocrine glands, it is important to determine the chemistry of these glands to elucidate chemobehavioral interactions. Since the exocrine secretions can also be species specific, these data are important to systematics.

The Dufour's or alkaline gland is associated with the sting apparatus and occupies a large portion of the female halictids abdomen. It varies considerably in size and shape from species to species (Lello, 1971). In the Halictinae (e.g., *Augochlora pura pura*) the gland is asymmetrically bilobed. Nomiine glands are crescent shaped, white (in fresh material) with a wrinkled surface that is highly tracheated. If the sac is ruptured under water, an odorless, colorless, immiscible fluid floats to the surface.

The chemistry of the Dufour's gland secretions is known for 25 species of Halictinae (Duffield et al., 1981; Hefetz et al., 1978; Bergström and Tengö, 1979). Their secretions contain different combinations of compounds from the following: straight-chained hydrocarbons, a homologous series of even-numbered saturated and unsaturated macrocyclic lactones (C_{18}–C_{26}), and a homologous series of esters formed from branched C_5-alkenols and even carbon fatty acids.

The chemistry of the Dufour's gland for the subfamily Nomiinae has not previously been reported. The genus *Nomia* is a large diverse assemblage of bees with most of the species distributed in tropical Africa, Asia, and Australia (Michener, 1979). The group is represented in the United States by 26 species (Hurd, 1979) divided among four subgenera. One of the species, *Nomia melanderi* or the "alkali bee," is a valuable pollinator of alfalfa which is managed using artificial nesting sites. Here we show that the Dufour's glands of *Nomia* (*Dieunomia*) *heteropoda, N.* (*Epinomia*) *nevadensis, N.* (*Epinomia*) *triangulifera*, and *N.* (*Tetrazonata*) *tetrazonata*[6] contain a homologous series of saturated macrocyclic lactones and a series of isopentenyl esters, two of the latter being newly reported exocrine products of halictid bees. We also discuss the function of the Dufour's gland secretions in these bees and their significance in apoid systematics.

METHODS AND MATERIALS

Nomia nevadensis and *N. tetrazonata* were collected at flowers near Portal, Arizona, during August 1980. *Nomia triangulifera* was collected near

[6]The species identified as *N.* (*Tetrazonata*) *tetrazonata* may be *N.* (*Curvinomia*) *augustitibialis*. Current keys do not resolve these species satisfactorily.

Lawrence, Kansas, during September 1980, and *N. heteropoda* was collected at *Helianthus* flowers during July 1980 near Havana, Illinois.

Individual specimens were placed in separate shell vials and stored in an ice chest prior to transport. The Dufour's glands were excised underwater and extracted with methylene chloride. Extracts contained from two to more than 30 glands. Individual variation within each species is currently being investigated.

The extracts were analyzed on a Finnigan 3200 computerized gas chromatograph–mass spectrometer (GC-MS) utilizing a 2-m × 1-mm 3% OV-17 on Supelcoport 60/80 column, temperature programmed from 60 to 300° C at 10° C/min. Individual compounds were identified by comparison of their mass spectra and retention times either with those of standard compounds or with previously published data. The isopentenyl esters used as standards were synthesized from the corresponding fatty acids and either 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol using *N,N'*-carbonyl diimidazole following the procedures of Staab (1962).

RESULTS

Chemistry. Dufour's glands were analyzed from four species of *Nomia* listed in Table 1. These contain even-numbered, saturated macrocyclic lactones, together with isopentenyl even-carbon fatty acid esters. While none of the five identified lactones appear in all species, three of the six esters are found in all four species. The relative amounts of the lactones and esters vary from species to species. In all species, the C_{18} and C_{20} isopentenyl esters are major constituents in the extracts. A typical gas chromatogram of the Dufour's gland extract of *Nomia nevadensis* is shown in Figure 1. The major compounds are the C_{18}, C_{20}, C_{22} isopentenyl esters and the C_{22} and C_{24} lactones. Traces of the C_{20} and C_{26} lactones as well as the C_{14}, C_{16}, and C_{24} esters are also present.

The Dufour's chemistry of *N. triangulifera* is very similar to that of *N. nevadensis* but lacks the C_{26} lactone and the C_{14} ester. That of *N. tetrazonata* is the most distinctive of the four species analyzed, while the chemistry of *N. heteropoda* seems to be intermediate in complexity.

All lactones exhibit well-defined mass spectra which are useful in the identification of trace constituents. Thus the C_{20} lactone exhibits a readily detectable molecular ion at m/z 310 (5%) with a loss of water to m/z 292 (5%). The next largest ion is at m/z 250 (M-60) (CH_3CO_2H) with a smaller ion at m/z 264 (M-46) followed by clusters of unsaturated ions at 125, 111, 97, 83, 69, 55, and 41. This type of fragmentation is seen in the other lactones as well, with the base peak appearing at either m/z 41 or 55.

TABLE 1. COMPOUNDS IDENTIFIED IN DUFOUR'S GLAND EXTRACTS OF NORTH AMERICAN *Nomia* BEES[a]

Compound	Species and Subgenus			
	N. (Dieunomia) heteropoda	*N. (Epinomia) nevadensis*	*N. (Epinomia) triangulifera*	*N. (Tetrazonata) tetrazonata*[6]
18-Octadecanolide	tr	–	tr	tr
20-Eicosanolide	+	tr	tr	–
22-Docosanolide	tr	M	M	–
24-Tetracosanolide	–	M	M	–
26-Hexacosanolide	–	tr	–	–
3-Methyl-2(3)-buten-1-yl tetradecanoate	–	tr	–	–
3-Methyl-2(3)-buten-1-yl hexadecanoate	–	+	M	–
3-Methyl-2(3)-buten-1-yl octadecanoate	M	M	M	M
3-Methyl-2(3)-buten-1-yl eicosanoate	M	M	M	M
3-Methyl-2(3)-buten-1-yl docosanoate	+	M	M	+
3-Methyl-2(3)-buten-1-yl tetracosanoate	–	tr	tr	–
No. glands	30+	6	3	2

[a]M = greater than 10% of the total volatiles, + = 5–10% of the total volatiles, tr = less than 1% of the total volatiles, – = not detected.

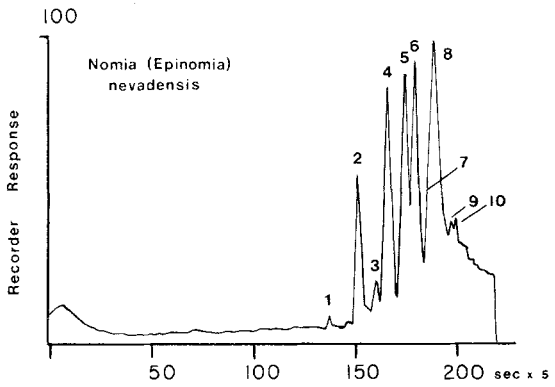

FIG. 1. Gas chromatogram of *Nomia (Epinomia) nevadensis* Dufour's gland extract: 1 = 3-methyl-2(3)-buten-1-yl tetradecanoate (mol wt 296), 2 = 3-methyl-2(3)-buten-1-yl hexadecanoate (mol wt 324), 3 = 20-Eicosanolide (mol wt 310), 4 = 3-Methyl-2(3)-buten-1-yl octadecanoate (mol wt) 352), 5 = 22-docosanolide (mol wt 338), 6 = 3-methyl-2(3)-buten-1-yl eicosanoate (mol wt 380), 7 = 24-tetracosanolide (mol wt 366), 8 = 3-methyl-2(3)-buten-1-yl docosanoate (mol wt 408), 9 = 26-hexadecanolide (mol wt 394), 10 = 3-methyl-2(3)-buten-1-yl tetracosanoate (mol wt 436).

All species contain mixtures of a homologous series of semiterpenoid esters with molecular weights of 296, 324, 352, 380, 408, and 436 (Table 1). Each of these exhibits a base peak at m/z 68 with an appreciable peak at 69. The molecular ion in each instance loses 85 amu, indicating a possible relationship between the base peak of 68 and this loss of 85. On the assumption that the fragment at M-85 constitutes an acylium ion ($C_{13}H_{27}CO^+$, $C_{15}H_{31}CO^+$, $C_{17}H_{35}CO^+$, $C_{19}H_{39}CO^+$, $C_{21}H_{43}CO^+$, and $C_{23}H_{47}CO^+$) and that the 85 fragment consists of an isopentenyl group, a series of 3-methyl-2-(and 3-) buten-1-yl esters were prepared. Comparison of the synthetic material with the natural product indicated that these nomiine species contain a mixture of 3-methyl-2-buten-1-yl and 3-methyl-3-buten-1-yl esters (with the 3 isomer dominating) in addition to the lactones. Although the isomeric esters coeluted on a temperature-programed OV-17 column, the intensity of the 69 peak relative to the 68 peak differed markedly in the two isomers. The natural material exhibited a 69 peak whose intensity was significantly greater than the 69 peak of the 3-methyl-3-buten-1-yl but significantly less than the 69 peak of the 3-methyl-2-buten-1-yl isomer. Isothermal gas chromatography on 3% OV-17 allowed separation of the isomeric mixtures and verification that the original peak was a composite of the two isomeric esters, the relative amounts varying from species to species.

Glandular Morphology. The relative size of the Dufour's gland in

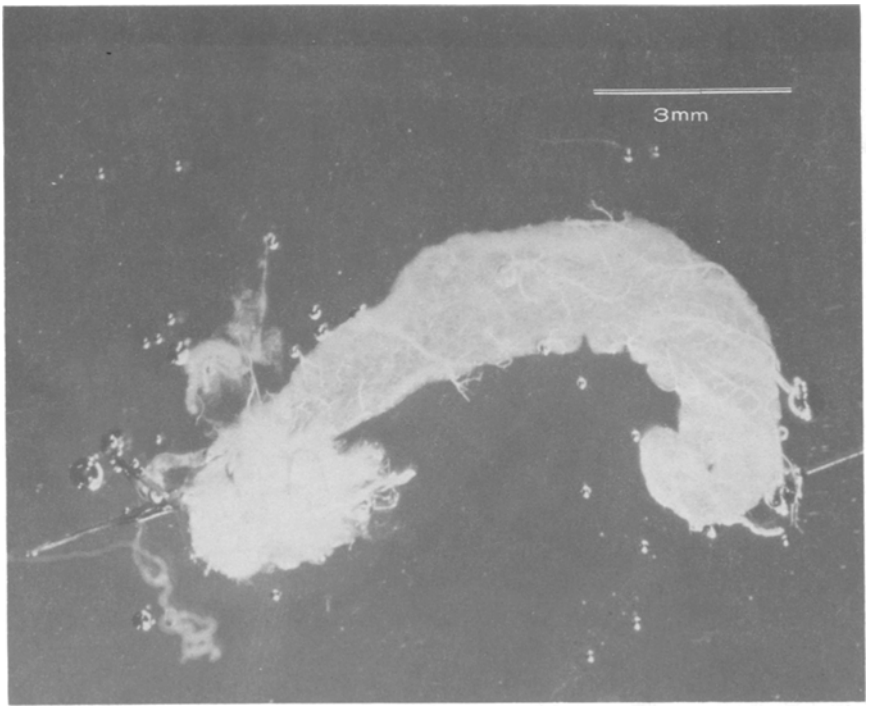

FIG. 2. Excised Dufour's gland of *Nomia* (*Dieunomia*) *heteropoda*.

actively nesting *Nomia* is dependent upon the species. In *Nomia heteropoda* the glandular reservoir is visible as a C-shaped, saclike structure about 13 mm in length (Figure 2). In occupies approximately one half of the abdomen. The gland is constricted where it attaches to the sting shaft. A dense matrix of tracheae envelop the gland, indicative of its intense biosynthetic activity.

DISCUSSION

The chemistry of the Dufour's gland extracts has been studied in several families of bees. Based on the chemistry of the Dufour's glands of 22 andrenids (Andrenidae), three distinct groupings of compounds are present: subgenus *Andrena* dominated by geranyl octanoate, numerous subgenera dominated by farnesyl hexanoate, and species which do not produce terpenoid esters (Fernandes et al., 1981). Esters including octadecyl butanoate dominate the Dufour's secretions of *Melitta* (Melittidae) species (Tengö and Bergström, 1976). Species in the Anthophoridae vary considerably. Those of *Melissodes*

are dominated by acetates (Batra and Hefetz, 1979) and *Anthophora* by triglycerides (Norden et al., 1978).

Dufour's gland secretions of the Colletidae: Hylaeinae (*Hylaeus*) (Duffield et al., 1980) and Colletinae (*Colletes*) Hefetz et al., 1979) and eight genera in the Halictidae (Halictinae) are dominated by a homologous series of saturated and unsaturated lactones ranging from C_{16} to C_{26} (Andersson et al., 1966; Bergström, 1974; Bergström and Tengö, 1979; Hefetz et al., 1978; Duffield et al., 1981). In addition, some species in the Halictidae contain a homologous series of isopentenyl esters of even carbon fatty acids (Duffield et al., 1981).

Systematic Implications. Although bees have proved to be a rich source of natural products, the exocrine chemistry of bees is a relatively unexplored area of chemical ecology. Among the primitive or short-tongued bees, Dufour's gland secretions have been analyzed for representatives of six of the 16 subfamilies. All species so far examined in the Halictidae (Halictinae) and Colletidae (*Colletes, Hylaeus*) contain macrocyclic lactones. Esters characterize the Dufour's gland secretions of representatives of the Andrenidae (*Andrena*) and Melittidae (*Melitta*). Chemical analyses of representatives of the remaining subfamilies could help clarify their systematic relationships.

The Halictidae is composed of three subfamilies. In all 25 of the Halictinae analyzed, the Dufour's glands contain saturated lactones along with traces of isopentenyl esters in some species. In contrast, *Nomia* has lactones and isopentenyl esters in equal quantities. It is significant to note that no unsaturated lactones have been detected in *Nomia*; in the Halictinae, both saturated and unsaturated lactones are present. Each of the four species of *Nomia* seems to exhibit species-specific blends, although the possibility of intraspecific variation (age, locality, etc.), cannot be excluded. The specificity is in contrast to the Halictinae, where the Dufour's gland chemistry is not species specific. The chemistry of the third subfamily, the Dufoureinae, is as yet unknown, but is currently under investigation.

Function. In the halictid bees the Dufour's secretions appear to generate the waxlike, waterproof brood cell linings (Michener, 1974) typical of many species. In *Augochlora pura pura* (Duffield et al., 1981) and *Lasioglossum albipes* (Cane, 1981), for example, brood cell linings contain the same constituents as the Dufour's gland. Similar evidence has been reported for *Colletes* (Hefetz et al., 1979; Albans et al., 1980), *Anthophora* (Norden et al., 1980), and *Andrena* (Cane, 1981). The halictids also incorporate the Dufour's secretion into the pollen ball (Duffield et al., 1981) as in *Anthophora* (Norden et al., 1980).

Nomia species line their cells with a waxlike covering (Malyshev, 1935; Bohart and Cross, 1955; Batra, 1972; Cross and Bohart, 1960) which may be

dull or glossy. The chemical composition of these and of pollen balls is unknown but may contain Dufour's constituents.

Recent data show that Dufour's secretions of bees are incorporated into cell linings and pollen balls. It is obvious that the chemistry of the Dufour's gland, the brood cell lining, and the pollen balls in more bees must be determined, both to clarify the secretion's functions and to aid in systematics.

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IDENTIFICATION OF A FEMALE-PRODUCED SEX PHEROMONE OF THE WESTERN CORN ROOTWORM

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Abstract—A sex pheromone has been isolated and identified from virgin females of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte. The synthesized compound, racemic 8-methyl-2-decanol propanoate, was equal in attraction to the natural pheromone when tested in the field as a trap bait against three taxa of *Diabrotica* known to respond to pheromone extracts from female WCR. Five taxa (*D. virgifera virgifera*; *D. virgifera zeae* Krysan and Smith, Mexican corn rootworm; *D. longicornis barberi* Smith and Lawrence, northern corn rootworm; *D. longicornis longicornis* (Say); and *D. porracea* Harold) were attracted to traps baited with 8-methyl-2-decanol propanoate. The response of male northern corn rootworms (NCR) in the field peaked at a relatively low concentration of 8-methyl-2-decanol propanoate and then was severely reduced at the higher concentrations tested. Conversely, the response of male WCR in the field continued to increase up to the highest dose tested.

Key Words—Chrysomelidae, *Diabrotica*, western corn rootworm, northern corn rootworm, Mexican corn rootworm, sex pheromone.

INTRODUCTION

The presence of a potent pheromone system in virgin females of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, has been known for some time (Guss, 1976; Bartelt and Chiang, 1977). Eariler studies by Ball and Chaudhury (1973) suggested the existence of a pheromone in the WCR of very low potency. These authors used extracts from field-collected insects of unknown mating history, and it was shown that mated WCR females do not attract males under field trapping conditions (Bartelt and Chiang, 1977).

Pheromone extracts from female WCR have been shown to attract males of the northern corn rootworm (NCR), *Diabrotica longicornis barberi* Smith and Lawrence, and live virgin females of both species appear equally attractive to males of either species when placed in field traps. A temporal difference in response between males of the two species was noted (Guss, 1976; Bartelt and Chiang, 1977). A recently described subspecies of *D. virgifera*, the Mexican corn rootworm (MCR), *Diabrotica virgifera zeae* Krysan and Smith, also readily responds to pheromone extracts from the WCR (Krysan et al., 1980). In this case, the temporal response is virtually identical to that of the male WCR; it was shown that these two subspecies interbreed in nature.

This paper reports the isolation, identification, and synthesis of a sex pheromone from WCR females and observations on the response of wild males of several *Diabrotica* taxa to the synthetic attractant. This is the first identification of a sex pheromone from a representative of the family Chrysomelidae, although a number of taxa in this family are significant economic pests throughout North America and much of the world.

METHODS AND MATERIALS

Pheromone Collection and Bioassay. Insects used in this study came from a laboratory colony reared according to Branson et al. (1975). Virgin females were isolated from males within 24 hr of emergence and held in screened cages (30 × 30 × 30 cm) for 3 days before being placed in pheromone collection chambers.

Initially, pheromone was absorbed on filter paper that had been in contact with virgin female beetles. Filter papers were shredded and extracted with 25% ether in hexane to yield a crude pheromone solution (Guss, 1976). This solution was subjected to thin-layer chromatography (TLC) on silica gel G with 5% ether in hexane as the solvent. The area of the chromatogram between R_f 0.23 and 0.37 was the only area containing pheromone activity. This area was scraped from the plate and extracted with 50% hexane–ether, and the resultant solution was suitable for gas–liquid chromatography (GLC) after volume reduction by evaporation at atmospheric pressure.

A new method was devised to collect larger quantities of pheromone. Approximately 1500–2000 virgin females were confined in a 6.5-liter glass cylindrical chamber. Air was filtered through charcoal, passed over the insects at 2.5 liter/min, and then through a filter (5.0 cm ID × 8.0 cm) containing ca. 5 g of Porapak Q® for pheromone absorption (Byrne et al., 1975). During pheromone collection, which was continuous, the insects were provided water and a dry diet mixed with a small amount of honey (Guss et al., 1976). Twice each week dead insects were removed and replaced with fresh females. Once each week all insects were removed from the chamber, the chamber was washed, and a fresh Porapak Q filter was installed.

Laboratory bioassay and field trapping experiments were conducted essentially as described earlier (Guss, 1976).

For laboratory bioassay, 4 or 5 male WCR beetles were placed in a disposable Petri dish (150 × 15 mm) and allowed to become acclimated for 15 min. Then a test compound in 1–5 μl of hexane was applied to a filter paper chip (5 mm^2), and after solvent evaporation (about 10 min), the treated chip was placed in the Petri dish. Positive responses consisted of orientation of the beetles toward the chip, distinctive antennal waving, and copulatory behavior toward the other males.

For field studies involving comparison of equal amounts of natural or synthetic pheromone, traps consisting of inverted 360-ml plastic coated cups were placed in corn fields on top of wooden stakes such that the top of the trap was about first ear height or about 1 m from the ground. Pheromone in hexane containing 10% trioctanoin was dispensed from cylindrical cotton wicks (30 × 10 mm) attached to the top of the trap. The traps were coated with Stickem Special®.

For field studies involving several concentrations of synthetic pheromone, the pheromone was dispensed from red rubber septa. In this case, no trioctanoin was added to the pheromone solution.

Pheromone Purification. Crude pheromone was extracted from the Porapak Q by back-flushing with 10 ml of 25% ether in hexane or 25% ether in pentane. Subsequent Soxhlet extraction of the filter material with ether and GLC analysis of that extract indicated that only trace amounts of pheromone remained on the Porapak after the first extraction. The volume of the eluent was reduced by evaporation at atmospheric pressure, and the ether–hexane extract was subjected to micropreparative GLC without further purification.

Micropreparative GLC for isolation of the pheromone was performed on a Varian model 1400® gas chromatograph equipped with a flame ionization detector. Stainless-steel columns were packed with 30% DEGS on 60/80 mesh Chromosorb-W® (3.2 mm OD × 9.1 m) and 1.5% OV-101® on 100/120 mesh Chromosorb G-HP® (3.2 mm OD × 1.5 m). Inlet temperature was 185°C and 150°C, respectively; the detector temperature was 250°C. Column temperatures were: DEGS, 160°, and OV-101, 120°C isothermal. A glass column (2 mm ID × 2 m) packed with 6% Carbowax 20M® on 120/140 mesh Chromosorb W was installed in an identical gas chromatograph and operated at 160°C with an injector temperature of 210°C. Carrier gas (N_2) flow rate through all columns was 20 ml/min.

The chromatographs were modified to accommodate a 90:10 effluent splitter and external fraction collector (Brownlee and Silverstein, 1968). Dry ice and acetone was the coolant for the fraction collector. Fractions were collected in 1.5 × 305-mm capillary tubes and were subsequently eluted with a minimal volume of hexane.

Analytical GLC was performed on 0.25-mm (ID) glass capillary columns

(Heath et al., 1980). An SP2340® column, 66 m long, was placed in a Hewlett-Packard model 5710A® gas chromatograph equipped with a model 18740B split/splitless injector. This system was operated at a N_2 carrier gas flow of 9.5 cm/sec. The column temperature was maintained at 60°C for 2 min after injection and then programed at 32°/min to 120°C. The splitless delay was 60 sec. An OV-101 column, 31 m long, in a Varian model 1200® GC equipped with a split injection system was operated at a He carrier gas flow of 18 cm/sec, a column temperature of 150°C, and an inlet split ratio of 100:1. A cholesteryl cinnamate column, 20 m long, was operated at the same conditions in a Varian model 1400 GC equipped with a split injection system.

Preparative liquid chromatography was performed on a 1.27 × 25-cm stainless-steel column packed with 5 μm Licrosorb® SI60 (Heath et al., 1978). A Lab Data Control Constametric II G Pump® delivered the hexane–ether (99:1) mobile phase at 3 ml/min, and the eluting components were detected with a Waters model R401® differential refractometer.

Pheromone Identification. Alkaline hydrolysis, hydrogenation, and bromination were conducted on small amounts of pheromone samples partially purified by TLC. Alkaline hydrolysis was carried out in 2 ml of 2 N KOH in 50% ethanol. The mixture was refluxed for 2 hr in a boiling water bath. After cooling, 1 ml H_2O was added, and the mixture was extracted with 3 ml hexane. The extraction procedure was repeated after acidification of the reaction mixture with 0.4 ml 10 N H_2SO_4. Both extracts were bioassayed in the laboratory and field.

Catalytic hydrogenation was carried out in a laboratory constructed microhydrogenator (Farquhar et al., 1959) in methanol with platinum oxide as the catalyst. The mixture was agitated vigorously under an atmosphere of hydrogen for 2 hr at ambient temperature. After removal of the catalyst, the mixture was bioassayed in the laboratory and field.

Bromination was conducted in methylene chloride containing an excess of Br_2. The mixture was allowed to react overnight at ambient temperature. Following this, 1 ml of 1% sodium bisulfite was added and the mixture shaken to convert excess Br_2 to HBr. The organic layer was washed four times with 2 ml H_2O before being used for laboratory and field bioassays.

Mass spectral data were obtained with a Finnegan model 3200® mass spectrometer equipped with both chemical ionization and electron impact sources. A Varian model 1400 gas chromatograph equipped with a 5% OV-1 column, 2.2 mm (ID) × 2 m, served to introduce samples to the CI source. Methane was employed as reagent gas and GLC carrier gas. The EI source was served by a Finnegan model 9500® gas chromatograph equipped with an OV-1 column of the same dimensions. Helium was normally used as the carrier gas.

Hydrogenolysis was carried out in the injector of the gas chromatograph that served the EI source by the method of Beroza and Sarmiento (1963,

1964). About 6 cm of a 3.2-mm (ID) stainless-steel tube was filled with neutral Pd catalyst and placed in the injector port ahead of the OV-1 column. The catalyst was maintained at 225°C for the hydrogenolysis, and H_2 was used as the carrier gas at 30 ml/min.

RESULTS AND DISCUSSION

Early isolation studies were conducted with pheromone collected by the filter paper method. Difficulties encountered in rearing large numbers of insects coupled with the fact that the WCR apparently produces only small amounts of pheromone resulted in the collection of only nanogram quantities. Nevertheless, because the WCR male responds to nanogram quantities of the pheromone, both in the laboratory and in the field, it was possible to determine separation parameters by chromatographic fractionation coupled with laboratory and especially field bioassay.

The laboratory bioassay (Guss, 1976) was shown to be definitive for the presence of the pheromone when used in conjunction with TLC, i.e., a positive response with the laboratory bioassay always correlated with a positive response in the field. Erratic results, however, were obtained with the laboratory bioassay when testing GLC fractions, necessitating heavy reliance on field-trapping experiments to identify GLC fractions containing pheromone. Attempts to demonstrate pheromone activity in a greenhouse or large-chamber situation with laboratory-reared males failed.

Partially purified pheromone obtained from TLC was first subjected to GLC on 30% DEGS. Fractions were collected every 4 min and checked for the presence of pheromone by field bioassay. Only that fraction eluting between 20 and 24 min was attractive to male WCR. Subdivision of this area of the chromatogram and subsequent bioassay showed a single peak of activity corresponding to a retention index of 18.40 (Kovats, 1965).

Chromatography on 1.5% OV-101 of partially purified pheromone from TLC as well as rechromatography of the active fraction from DEGS again produced a single active fraction eluting between 20 and 24 min. The active peak on this column had a retention index of 14.55.

Alkaline hydrolysis of the partially purified pheromone from TLC by 15% KOH in ethanol completely destroyed pheromone activity, but catalytic hydrogenation or bromination had no apparent effect. These results suggested that the pheromone was an ester containing no olefinic bonds.

Improved rearing techniques increased the number of virgin females available for pheromone production to the point that it became feasible to undertake the isolation of microgram quantities of pheromone. Thus, the Porapak Q method was developed, and approximately 200,000 beetles over a 2-year span yielded about 10 μg of pheromone. The material extracted from Porapak Q was purified by sequential chromatography on DEGS and OV-

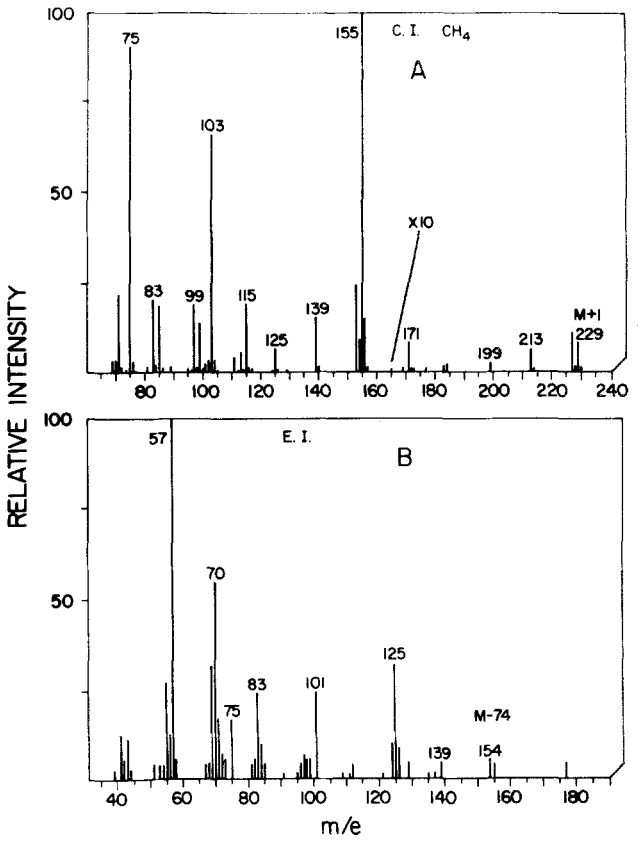

FIG. 1. (A) Chemical ionization (CH_4) mass spectrum of *D. virgifera* pheromone. (B) Electron impact mass spectrum of *D. virgifera* pheromone.

101 (see above) to yield a single fraction that was active in both laboratory and field bioassays. Male NCR were also attracted to those fractions attractive to the WCR. This was not unexpected since it had been shown earlier that extracts as well as live virgin females of either species attracted males of both species (Guss, 1976; Bartelt and Chiang, 1977).

Analysis on the OV-101 capillary column of the active material collected from the packed OV-101 column indicated that it contained about 5% of an impurity eluting immediately after the major peak. This impurity appeared as a shoulder on the major peak on the packed OV-101 column and could not be removed by chromatography on the other packed columns without sacrificing an unacceptable amount of the major peak which was known to be active.

The methane ionization mass spectrum of the major peak (Figure 1A) indicated this compound was a propionate ester of an 11-carbon saturated alcohol with diagnostic peaks at m/e 229 (M + 1), 227 (M − 1), 155 (M + 1 −

CH_3CH_2COOH), 75 ($CH_3CH_2COOH_2^+$), 103 ($CH_3CH_2COOHC_2H_5^+$), and 115 ($CH_3CH_2COOHC_3H_5^+$). The strong peaks, relative to M + 1, at 213 and 199 suggested the possibility of methyl substitution on the 3rd carbon from the hydrocarbon end of the alcohol moiety. The electron impact spectrum of this compound (Figure 1B) strengthened the evidence for a propionate with the peak at *m/e* 75, and the strong peak at *m/e* 101 indicated an alcohol function in the C-2 position of the chain. Jewett et al. (1976) suggested that a strong peak at *m/e* 101 is characteristic of a propionate function at the C-2 position and a branch at the C-3 position. However, the EI spectrum of authentic 2-undecanol propanoate, obtained under identical conditions, contained a peak at *m/e* 101 of about the same relative intensity as that found in the pheromone spectrum. The strong peak at 125 is characteristic of a methyl branch at either the C-3 or C-8 position of a 10-carbon chain, but its intensity suggested that a methyl at C-8 was more likely.

The mass spectrum of the product of hydrogenolysis of the pheromone in the gas chromatographic injector leading to the EI source is shown in Figure 2. The peaks at *m/e* 156 (M+) and 126 support the structure of 3-methyl decane for the hydrogenolysis product.

Thus all the mass spectral evidence supports the assignment of 8-methyl-2-decanol propanoate (I, Figure 3) for the structure of the pheromone, but 3-methyl-2-decanol propanoate could not be ruled out absolutely.

Synthesis of I. The synthesis of racemic 8-methyl-2-decanol propanoate, I (Figure 3) commenced with the reaction of methyl cyclopropyl ketone and ethyl magnesium bromide. The product was isomerized (Julia, 1961) producing the homoallylic bromide II (bp 76–82°C at 30 mm, rep. bp 76–80°C at 27

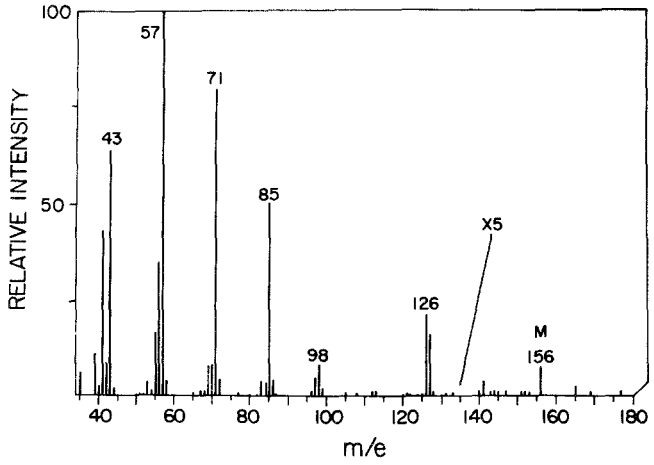

FIG. 2. Electron impact mass spectrum of hydrogenolysis product of *D. virgifera* pheromone.

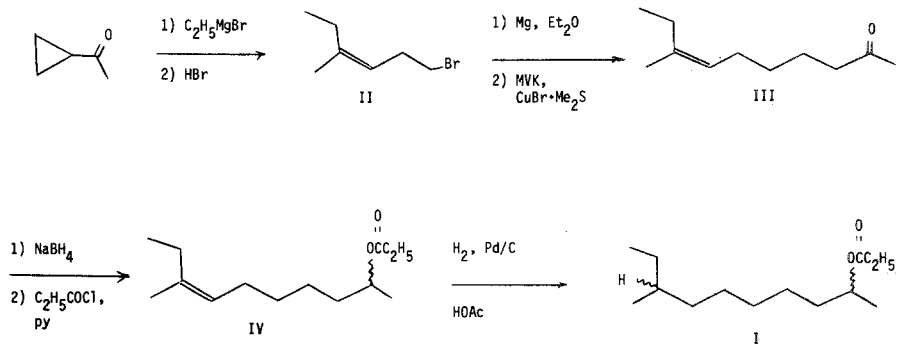

FIG. 3. Synthesis of racemic 8-methyl-2-decanol propanoate.

mm, yield: 74%). The bromide was converted into a Grignard reagent in ether and added to a stirred suspension of cuprous bromide–dimethyl sulfide (House et al., 1975) in an ethereal solution of freshly distilled methyl vinyl ketone. The ketonic product, III, was obtained pure after chromatography on silica gel separated it from the dimeric hydrocarbon formed from II; i.e., the Grignard preparation from II was beset by self-condensation. Although several alternative techniques and catalysts were examined, the study was not exhaustive. In addition, the procedure described provided a product free of the allylic alcohol that would (and generally did) derive from condensation of the Grignard reagent of II with the carbonyl group of methyl vinyl ketone. This inverse addition technique gave III in ca. 37% yield from II[IR(CCl_4) 1700 cm^{-1} (C=O)]. Ketone III was reduced with $NaBH_4$ in methanol to give the corresponding alcohol [IR (CCl_4) 3560 cm^{-1} (OH)] which was converted directly to the propionate ester IV using propionyl chloride in pyridine [bp 66°C (bath temp.) at 0.005 mm; IR (CCl_4) 1740 cm^{-1} (ester C=O); NMR (d_6-acetone) 5.0 (m, 1H, vinyl H), 2.28 (g, 2H, $J = 7$, $CH_3C\underline{H}_2CO_2$), 1.18 (d, 3H, $J = 7$, $C\underline{H}_3CHO$), 1.07 ppm (t, 3H, $J = 7$, $C\underline{H}_3CH_2CO_2$); yield ca. 80–90% from ketone III). The saturated ester I was then obtained by hydrogenation over 5% Pd/C in acetic acid [bp 60–65° C (bath temp.) at 0.005 mm; IR (CCl_4) 1740 cm^{-1}; NMR (d_6-acetone) 4.86 (m, 1H, $CH_3C\underline{H}O$), 2.26 (g, 2H, $J = 7$, $CH_3C\underline{H}_2CO_2$), 1.17 (d, 3H, $J = 7$, $CH_3C\underline{H}O$), 1.06 ppm (t, 3H, $J = 7$, $CH_3C\underline{H}_2CO_2$)].

The synthesized racemic 8-methyl-2-decanol propanoate was purified by high-performance liquid chromatography (HPLC) on Licrosorb SI60 and subsequently by gas chromatography on Carbowax 20M. Analysis of the material collected from Carbowax 20M by capillary gas chromatography on OV-101, SP2340, and cholesteryl cinnamate indicated that it was greater than 99% pure and identical in retention time to the major peak of the active pheromone fraction collected from OV-101. Both the EI and CI spectra of 8-methyl-2-decanol propanoate were identical to the respective spectra of the major peak of the natural material.

TABLE 1. COMPARISON OF 8-METHYL-2-DECANOL PROPANOATE (8-MDP) AND NATURAL WCR PHEROMONE

Species	Average number of insects trapped		
	8-MDP	WCR pheromone	Unbaited control
D. virgifera zeae (MCR)	173[a]	180[a]	12
D. virgifera virgifera (WCR)	1703[b]	1868[b]	19
D. longicornis barberi (NCR)	1455[b]	1339[b]	145

[a]Average of 4 traps containing about 10 ng active compound. Data collected for 24-hr period near Beeville, Texas.
[b]Average of 2 traps containing about 350 ng active compound. Data collected for continuous 12-day period near Brookings, South Dakota.

Bioassay of I. Laboratory bioassay of synthesized racemic 8-methyl-2-decanol propanoate (I) elicited behavior from male WCR identical to that observed with the natural pheromone. Field assay of I was first carried out in late May 1980 in cornfields near Beeville, Texas. The corn rootworm population in these fields was exclusively Mexican corn rootworm (MCR), *D. v. zeae*. Traps containing as little as 10 ng of I were highly attractive to the MCR compared to unbaited control traps.

Data in Table 1 show the comparative attractive properties of 99+% pure I and unpurified natural WCR pheromone toward the MCR under field conditions. The concentraton of I and natural pheromone was ca. 10 ng as determined by GLC. Because of time limitations, the data in Table 1 concerning the MCR are from a single 24-hr period; nevertheless, the average number of beetles caught by each treatment strongly suggests that I is the active component in pheromone extracts from WCR females responsible for attracting MCR males.

Comparison of 99+% pure I and >90% pure natural pheromone in the presence of native populations WCR was carried out at Brookings, South Dakota, in early August of 1980. Data in Table 1 show the results of trapping for a continuous 12-day period. About 350 ng of I or natural pheromone were placed in each trap. The data show that I and the natural pheromone are equally attractive at these levels to both the WCR and the NCR. The identity of the impurity in the isolated WCR pheromone fraction was not determined. Since the natural and synthetic pheromone elicited identical responses when tested at the same concentration, this impurity apparently does not interfere with trap captures. The possibility that this compound plays some as yet undefined role in WCR chemical communication or that other active components of the WCR pheromone exist cannot be ruled out. Laboratory bioassays of GLC fractions of the natural extract indicated that other compounds may play a role, as yet undefined, in the communication of the

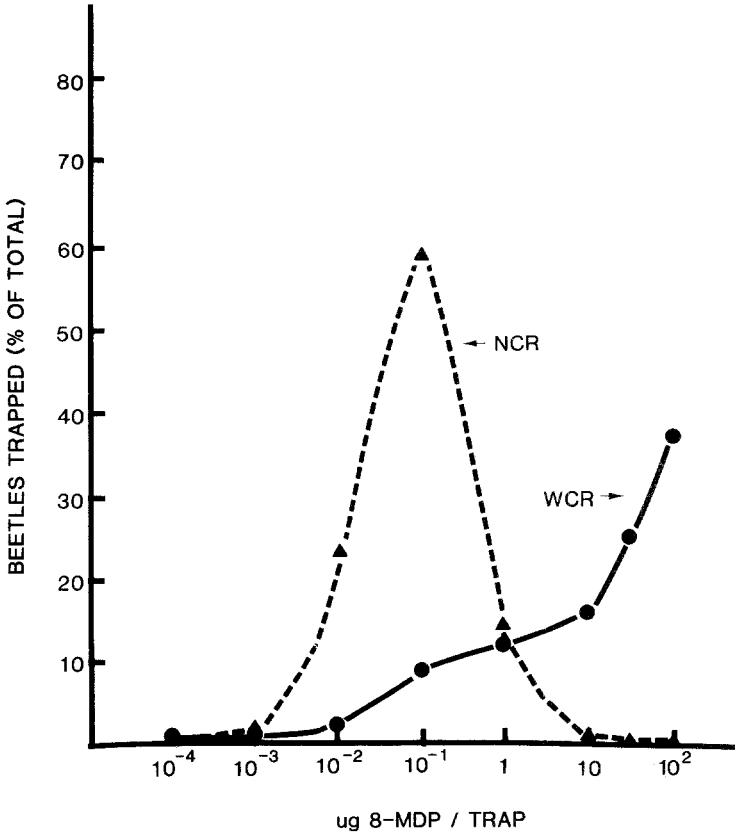

FIG. 4. Percentages of total WCR and NCR captured in field traps containing varying amounts of 8-methyl-2-decanol propanoate. Traps were located in a field containing a mixed population of WCR and NCR.

WCR. We therefore conclude that 8-methyl-2-decanol propanoate is identical to the major, if not the only, natural product produced by WCR females that is responsible for attraction of WCR males under field conditions.

Because only small amounts of natural pheromone were available for these studies, relatively small amounts of compounds were used in the comparative experiments. Another series of experiments was conducted in which a wide range of concentrations of I was placed in field traps in the presence of a mixed population of WCR and NCR. The results of this study, summarized in Figure 4, show that trap catches of WCR increased with increasing pheromone concentration while catches of the NCR peaked at a pheromone concentration of about 0.1 μg/trap and fell to zero at 10 μg/trap and above. The fact that the synthetic pheromone was racemic may have an

influence on the results obtained. The effects of individual stereoisomers or blends thereof await the availability of these compounds in pure form.

In addition to the taxa mentioned above, at least two other diabroticites are known to be attracted to traps baited with I. Thus, in trapping experiments in Mexico (State of Jalisco) males of *D. porracea* Harold were found in small but significant numbers in I baited traps (T.F. Branson, personal communication). This species never appeared in unbaited control traps, and intensive personal searches failed to produce a single free moving specimen of either sex, indicating the scarcity of these insects in the trap area. Also, males of *D. longicornis longicornis* (Say), which closely resemble those of the northern corn rootworm, were attracted to I baited traps placed in wild cucurbit patches in Kansas (J.L. Krysan, personal communication).

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BIOCIDAL ACTIVITY OF ALGAL TOXINS AGAINST IMMATURE MOSQUITOES

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Abstract—Extracts of two species of green algae, filamentous *Rhizoclonium hieroglyphicum* Kütz and a phytoplankton, *Chlorella ellipsoidea* Gerneck, obtained with solvents in the laboratory were assayed against *Aedes aegypti* L., *Culex quinquefasciatus* Say, and *Culiseta incidens* (Thomson). On extraction with petroleum ether, ground *R. hieroglyphicum* yielded an active crude extract which was chromatographed on a neutral alumina column and eluted consecutively with petroleum ether, benzene, and methanol. All three eluted fractions were found to induce significant mortality in test mosquito species. The benzene-eluted fraction was the least toxic. The methanol-eluted fraction was the most toxic to all species and exhibited juvenile hormone-like activity; it also caused morphogenetic changes in emerging adults. All three fractions delayed the rate of development of mosquito larvae by 2–5 days. Three supernatants of *C. ellipsoidea* obtained on different occasions were tested against first instars of *C. quinquefasciatus*. After the confirmation of their activity, all supernatants were extracted with diethyl ether, combined, and assayed against first- and fourth-stage larvae of the three mosquito species. The first-stage larvae of *C. quinquefasciatus* and *C. incidens* were approximately three times more suspectible than those of *A. aegypti*. However, fourth-stage larvae of the former two species were about twice as susceptible to the extract as those of the latter species. Dead first-stage larvae of all the species had a shrunken appearance. In general, *C. ellipsoidea* extract was quicker acting than that of *R. hieroglyphicum*.

Key Words—*Rhizoclonium hieroglyphicum*, *Chlorella ellipsoidae*, algae, algal toxins, mosquito larvicides, *Aedes aegypti*, *Culex quinquefasciatus*, *Culiseta incidens*, Diptera, Culicidae.

INTRODUCTION

As autotrophs, algae generally play a significant role in the maintenance of homeostasis in aquatic ecosystems. However, some of the algal species have

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been known to induce deleterious effects in some components of the aquatic fauna. Cases of acute poisoning of fish and waterfowl caused by the toxic blooms of blue-green algae have been reported (Prescott, 1948; Ingram and Prescott, 1954; Davidson, 1959). It has been documented that Cyanophyceae produced toxins which were proven to be lethal to a wide variety of organisms in aquatic systems (Stangenberg, 1968; Michel et al., 1972; Andreyuk et al., 1975). Isolation of the toxic factors from various algal species has been reported in several studies (Bishop et al., 1959; Dillenberg and Dehnel, 1961; Amonkar, 1969; Topachevsky, 1975).

Like blue-green algae, green algae have also been implicated in reducing zooplankton populations. Ryther (1954) and Arnold (1971) reported the inimical action of *Chlorella vulgaris* on water fleas, *Daphnia* spp. Amonkar (1969) and Lalonde et al. (1980) demonstrated that *Cladophora glomerata* Kütz induced mortality in several species of mosquito larvae.

In our recent studies (Dhillon and Mulla, 1980), a green phytoplankton alga, *Chlorella ellipsoidea* Gerneck, was found to exhibit toxic effects in mosquitoes breeding in cemetery vases. The density of mosquito larvae in vases with *C. ellipsoidea* was found to be substantially and signficantly lower than that in vases without the alga. We also noticed that the blooms of another green filamentous alga, *Rhizoclonium hieroglyphicum* Kütz, growing at the bottom of a lake caused a considerable reduction in chironomid midge populations in a residential-recreational lake in southern California (Mulla and Dhillon, unpublished data). We therefore hypothesized that the low densities of mosquitoes in cemetery vases and chironomid midges in the lake benthos might be due to the toxic principles produced by these algae in the immediate environment. In an attempt to prove this hypothesis, we conducted numerous biological investigations on these two species of algae. The objectives of the present study, a continuation of the earlier ones, were the isolation of toxic substances from these two algal species and the evaluation of their biological activity against several species of mosquitoes.

METHODS AND MATERIALS

Isolation. Studies on the evaluation of the toxic entities of the two algal species, *R. hieroglyphicum* and *C. ellipsoidea*, against several species of mosquitoes were carried out under laboratory conditions. The methods of extractions and bioassay are described below.

The filamentous alga, *R. hieroglyphicum*, growing on the lake bottom was collected from Lake Calabasas, California, washed several times with tap water, and air-dried at room temperature. The filaments were cut into smaller pieces and pulverized in a meat chopper (Universal #323). The ground alga (450 g) was gently refluxed in a mixture of benzene and methanol (3 : 1, 2 liter)

for 2 hr. After cooling, the mixture was filtered and washed with a mixture of benzene and methanol (3 : 1, 1 liter). The filtrate and the washings were combined, and the solvents were removed by means of a rotary evaporator. The residue obtained from this process was dissolved in petroleum ether (90 ml). After being chilled for 4 hr in a refrigerator, the mixture was filtered and washed with the same solvent (25 ml 2 ×). The combined filtrate and washings from this process were evaporated to dryness to obtain a crude extract (8.4 g). A portion of this extract was assayed at 30.0 mg extract/100 ml H_2O against laboratory-reared first-stage larvae of the mosquito, *Culex quinquefasciatus* Say. Most of the remaining crude extract (8.0 g) was chromatographed on a neutral alumina column (320 g). Two liters of each eluant were used in the following sequence: petroleum ether, benzene, and methanol. The eluates were evaporated to dryness, and each of the three residues was dissolved in acetone (20 ml) and then assayed at two different concentrations (1.0 and 0.5 ml/100 ml H_2O) against first-stage larvae of the mosquitoes *C. quinquefasciatus*, *Culiseta incidens* (Thompson), and *Aedes aegypti* L.

A similar procedure was followed to conduct the entire isolation process without using the ground alga. The product, if any, was subjected to bioassay tests as control.

Algal suspensions of unicellular *C. ellipsoidea*, collected from cemetery vases of fir and arbor lawns of a cemetery (Rose Hills Memorial Park, Whittier, California), were sieved through 100-mesh screen. The algal suspension was then centrifuged (Schnell Centrifuge CEPA, type 41), and the supernatant was collected in 5-gal glass jars. A portion of this supernatant was assayed for activity against first-stage larvae of *C. quinquefasciatus*, and the rest of the supernatant in the jars was transferred to a cold room for storage. Upon confirmation of the biocidal activity, the supernatant was then removed from the cold room 6 hr prior to the extraction process. The supernatant (1 liter) was extracted with diethyl ether (300 ml 1 × , 150 ml 2 ×). The ether layers were combined and dried over $MgSO_4$ for 4 hr. After filtration, the ether solution was evaporated, and the residue thus obtained was dissolved in acetone.

A total of 71 liters of biologically active supernatants, obtained from algal suspensions on three different occasions, were extracted. All three extracts obtained at different times were tested separately at three concentrations against first-stage larvae of *C. quinquefasciatus*. The concentrations tested were 1 × , 5 × , and 10 × (1 × = amount of extract equivalent to 100 ml of the original supernatant). After confirmation of the bioactivity of the extracts separately, the extracts were combined and tested again against the first- and fourth-stage larvae of *C. quinquefasciatus*, *A. aegypti*, and *C. incidens*.

A similar procedure was followed for the water obtained from cemetery vases containing no algae and also for the laboratory tap water to serve as blank checks.

Bioassays. The procedure for bioassays described by Mulla et al. (1974) was followed. Twenty first- or fourth-stage larvae were placed in glass bowls (11-cm diam, 300-ml capacity) containing 100 ml tap water. A desired concentration of the extract was added to each bowl to produce a significant response. To test the supernatants, 100 ml was used without adding extract or water. Treatments and checks were replicated three times. Mortality was recorded every other day and observations were continued until all larvae were dead or all mosquitoes had emerged. Therefore, all means represent acute or delayed mortality including that in the emerging adults. Data were analyzed on a Statiscian Compucorp 145 E, and signficance of means between or among the treatments was compared by using the Duncan's multiple-range test ($P = 0.01$).

RESULTS AND DISCUSSION

Upon extraction with a benzene–methanol mixture and subsequent petroleum ether extraction, 450 g of the pulverized alga, *R. hieroglyphicum*, gave 8.4 g or a "crude petroleum ether extract." After column chromatography, 8.0 g of the crude petroleum ether extract yielded 82, 73, and 2330 mg of petroleum ether-, benzene-, and methanol-eluted fractions, respectively. The recovery rate of this chromatographic process was only 31%.

Exposure of first-stage *C. quinquefasciatus* larvae to the extract (30 mg/ 100 ml H_2O) resulted in 13, 60, and 12% larval, pupal, and adult mortality, respectively (total mortality of 85%), thus showing that the crude extract caused delayed mortality in this species and that the bulk of the mortality was in the pupal stage.

The toxicity of the three eluted fractions against first-stage larvae of *C. quinquefasciatus*, *C. incidens*, and *A. aegypti* is presented in Table 1. The petroleum ether-eluted fraction produced substantial larval mortalities in all three mosquito species at the higher concentration (1.0 ml/ 100 ml H_2O). At the lower concentration (0.5 ml/ 100 ml H_2O), this fraction was effective against *C. incidens* and *A. aegypti* but ineffective against *C. quinquefasciatus*. Of the three species tested, *C. incidens* was found to be the most susceptible and *C. quinquefasciatus* the least susceptible at both dosages. At the higher concentration, of the mortality of *C. quinquefasciatus* and *A. aegypti* was induced 3–5 days after the treatment, whereas in *C. incidens*, it occurred within 2 days.

The benzene-eluted fraction was found to be ineffective against *C. quinquefasciatus* (Table 1). However, the other two species of mosquitoes

were affected significantly. At both concentrations, the toxic effects of this fraction were more pronounced on *C. incidens* (75 and 32% mortality) than *A. aegypti* larvae (42 and 18%). Mortality in these two species occurred during almost the same period (within 2–5 days) as shown by the petroleum ether-eluted fraction. No significant delayed mortality in pupal or adult stages was noted. In general, this fraction was less potent than the petroleum ether fraction.

The methanol-eluted fraction at both concentrations exhibited a highly significant activity against all test species (Table 1). Exposure of first-stage larvae to the higher concentration resulted in 98% larval mortality in *C. incidens*. Mortality in both larvae (58%) and pupae (42%) of *A. aegypti* and in all the stages of *C. quinquefasciatus* was noted at this higher concentration. At the lower concentration (0.5 ml/100 ml H_2O), however, this fraction caused mortality in larval, pupal, and adult stages of all three test species. In general, mortality was highest in adult stages of these mosquitoes. Adult mortality was 52% in *C. quinquefasciatus*, 42% in *C. incidens*, and 33% in *A. aegypti*. This fraction at both concentrations induced little, if any, larval mortality in *A. aegypti* and *C. quinquefasciatus* during the first 5 days after treatment. The higher concentration of this fraction caused a substantial larval mortality in *C. incidens* 2–5 days after treatment, whereas the lower concentration showed very little activity during this period (2–5 days) against this species.

Synthetic insect growth regulators (IGRs) have been shown to cause mortality in pupal and adult stages when mosquito larval instars were exposed to these compounds (Mulla et al., 1974). In the present study the methanol-eluted fraction also demonstrated activity similar to that of the IGRs. In various other studies on the activity of algae, it has been documented that extracts obtained from *Chara globularis* (Angerilli and Beirne, 1974) and *Cladophora glomerata* (Amonkar, 1969) exhibited IGR-like activity in mosquitoes.

The methanol-eluted fraction was also found to cause various abnormalities in ecloding adults. The metathoracic legs of emerging adults were found to be attached to the pupal exuviae (Figure 1). Partial eclosion of adults was noticed to be another occurrence due to the activity of this fraction. Some of the adults after eclosion were incapable of flying and eventually died within a short period after emergence (4–6 hr). In addition to the above, in most of the emerged or partially emerged adults, deformities, such as cohesions of antennae, legs, or wings were the most frequent occurrences. In some mosquitoes, the costal and anal veins of wings were unusually swollen and filled with fluid.

In general, the petroleum ether- and benzene-eluted fractions were primarily larvicidal, whereas the methanol-eluted fraction, in addition to its larvicidal activity, also showed delayed activity in subsequent stages. Unlike the methanol-eluted fraction, the petroleum ether and benzene fractions did

TABLE 1. EVALUATION OF VARIOUS FRACTIONS OF *Rhizoclonium hieroglyphicum* EXTRACT AGAINST FIRST-STAGE LARVAE OF THREE SPECIES OF MOSQUITOES IN THE LABORATORY

Dosage (ml/100 ml H_2O)	Cumulative mortality (%) of different stages[a] by various fractions											
	Petroleum ether[b]			Benzene[b]			Methanol[b]			Check[b]		
	L	P	A	L	P	A	L	P	A	L	P	A
Culex quinquefasciatus[c]												
1.0	45 d	0	0	6 ab	3 a	2 a	17 c	12 bc	71 e	3 a	0	0
0.5	6 ab	0	2 a	2 a	0	0	7 ab	13 bc	52 d	2 a	1 a	1 a
Culiseta incidens[c]												
1.0	85 f	5 a	0	75 e	3 a	0	98 g	0	0	5 a	0	0
0.5	48 d	1 a	1 a	32 c	5 a	2 a	25 bc	23 b	42 d	5 a	0	1 a
Aedes aegypti[c]												
1.0	73 h	5 ab	0	42 f	0	0	58 g	42 f	0	7 ab	0	0
0.5	32 ef	7 ab	1 e	18 cd	0	1 a	15 bcd	25 de	33 ef	8 abc	0	1 a

[a] L = larvae, P = pupae, A = adults.
[b] 1.0 ml of petroleum ether, benzene, and methanol fraction contains 4.0, 3.5, and 116.5 mg of extract, respectively.
[c] Data analyzed separately for each species; means followed by the same letters are not significantly different from one another, new Duncan's multiple range test $P = 0.01$.

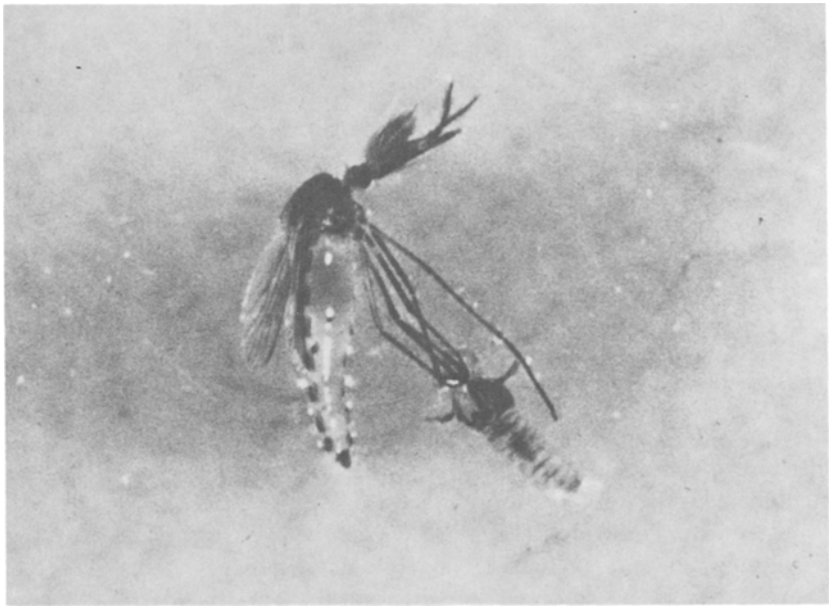

FIG. 1. Deformation caused by the methanol-eluted fraction in *C. quinquefasciatus* (meso-and metathoracic legs attached to the pupal exuvia).

not cause any deformities. All the fractions delayed the rate of development of mosquito larvae from 2 to 5 days, with the methanol-eluted fraction being the most effective in delaying the development.

The supernatants, obtained from algal suspensions of *C. ellipsoidea* which were collected on three different occasions, were extracted with diethyl ether. The three supernatants of 19, 22, and 30 liters, after extraction, yielded residues of 17.4, 73.0, and 323.5 mg, respectively.

The activity shown by three supernatants of *C. ellipsoidea* against *C. quinquefasciatus* is given in Table 2. The activity of supernatant 1 was the highest (92% mortality) and was not significantly different from that of supernatant 3 (76%). Supernatant 2 caused the lowest mortality (48%) in mosquito larvae. It indicates that supernatants 1 and 3 had higher bioactivity against mosquito larvae than had supernatant 2.

Data on the evaluation of the extracts obtained from these supernatants, each tested at three different concentrations against *C. quinquefasciatus*, are given in Table 3. The lowest concentration (1 ×) of these extracts was found ineffective, whereas the highest concentration (10 ×) produced acute toxic effects causing complete mortality of the larvae within 8 hr after the treatment. Nonetheless, at 5 × concentration, extracts 1 and 3 produced 98 and 93% mortality, respectively, as compared with extract 2 which caused 80%

TABLE 2. BIOLOGICAL ACTIVITY OF SUPERNATANTS OBTAINED FROM *Chlorella ellipsoidea* COLLECTED[a] ON THREE DIFFERENT OCCASIONS, AS EVALUATED AGAINST FIRST INSTARS OF *C. quinquefasciatus*

	Cumulative mortality (%) by supernatant		
	No. 1	No. 2	No. 3
Supernatant	92 b	48 a	76 b
Check[b]	8 a	10 a	8 a

[a] *C. ellipsoidea* was collected from vases of Rose Hills Memorial Park, Whittier, California.
[b] Check consists of water collected from nonalgal vases. Means followed by the same letter in the rows are not significantly different (new Duncan's multiple-range test $P = 0.01$).

mortality of *C. quinquefasciatus*. The toxicities shown by these extracts are almost in agreement with the mortality data of their respective supernatants tested against this mosquito species (see Table 2).

After testing individually against *C. quinquefasciatus*, the three extracts were combined and evaluated for biological activity against the three mosquito species. Based on LC_{50} and LC_{90} values presented in Table 4, the combined extract was found to be more toxic to *C. quinquefasciatus* and *C. incidens* than to *A. aegypti*. The first instars of both *C. quinquefasciatus* and *C. incidens* were about three times more susceptible to the extract than were those of *A. aegypti* at both LC_{50} and LC_{90} levels. The fourth instars of the two former species were only about twice as susceptible to the extract as the latter species. The data in Table 4 also indicate that the first-stage larvae of *C. quinquefasciatus*, *C. incidens*, and *A. aegypti* were about 4, 5, and 3.5 times

TABLE 3. EVALUATING OF VARIOUS DIETHYL ETHER EXTRACTS OF *C. ellipsoidea* SUPERNATANTS AT DIFFERENT CONCENTRATIONS AGAINST FIRST INSTARS OF *Culex quinquefasciatus*

	Cumulative mortality (%) by different extracts[a]		
Rate	Extract 1	Extract 2	Extract 3
1X[b]	7 ab	5 ab	20 b
5X[c]	98 d	80 c	93 cd
10X	100 d	100 d	100 d
Check	3 a	7 ab	2 a

[a] Extracts 1, 2, and 3 have been obtained from supernatants 1, 2, and 3 respectively (see Table 2 for supernatant sources).
[b] 1X = amount of extract(s) equivalent to 100 ml of supernatant before extraction.
[c] At 5X rate, extracts 1, 2, and 3 contained 0.45, 1.65, and 5.39 mg/100 ml H_2O, respectively. Means followed by the same letters are not significantly different from one another (Duncan's multiple-range test $P = 0.01$).

TABLE 4. ACTIVITY OF *Chlorella ellipsoidea* EXTRACT[a] AGAINST FIRST- AND FOURTH-INSTAR LARVAE OF THREE SPECIES OF MOSQUITOES

Larval stage	LC_{50} (ppm)[b]	LC_{90} (ppm)[b]	r	Slope
Culex quinquefasciatus				
1st	9	15	0.958	5.470
4th	38	59	0.978	6.623
Culiseta incidens				
1st	7	18	0.954	3.097
4th	38	68	0.992	5.091
Aedes aegypti				
1st	24	47	0.986	4.488
4th	85	141	0.983	5.875

[a]Extracts 1, 2, and 3 were combined and then tested.
[b]Concentrations are calculated based on the amount of extract in the solutions. Data analyzed using log-probit regression analysis on Statistician Compucorp 145E.

more susceptible respectively, than their fourth-stage larvae at LC_{50} level. At LC_{90} level the first-stage larvae of *C. quinquefasciatus*, *C. incidens*, and *A. aegypti* were about 4, 4, and 3 times more suspectible than their respective fourth instars. Of the three species tested, *A. aegypti* was relatively tolerant of *C. ellipsoidea* extract as compared to *C. quinquefasciatus* and *C. incidens*. At higher concentration, most of the mortality was produced within 24 hr, whereas at the lower concentration it occurred within 72 hr.

The *Chlorella* extract, like that of *R. hieroglyphicum*, also induced some morphological abnormalities in treated mosquitoes. The dead first-stage larvae of all test mosquito species showed a shrunken appearance. No such phenomenon was observed in dead fourth-stage larvae or in controls.

The extracts of water obtained from cemetery vases devoid of *C. ellipsoidea* and of tap water did not induce any significant mortality.

The above study reveals that both *R. hieroglyphicum* and *C. ellipsoidea* possess biocidal activity against the test mosquito species. The bioactive extract of *R. hieroglyphicum* exhibited juvenile hormone-like activity and was slower acting than that of *C. ellipsoidea*. The active principles of the former algal species were more toxic to *C. incidens* than to *C. quinquefasciatus*. Both mosquito species, however, were equally susceptible to the *Chlorella* extract. *A. aegypti* was found relatively more tolerant of *C. ellipsoidea* extract than that of *R. hieroglyphicum*.

Dhillon and Mulla (1980) demonstrated that the presence of *C. ellipsoidea* reduced mosquito larval population in the field in the cemetery vases. It appears from the present study that the low densities of mosquito larvae in vases were probably due to the biocidal activity of toxins produced by *C. ellipsoidea*. Likewise, it should be pointed out that the low midge

population in the lake bottom (Mulla and Dhillon, unpublished data) was likely due to the activity of toxins elaborated by *R. hieroglyphicum*.

Thus, at this stage, the bioactive compounds of these algal species show good potential as mosquito larvicides. Further studies are required to investigate the chemical identity of these toxic principles.

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NASONOV PHEROMONE OF THE HONEYBEE. *Apis mellifera* L. (HYMENOPTERA, APIDAE) IV. Comparative Electroantennogram Responses

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Abstract—Electroantennogram (EAG) responses from worker honeybee antennae were obtained for each Nasonov component. Response amplitudes to 10 μg of components correlated well with reported relative abilities to attract foragers in the field. EAG responses of worker, queen, and drone antennae to natural pheromone were consistently greater than to synthetic pheromone, a difference only partly explained by enzymic conversion of geraniol to (*E*)-citral during preparation of natural extracts.

Key Words—Honeybee, *Apis mellifera*, Hymenoptera, Apidae, electroantennography, Nasonov pheromone, multicomponent pheromone, enzymic oxidation, behavior, foraging.

INTRODUCTION

Electroantennography (EAG) has been much used to study pheromones of Lepidoptera and Coleoptera but less for those of Hymenoptera. In examining the Nasonov pheromone of the honeybee, a technique was devised to record EAG responses to it and its components so that physiological responses could be compared with behavioral responses in the field and the value of the EAG technique for honeybee pheromone research assessed.

The Nasonov gland of the worker honeybee secretes into a groove in the seventh dorsal abdominal tergite (Zoubarev, 1883; McIndoo, 1914). Workers expose the groove when foraging either at a source of water (Free and Williams, 1970) or artificial food, such as a dish of sugar syrup (von Frisch, 1923; Free and Williams, 1972). On exposure the secretion releases a volatile pheromone that attracts other workers.

The Nasonov pheromone comprises seven components, (*Z*)-citral, (*E*)-

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citral, nerol, geraniol, nerolic acid, geranic acid, and (*E, E*)-farnesol (Pickett et al., 1980), each attractive to foraging honeybees. A mixture of components in the proportions present in the honeybee is as attractive as the natural secretion, each component contributing to the attractiveness of the mixture (Williams et al., 1981). The composition of the pheromone during release is maintained by a highly specific enzyme system in the Nasonov gland that converts the major component, geraniol, into the more volatile (*E*)-citral (Pickett et al., 1981).

EAG responses to the Nasonov pheromone have not previously been reported. However, Kaissling and Renner (1968) recorded alternating current responses from single-pore plates of worker, queen, and drone antennae to scent from the Nasonov gland, and Beetsma and Schoonhoven (1966) and Vareschi (1971) recorded responses from worker antennae to geraniol, citral, and nerol.

Here responses to serial dilutions of each Nasonov component and to a synthetic mixture of components compared with those of the natural secretion are reported.

METHODS AND MATERIALS

Electroantennography (EAG). The right antenna of a honeybee was excised at the proximal end of the scape and laid on a block of plasticine. A glass capillary Ag–AgCl microelectrode, filled with Pringle's saline (Pringle, 1938) was inserted into the cut end of the scape and grounded. A similar electrode, mounted on a probe (Bioelectrics PAD 1) was inserted into the outer side of the second distal segment. With electrodes in position the total resistance at the input was 6–19 MΩ.

The test chemical in solvent (usually 10 μl hexane) was applied to the glass interior of a Pasteur pipet, positioned so that its tip was approximately 10 mm from the second segment of the antenna. The other end of the pipet was connected to a glass syringe (2 ml). To treat the antenna with the test chemical, air (1 ml) was ejected from the glass syringe through the Pasteur pipet after the solvent had evaporated. Each series of chemicals was tested on 4–8 antennae, starting with a solvent blank (10 μl hexane) and followed by synthetic chemicals or natural extracts in ascending concentrations to minimize adaptation. Odors were extracted from the vicinity of the preparation by a fan.

The output from the probe was monitored with a preamplifier (Palmer 8121) and oscilloscope (Devices 3121) and permanently recorded (Devices M2 with preamplifier DC 6).

The EAG response to mechanical and chemical stimulation was a rapid negative change in potential at the recording electrode, relative to the

indifferent electrode, followed by a return to near the original potential. The amplitude (in mV) of the initial response was recorded.

Chemicals. The preparation and purification of the chemicals used has been described (Pickett et al., 1980). For EAG responses from worker antennae to serial dilutions of each Nasonov component, portions (10 μl) of solutions of the components (10^{-2}–10^{3} μg in 10 μl hexane) were used to dose the pipets.

The synthetic mixture of components (*Z*)-citral–(*E*)-citral–nerol–geraniol–nerolic acid–geranic acid–(*E*,*E*)-farnesol, 1:1:1:100:78:11:44, was dissolved in hexane so that each dose (10 μl of solution) contained 0.1, 1, or 10 Nasonov gland equivalents (see Pickett et al., 1980). To assess the effects of enzymic conversion of geraniol to (*E*)-citral, and of fixatives, some solutions of synthetic mixture contained six or twelve times the amount of (*E*)-citral or 1% paraffin oil.

Natural pheromone was obtained from the excised Nasonov glands of 20 worker honeybees killed by chilling at −10° C. Glands were crushed in hexane (300 μl), and portions of the extract (1.5, 15.0, or 150 μl) containing 0.1, 1, or 10 gland extracts of natural pheromone, were tested.

RESULTS AND DISCUSSION

EAG Responses of Worker Antennae to Serial Dilutions of Nasonov Components. Figure 1 shows responses for each component. The amplitude of response to (*Z*)-citral, geraniol, geranic acid, nerolic acid, and (*E*,*E*)-farnesol increased as the source concentration of the component rose, but high source concentration of (*E*)-citral and nerol gave diminished responses. Thus responses to 10^{2} and 10^{3} μg (*E*)-citral were smaller than to 10 μg (*E*)-citral, and response to 10^{3} μg nerol was smaller than to 10^{2} μg nerol, probably because of adaptation of antennae by saturation of acceptor sites by doses applied previously to the antennae. Responses to other components, particularly at the higher source concentrations may have been similarly lowered by adaptation.

No adaptation to 10 μg source concentration of any component was apparent from the dose-response curves. Mean responses to 10 μg component, were, in order of decreasing amplitude (mV), (*E*)-citral, 0.46; geranic acid, 0.44; (*E*,*E*)-farnesol, 0.39; geraniol, 0.34; nerol, 0.29; nerolic acid, 0.26; and (*Z*)-citral, 0.23; correlating well (Spearman's coefficient of rank correlation $r = 0.857$, $P < 0.05$) with behavioral responses of foraging honeybees in the field (Williams et al., 1981). (*E*)-Citral and geranic acid were the most attractive of the components to foragers, and their omission from a synthetic mixture of components diminished its attractiveness most (Williams et al., 1981). Further evidence for the importance of these two components in the

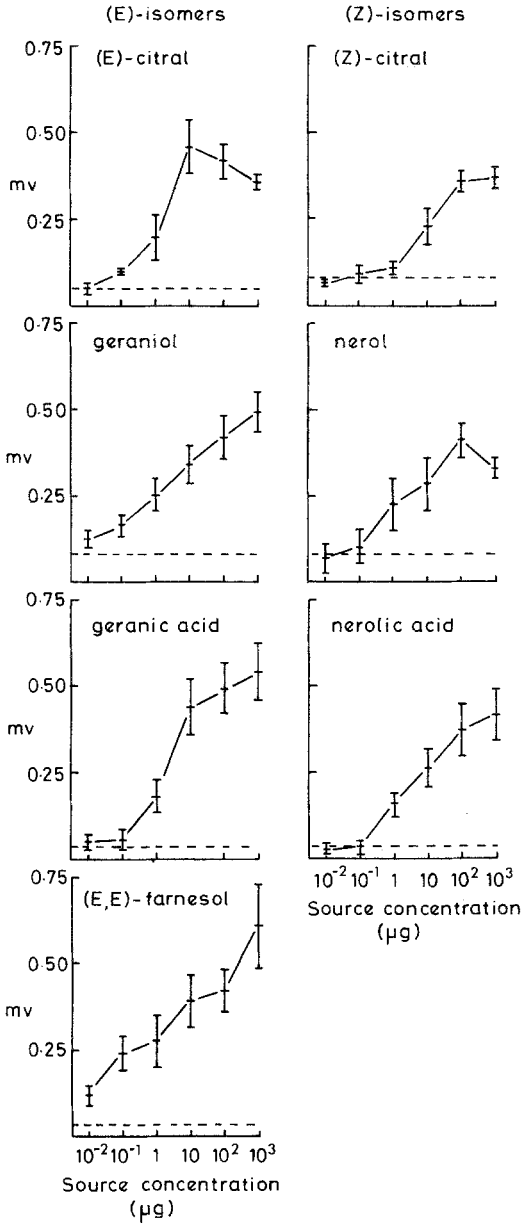

FIG. 1. Mean electroantennographic (EAG) responses of worker honeybee antennae ($N = 5$) to serial dilutions of components of the Nasonov pheromone. (Dotted lines indicate response to hexane blank, bars indicate SEM).

Nasonov pheromone is the presence of a highly specific enzyme system in the Nasonov gland that converts the major component, geraniol, into the more volatile (*E*)-citral and geranic acid, thus maintaining pheromone composition during release (Pickett et al., 1981).

EAG responses to the *E* isomers, (*E*)-citral, geraniol, and geranic acid, were greater throughout most of the concentration range tested than to the corresponding *Z* isomers, (*Z*)-citral, nerol, and nerolic acid. This probably arose from differences in chemical structure and not volatility because *E* isomers are less volatile than the *Z* isomers and hence the actual dose at the antenna is less concentrated. In the field, the *Z* isomers, (*Z*)-citral, nerol, and nerolic acid, also contributed less than the *E* isomers, (*E*)-citral, geraniol, and geranic acid, to the attractiveness of the synthetic Nasonov pheromone to foragers (Williams et al., 1981).

(*E*, *E*)-Farnesol gave the third largest EAG response at 10 μg source concentration and was also the third most important of the components to the synthetic mixture in attracting foragers. Geraniol, however, elicited a larger EAG response than the three *Z* isomers but was relatively unimportant (sixth) to the activity of the synthetic pheromone in the field.

The relative amplitude of the EAG responses at 10 μg, the highest source concentration tested at which no adaptation was apparent, may reflect the relative number of receptor cells for each Nasonov component, if dissimilar components elicit generator potentials of similar magnitude. The poor correlation of EAG responses to low concentrations of components (10^{-3}–1

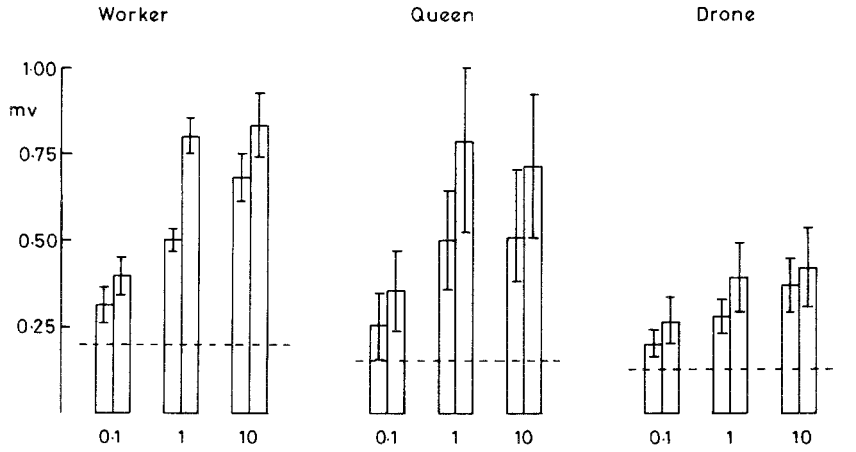

FIG. 2. Mean electroantennographic (EAG) responses of worker ($N = 5$), queen ($N = 4$), and drone ($N = 5$) honeybee antennae to 0.1, 1, and 10 gland equivalents of synthetic (first histogram) and natural Nasonov pheromone. (Dotted lines indicate response to hexane blank; bars indicate SEM).

μg) to field responses was perhaps explained by the few receptor cells stimulated.

The relative importance of the Nasonov components as assessed by electrophysiological and behavioral responses may indicate the evolutionary development of the pheromone. Possibly the first pheromonal component was geraniol. Then the development of the enzyme system for conversion of geraniol to (*E*)-citral and geranic acid (Pickett et al., 1981) may have led to increased specificity. This introduction of the *E* isomers might then have been followed by elaboration of the *Z* isomers, nerol, (*Z*)-citral, and nerolic acid by isomerase activity.

EAG Responses of Worker, Queen, and Drone Antennae to Natural and Synthetic Nasonov Pheromone. Figure 2 gives EAG responses of worker, queen (mated), and drone antennae to natural and synthetic Nasonov pheromone. Responses from worker and queen antennae were similar, presumably because they have similar numbers of pore plates and receptor cells for the Nasonov components. However, drone antennae gave smaller responses despite having more than seven times as many pore plates per antenna (Esslen and Kaissling, 1976), suggesting that relatively few of their pore plates have receptor cells that respond to Nasonov pheromone. The ability of queens and drones to detect the Nasonov pheromone may enable them to fly with an airborne swarm, and to cluster with the swarm when it settles.

Responses to synthetic pheromone were consistently smaller than those to natural pheromone; this was not because of adaptation for the synthetic pheromone was always tested before the natural pheromone. This contrasts with the results from behavioral bioassays, in which natural and synthetic pheromone attracted foragers equally (Williams et al., 1981), and may have resulted from enzymic conversion of geraniol to the more active (*E*)-citral during the time (30–60 min) between the excision and testing of glandular extracts. GC analysis (Pickett et al., 1980) of glandular extract showed that after 30 min the relative amount of (*E*)-citral present had increased sixfold.

Additional (*E*)-citral increased mean response amplitude slightly (Figure 3); for example, seven of the eight antennae tested gave larger responses to synthetic pheromone with 12× (*E*)-citral at both 0.1 and 1 gland equivalent source concentrations. However, responses were still not as large as to natural pheromone. Addition of paraffin oil diminished responses, presumably because it slowed release of chemicals to the air stimulating the antenna. Enzymic conversion of geraniol to (*E*)-citral was therefore probably only partly responsible for the increased EAG activity of the natural pheromone compared with synthetic pheromone, the remainder probably arising from active compounds not originally in the Nasonov gland, e.g., the alarm pheromone component (*Z*)-11-eicosen-1-ol (Pickett et al., 1982).

Thus, although the amplitude of EAG responses to individual Nasonov

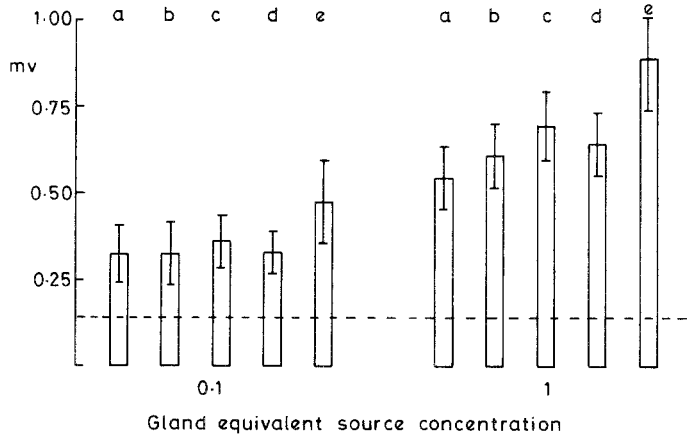

FIG. 3. Mean electroantennographic (EAG) responses of worker honeybee antennae ($N = 8$) to synthetic Nasonov pheromone (a), synthetic pheromone with sixfold (*E*)-citral (b), synthetic pheromone with 12-fold (*E*)-citral (c), synthetic pheromone with 12-fold (*E*)-citral and 1% paraffin oil (d), and natural pheromone (e). (Dotted lines indicate response to hexane blank; bars indicate SEM).

components correlated to some extent with behavioral responses, EAG but not behavioral responses to synthetic pheromone were consistently smaller than to natural pheromone. Hence conclusions from physiological assays must be confirmed in the field.

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Book Review

Management of Insect Pests with Semiochemicals: Concepts and Practice. E.R. Mitchell (ed.). New York: Plenum Press, 1981. U.S. $59.50, 514 pp.

Although about a dozen books have been written on the topic of semiochemicals, none focuses so sharply on practical uses of semiochemicals and underlying concepts as this most recent book: *Management of Insect Pests with Semiochemicals: Concepts and Practice*. The book constitutes the proceedings of an international colloquium on management of insect pests with semiochemicals, held March 23–28, 1980, in Gainesville, Florida.

E.R. Mitchell has skillfully edited the 36 contributions into five sections that correspond to the sessions of the colloquium as follows: I. Biomonitoring; II. Mass Trapping; III. Mating Disruption; IV. Formulation, Toxicology, and Registration; and V. Oviposition Disruptants and Antiaggregants. Each of the first four sections is followed by a summary of the recommendations from a panel discussion on future thrusts needed to foster the further development and practical use of the technology and of the underlying science. These summaries should be of particular interest to research administrators and to funding agencies.

Section I, Biomonitoring, includes contributions ranging from summaries of the manner and extent of use of semiochemicals in governmental and commercial programs to accounts of efforts to correlate numbers of insects caught in traps to population densities and to the need for treatment. Such correlations are strongly affected by the season, population density, host species and phenology, weather, etc. Therefore, the use of semiochemicals is most readily adapted to quarantine and food processing situations in which implementation of suppressive measures may be based solely on the presence of the pest. Only a few examples were given of the use of trap data in situations in which pest control decisions are made on the basis of pest density and on the likelihood that pest density will continue to increase. Further, none of the chapters deal in depth with the possibilities provided by using trap data in predictive pest–crop models and by computer processing of data obtained over a region to yield a synopsis of the pest situation.

Section II, Mass Trapping, provides fascinating accounts of mass

trapping programs involving tephritid fruit flies, the western pine beetle *Dendroctonus brevicomis*, the elm bark beetle, *Scolytus multistriatus*, ambrosia beetles, *Gnathotrichus*, sp. and *Trypodendron* sp., the spruce bark beetle *Ips typographus*, the Japanese beetle, *Popillia japonica*, and boll weevil, *Anthonomus grandis*. For example, the program on *Ips typographus* in Scandanavia is intended to protect overmature forest stands until they can be harvested. For this purpose 600,000 pheromone baited traps were developed on 4 million hectares in Norway and 320,000 traps were deployed in Sweden. The program is believed to have prevented the increase in tree mortality that had been predicted.

Quantitative interpretation of trap catch data is dealt with extensively in the chapter on boll weevil trapping. A computer simulation Monte Carlo model was developed in which the most important variables that govern the efficiency of traps are: (1) relative attractiveness of the pheromone bait to that of the pheromone produced by a wild insect; (2) number and distribution of traps in relation to numbers and distribution of pheromone-producing insects and of mate-seeking insects; (3) size of areas in which a trap and a competing insect can elicit a response by the mate-seeking sex; and (4) the actual efficiency of the trap in capturing individuals that respond. This model was used to interpret data from traps. For example, the study indicates that the probability that a trap will detect a single unmated boll weevil in 0.4 hectares of cotton is 94%. In addition, the study indicates that boll weevil populations can be suppressed in the F_1 generation by deploying 4–5 traps per 0.4 hectare. Thus, mass trapping can provide quantitative data on pest distribution and density in addition to suppressing the pest population.

Chapters in Section III, Mating Disruption, provide overviews of current status and future prospects for this approach, and also treat diverse topics such as the mechanisms whereby communication and mating are disrupted, methods for discovering mating disruptants, and methods for evaluating controlled-release formulations in the field. Successful practical applications of mating disruption technology are described for the pink bollworm, *Pectinophora gossypiella*; the grape moth, *Eupoecilia ambiguella*; western pine shoot moth, *Eucosma sonomana*. Both promising and discouraging results are described for a variety of other species.

Against the southern pine beetle, *Dendroctonus frontalis*, attractants are thought to have promise for managing small infestations, whereas inhibitors are thought to have promise for aerial application against large and currently unmanageable infestations. Camouflaging of the pheromone plumes of calling insects by plumes from artificial sources of the pheromone is believed to be an important mechanism of mating disruption in several lepidopteran species. The panel suggested that mating disruption would be facilitated if suitable formulations could be devised for application with existing spray

equipment. Also, moderate-scale commercial pheromone synthesis procedures are needed to reduce costs.

In Section IV, Formulation, Toxicology, and Registration, the notion that the registration process is still a serious constraint to full utilization of semiochemicals in the U.S.A. is rebutted. Moreover, the intention of the Environmental Protection Agency to implement special guidelines to facilitate the registration of biorational pesticides is stated. Since registration of semiochemicals is an international concern, coordination of the U.S. thrust with special studies sponsored by NATO and World Health Organization is urged. Even though the participants agreed that attitudes toward registration of semiochemicals should be more favorable, they nevertheless recognized the need for data to ensure that candidate materials for registration be free of adverse effects on man and on the environment.

Section V, Oviposition Disruptants and Antiaggregants, discusses pioneering efforts to determine the nature and possible utility of a variety of chemical factors that prevent the overutilization of a host resource by an insect pest. In 33 pest species belonging to 6 orders and 16 families, there is evidence that epideictic pheromones elicit dispersal of conspecifics away from food resources already occupied at densities near or at the upper end of the optimal range.

Oviposition-deterring pheromones may have an intriguing potential. Unlike the volatile sex pheromones, oviposition-deterring pheromones are persistent and, like insecticides, they may be useful in protecting commodities in close proximity to sources of gravid females. Moreover, these pheromones appear to be perfectly amenable to integration into pest management systems that involve mass trapping, release of sterile males, cultural measures, etc.

One chapter is devoted to the use of semiochemicals in enhancing the effectiveness of entomophagous insects.

The book has appeared at a time when interest and opportunities for developing the practical use of semiochemicals have reached unprecedented levels. The limited overlap and divergent views that exist between chapters are particularly beneficial to scientists and scholars who are pioneering in the use of semiochemicals. Thus, the book will be a very valuable resource on specific topics and can serve as a text for advanced students only. Most specialists will be pleased with the book and feel compelled to possess a desk copy.

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PLANT-DETERMINED VARIATION IN THE CARDENOLIDE CONTENT, THIN-LAYER CHROMATOGRAPHY PROFILES, AND EMETIC POTENCY OF MONARCH BUTTERFLIES, *Danaus plexippus*[1] REARED ON THE MILKWEED, *Asclepias eriocarpa*[2] IN CALIFORNIA[3]

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Abstract—This paper is the first in a series on cardenolide fingerprinting of the monarch butterfly. New methodologies are presented which allow both qualitative and quantitative descriptions of the constituent cardenolides which these insects derive in the wild from specific *Asclepias* foodplants. Analyses of thin-layer chromatographic profiles of *Asclepias eriocarpa* cardenolides in 85 individual plant–butterfly pairs collected at six widely separate localities in California indicate a relatively invariant pattern of 16–20 distinct cardenolides which we here define as the *Asclepias eriocarpa* cardenolide fingerprint profile. Cardenolide concentrations vary widely in the plant samples, but monarchs appear able to regulate total storage by increasing their concentrations relative to their larval host plant when reared on plants containing low concentrations, and vice versa. Forced-feeding of blue jays with powdered butterfly and plant material and with one of the constituent plant cardenolides, labriformin, established that the *A. eriocarpa* cardenolides are extremely emetic, and that monarchs which have

[1]Lepidoptera: Danaidae.
[2]Apocynales: Asclepiadaceae.
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fed on this plant contain an average of 16 emetic-dose fifty (ED_{50}) units. The relatively nonpolar labriformin and labriformidin in the plant are not stored by the monarch but are metabolized in vivo to desglucosyrioside which the butterfly does store. This is chemically analogous to the way in which monarchs and grasshoppers metabolize another series of milkweed cardenolides, those found in *A. curassavica*. It appears that the sugar moiety through functionality at C-3′ determines which cardenolides are metabolized and which are stored. The monarch also appears able to store several low R_f cardenolides from *A. eriocarpa* without altering them. Differences in the sequestering process in monarchs and milkweed bugs (*Oncopeltus*) may be less than emphasized in the literature. The monarch is seen as a central organism involved in a coevolutionary triad simultaneously affecting and affected by both its avian predators and the secondary chemistry of the milkweeds with which it is intimately involved.

Key Words—*Danaus plexippus*, Danaidae, monarch butterflies, *Asclepias eriocarpa*, Asclepiadaceae, milkweeds, coevolution, thin-layer chromatography, glycosides, cardenolides, cardenolide fingerprints, chemical defense, chemical ecology, labriformin, labriformidin, desglucosyrioside, emesis, ecological chemistry, plant–insect interactions.

INTRODUCTION

Monarch butterflies, *Danaus plexippus* L., are able, in their larval stage, to sequester cardiac glycosides from milkweed plants (Asclepiadaceae) which they store and utilize for chemical defense against vertebrate predators (review in Roeske, et al., 1976). Chemical analyses of wild-caught monarchs from eastern and western North America and in Mexico have established wide variation in their gross cardenolide content. Moreover, by using the blue jay (*Cyanocitta cristata bromia* Oberholser) as an experimental predator, it was found that the emetic potencies of the butterflies varied in proportion to the amount of cardenolide they contained (Brower, et al., 1972; Brower and Moffitt, 1974; Fink and Brower, 1981). However, at comparable levels of cardenolide content, the butterflies from overwintering areas in California were much more emetic than the others. Another difference between the eastern and western butterflies was also found: the relationship of cardenolide content and emetic potency was more variable in the California sample. It was hypothesized that a common explanation for both of these differences could be traced to the larval foodplants of the butterflies; the milkweed species in the west are completely distinct from those in the east. It seemed reasonable that their cardenolides would also differ and thereby impart these differences to the butterflies.

Thin-layer chromatographic analyses (TLC) of individual butterflies confirmed different cardenolide profiles in the eastern and western butterflies and also suggested a reasonable explanation for the more heterogeneous

relationship found between the emetic potency and gross cardenolide content of the California as compared to the Massachusetts samples. The latter produced qualitative TLC patterns which differed only slightly, whereas the California butterflies exhibited five distinct TLC patterns (Roeske et al., 1976). Further comparisons of the TLC patterns of the cardenolides in the butterflies with those in the leaves of several of the California milkweeds suggested that the different *Asclepias* species eaten by the monarch are responsible for the distinctive TLC profiles, i.e., it might be possible to "fingerprint" individual wild-captured monarch butterflies to the species of *Asclepias* previously ingested in the wild by their larvae.

Clearly, if fingerprinting is to be successful, the cardenolide profile of each species of milkweed must be distinct and must also be mirrored within reasonable quantitative limits by the butterflies. To examine these possibilities, we have analyzed the cardenolide content of several hundred individual adult monarchs collected as mature larvae or chrysalids on individual plants of seven milkweed species growing naturally in a diversity of geographic locations in California.

In this paper we present our basic methodology and the results of our first large-scale quantitative biogeographic analysis of the cardenolides in monarch butterflies collected on *Asclepias eriocarpa* Benth., a widely distributed California milkweed (Figure 1). Subsequent papers will present similar studies of monarchs in relation to six other California milkweeds, including *A. speciosa* Torr., *A. cordifolia* (Benth.) Jepson, *A. vestita* Hook and Arn., *A. erosa* Torr., *A. californica* Greene, and *A. fascicularis* Dcne.

Our specific aims in this series of papers are to answer the following questions: (1) To what extent is individual variation in the gross cardenolide content of wild butterflies determined by individual variation in the gross cardenolide content of the species of the larval host milkweed plant they ate in their natural habitat? (2) To what extent and with what fidelity do the butterflies mirror the TLC profiles of the cardenolides in each milkweed species? (3) How constant is the plant-determined butterfly cardenolide profile, both qualitatively and quantitatively, for both sexes of the butterfly and over the geographic range of the milkweed species? (4) What are the emetic potencies of monarchs reared on the various milkweed species, and do these correspond to the emetic potencies of the butterflies found in natural populations? and (5) How do the different emetic potencies relate to the cardenolide profiles in the various milkweed species and to the butterflies reared upon them?

If there is a sufficiently defined relationship between the butterflies and the plants which is retained throughout the life of the adult butterflies, it will be possible to answer a number of important questions about their ecological chemistry. First, we should be able to fingerprint the adult butterflies from

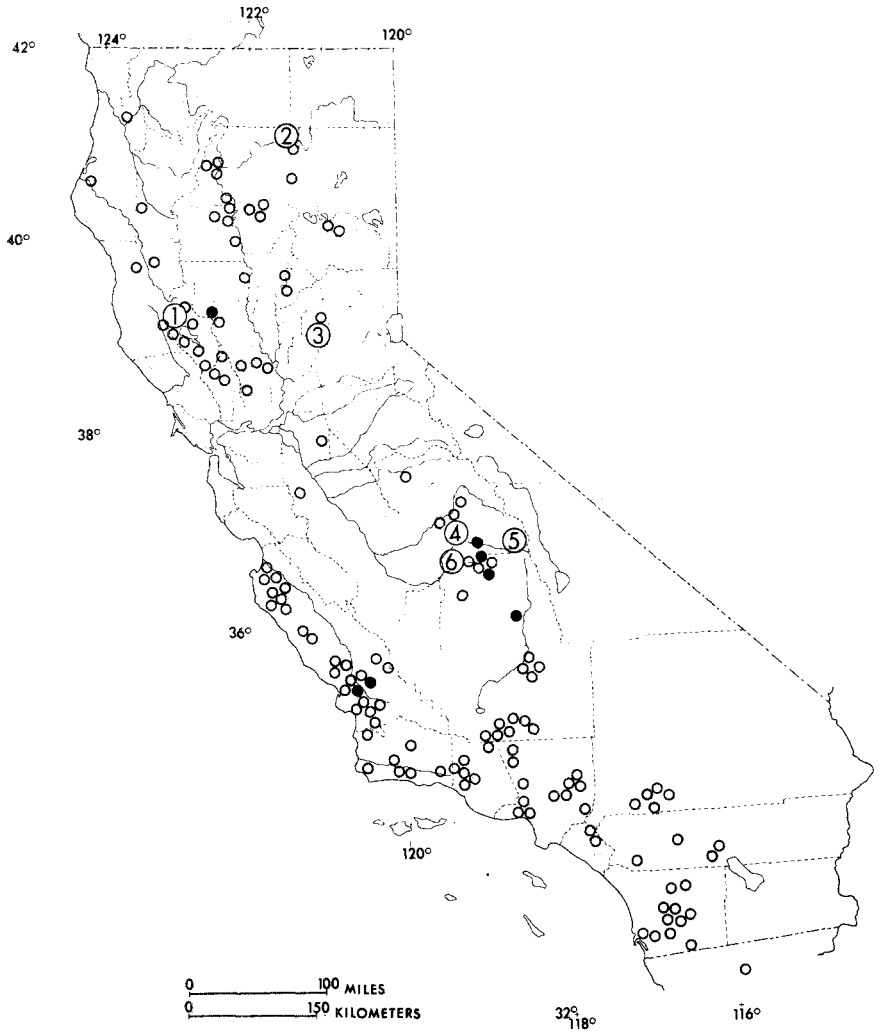


FIG. 1. The known geographic distribution of the milkweed *Asclepias eriocarpa* and the collection sites of the material analyzed in the study. The numbered open circles are the six localities where the 172 samples of monarch butterflies and their corresponding individual *A. eriocarpa* food plants were collected. The black circles are additional localities where feeding larvae were observed. Area 1 is in the North Coast Range, area 2 is in the montane zone of the southern Cascades, area 3 is in the foothills of the northern Sierra Nevada, area 4 is in the foothills of the southern Sierra Nevada, area 5 is in the montane zone of the southern Sierra Nevada, and area 6 is in the King's River floodplain, also in the southern Sierra Nevada.

wild populations and determine which species of milkweed each individual butterfly had consumed in its larval stage. Then, because of the extraordinary migration and overwintering behavior of the species (Urquhart and Urquhart, 1976, 1979; Brower 1977; Brower, et al., 1977; Tuskes and Brower, 1978), it may eventually be possible to assess quantitatively the extent to which individual species within the entire North American milkweek flora are utilized as larval food sources. It will also be possible to determine both qualitatively and quantitatively how different kinds and combinations of cardenolides found in the various milkweeds are selectively sequestered and stored by the monarch. Once these patterns are known, it will be feasible to investigate how selectivity of the compounds is related to their chemistry (Seiber et al., 1980), to their presumed differential negative physiological effects upon the butterflies (Brower and Glazier, 1975; Seiber et al., 1980) and to their differential pharmacological effects on vertebrates (Benson et al., 1979; Fink and Brower, 1981).

Definitive answers to these questions will provide a model system from which it will be possible to deduce how the secondary plant chemistry has developed through coevolution involving a multiplicity of interactive links in the community food chain, including the plants, their herbivores, and the vertebrate predators of the herbivores (Ehrlich, 1970; Brower, 1970: Gilbert and Raven, 1973; Atsatt and O'Dowd, 1976; Harborne, 1977; Levin and York, 1978; Cronin et al., 1978; Price et al., 1980).

METHODS AND MATERIALS

Collecting Methods.

A mobile laboratory was outfitted to collect immature monarchs (*Danaus plexippus plexippus* L., western U.S.A. population) from individual milkweeds over an extensive area of California in the summer of 1975. Populations of milkweeds were located and each plant was individually searched for larvae or chrysalids. If a chrysalid were found, it was removed from the plant along with a sample of leaves from the same stalk the larva had fed on, or from the same area of an adjacent stalk growing out of the same root. The leaves (frequently including immature and occasionally mature inflorescenses) were picked off and placed in a plastic bag with a code number and frozen within minutes on dry ice in a styrofoam chest. The chrysalid was given the same code number, kept under ambient conditions and allowed to emerge in a screen hatching chamber. Within 12 hr after hatching, each adult butterfly was placed in a glassine envelope, given a code number, and frozen on dry ice. All material was kept frozen on dry ice or in a freezer until oven dried and prepared for spectroanalysis.

Late fifth instar larvae were also collected and reared individually on leaves matched as to position with those which were frozen. These were stored in a plastic bag in a refrigerator in the mobile laboratory and fed to the larvae for up to three days following their initial collection. In some localities it was necessary to enclose earlier instar larvae on individual plants with nylon net bags and return to collect them as late 5th instar larvae or chrysalids, along with the sample of leaves. This was generally successful and avoided dipteran parasites. However, some larvae or chrysalids were removed, probably by rodents, through holes chewed in the bags.

Our cardenolide analyses require the larvae to eat the individual plant on which they are collected. This was assured in the bagged samples and facilitated for the others by the fact that monarch larvae in California show far greater fidelity to their individual plants upon which they nearly always form their chrysalids. This contrasts with larvae in the northeastern U.S. which usually wander off their plants to pupate on adjacent vegetation, frequently low in the grass (Brower, personal observations). Furthermore, because rain seldom occurs in the California *Asclepias* habitats during the monarch's breeding season, fecal pellets from several instars accumulate at the leaf bases. By selectively collecting larvae or chrysalids from individual plants with clear evidence of eating (chewed leaves and fecal pellets), together with the bagging, we are confident that nearly all our samples met the required matching criterion.

Collection Sites, Dates, and Geographic Distribution of Asclepias eriocarpa

Figure 1 shows the distribution of *Asclepias eriocarpa* in California based on Woodson (1954), field observations (S.P. Lynch), and data from herbarium specimens in all major University of California collections, the Rancho Santa Anna Botanical Garden, the California Academy of Sciences, the Jepson and Dudley herbaria in California, the University of New Mexico, Arizona State University, the University of Mexico (U.N.A.M.), the Universitario Polytechnico National, the Missouri Botanical Garden, and the Field Museum in Chicago.

According to Woodson (1954), *A. eriocarpa* is almost exclusively a California species but extends southward into northern Baja California. (We believe that its recorded occurrence in southeastern Nevada is erroneous.) It is abundant in numerous areas in California, and occurs in open habitats in many different plant communities. In disturbed areas such as roadsides, floodplains, and pastures, it often grows in dense stands.

Our total sample size is 172 (where 171 is indicated the discrepancy is due to one missing measurement). Six sampling areas were chosen to represent a diversity of localities over most of the plant's range. These include sites in the northern Coast Range, the southern Cascades, and the northern and

southern Sierra Nevada. Early (June) and late (August) samples were taken at area 6. The locations, site descriptions, and inclusive sample numbers and dates of collection (all in 1975) for the six areas are as follows:

Area 1 (North Coast Range) is located in the North Coast Range in Lake County, just northwest of Upper Lake on Bachelor Valley Road at an elevation of 410 m. Numerous plants were found at the edge of the small floodplain of Cooper Creek and in an adjacent clearing in the oak woodland surrounding the site. Sample numbers and dates of collections include Nos. 676–711, September 23. No. 47, also included here, was collected on June 17 at a similar site near Leesville in Colusa County.

Area 2 (Southern Cascades) is located in the Southern Cascade Ranges (of volcanic origin) in Lassen County at the eastern end of the Fall River Valley at an elevation of 1050 m. Numerous plants were found in the rocky meadows and scrub at the base of Big Valley Pass. Sample numbers and dates include Nos. 313–318, July 28; 664–672, September 14.

Area 3 (northern Sierra Nevada, foothills) is located in the foothills of the northern Sierra Nevada at the boundary of Placer and Nevada counties, just north of Auburn. The plants were located in open pasture adjacent to highway 49 at an elevation of about 425 m. Sample numbers and dates include Nos. 124–144, July 1; 188–204, July 12; 299, July 26; and 635, September 13.

Area 4 (southern Sierra Nevada, foothills) is located in Fresno County in the foothills of the southern Sierra Nevada between Tollhouse and Pine Flat Reservoir. The plants in this area were in open fields surrounded by blue oak (*Quercus douglasii*, H. and A.) woodland and along roadsides at elevations between 300 and 600 m. Sample numbers and dates include Nos. 54–77, June 25–26.

Area 5 (southern Sierra Nevada, montane) is located in Cedar Grove in Kings Canyon National Park, Fresno County. The plants were found in clearings in an open *Pinus ponderosa* Laws. forest community along the Kings River at an elevation of 1450 m. Sample numbers and collection dates include Nos. 272–285, July 20; 393–408, August 3.

Area 6 (southern Sierra Nevada, floodplain) is in Fresno County on Elwood Road along the floodplains of the Kings River and Mill Creek at an elevation of 230 m. Many plants were found along the roadside and in clearings in blue oak woodland. Sample numbers and dates include Nos. 29–34, June 10; 78–84, June 26; 378–380, August 2 and 607–627, August 27.

Analytical Procedures

Gross Cardenolide Content. The individual plant and butterfly specimens were analyzed for gross cardenolide content by a spectrophotometric assay based upon the base-catalyzed reaction of cardenolide with 2,2′, 4,4′-tetranitrodiphenyl (TNDP) as described in Brower et al. (1972), as modified

by Brower et al. (1975). The results are reported in μg (equivalent to digitoxin) per 0.1 g dry weight of material analyzed. These data cannot be converted to the exact amounts of cardenolides actually present in the milkweeds, since the molar absorptivities of the TNDP complex of all the *A. eriocarpa* cardenolides are not known. However, the estimates of cardenolide as μg/0.1 g dry weight and total μg are comparable to other assay results obtained using the readily available digitoxin standard (Roeske et al., 1976).

Cleanup Prior to Thin-Layer Chromatography. In order to remove pigments from the samples, a lead acetate precipitation method was developed using a procedure similar to Rowson's (1957) for *Digitalis* leaf tinctures. The method is as follows: Three ml of the extracts of the same plants and butterflies remaining after the spectroassay were evaporated to dryness under a nitrogen jet in 5-dram vials on a hotplate held between 60 and 70° C. These dried individual residues were stored in a freezer except while en route via air mail to Davis from Amherst. At Davis, about 3 ml of 95% ethanol was used to dissolve and transfer each sample to a 15-ml screw-cap test tube, which was then blown down with N_2 in a hot water bath (N-Evap, Organomation Associates, Shrewsbury, Massachusetts). Each residue was then dissolved in 1 ml of 95% ethanol, to which was added 2 ml of a 5% aqueous lead acetate solution. The contents were mixed for 30 sec by vortexing (Vari-Whirl Mixer, Van Waters and Rogers Co., New York), and the tubes were then placed in an ice bath for 20 min (see below). Following centrifugation at moderate speed for 10 min, the supernatant was decanted to a second screw-cap tube containing about 0.5 ml of granular ammonium sulfate and vortexed to precipitate the excess lead which had not complexed with pigments and other interfering materials. The precipitate from the lead acetate treatment was rinsed with 2 ml of a 1 : 2 solution of 95% ethanol–water; the rinse supernatant, separated by centrifugation, was combined in the ammonium sulfate treatment tube and the remaining residue was discarded. The combined supernatants and ammonium sulfate were mixed by vortexing and then centrifuged. This supernatant was decanted to a third 15-ml screw-cap test tube; the residue was rinsed with 2 ml of a 1 : 2 solution of 95% ethanol–water saturated with ammonium sulfate, and after centrifugation, the rinse solution was combined with the supernatant. This solution was extracted by vortexing with two separate 2-ml portions of chloroform. Each of the two chloroform extract layers was transferred by Pasteur pipet and filtered into a 5-ml screw-cap vial through about 1 g of anhydrous sodium sulfate placed on top of Pyrex glass wool in a small funnel. The filter was rinsed with a final 2 ml of chloroform. The solution in the vial was evaporated with N_2 (N-Evap) after each 2-ml aliquot was added. The vial was stored in a freezer for later use in spotting the TLC plates.

This method, summarized in Figure 2, provided substantially improved cardenolide recovery compared to a prior cleanup using solvent partitioning

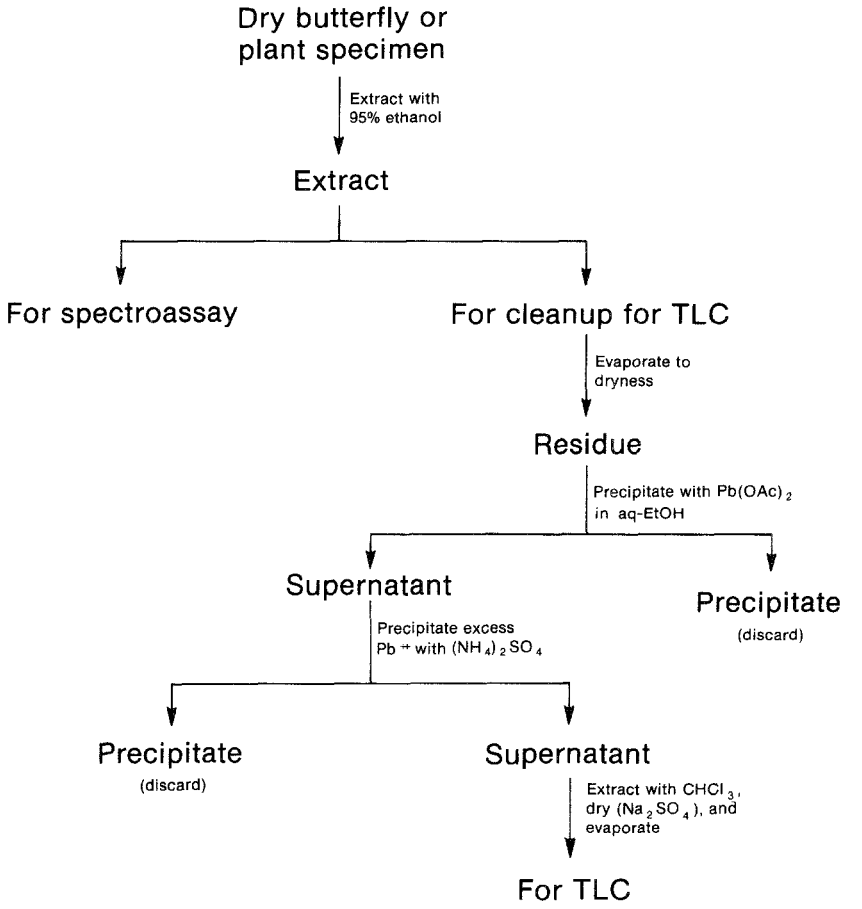


FIG. 2. Sample preparation flow chart for the spectroassay and thin-layer chromatography (TLC) analyses. For further details, see text.

(Roeske et al., 1976). Based on TNDP spectroassay, uncleaned *A. eriocarpa* plant extracts had a mean cardenolide concentration of 23.91×10^{-5} M (95% confidence limits = 0.53, $N = 5$, $s = 0.42$), the cleaned-up, 22.39×10^{-5} M (95% confidence limits = 0.80, $N = 5$, $s = 0.64$), giving a recovery efficiency of 94%. The uncleaned *A. eriocarpa* butterfly extract had a mean concentration of 5.42×10^{-5} M (95% confidence limits = 0.71, $N = 5$, $s = 0.57$) and after cleanup 4.47×10^{-5} M (95% confidence limits = 0.31, $s = 0.25$), giving a recovery efficiency of 82%. (The 95% confidence intervals are calculated according to Dean and Dixon, 1951).

During previous work on the isolation of three major cardenolides from *A. eriocarpa* (labriformin, labriformidin, and desglucosyrioside, see below),

we observed that treating a solution of pure labriformin with 5% lead acetate for 1 hr at room temperature resulted in partial conversion to labriformidin. However, by shortening the time to 20 min and by using an ice bath during this portion of the procedure, we reduced the conversion to less than 10%, as estimated visually from TLC analysis. With this modification the TLC profiles of uncleaned and cleaned samples were virtually identical.

Thin-Layer Chromatography (TLC). After cleanup, each sample residue was dissolved in a particular microliter amount of chloroform such that a constant 20 μl of chloroform solution spotted (with a Brinkmann 5-μl spotting capillary tube) would contain 25, 50, or 75 μg of digitoxin equivalents for the butterfly extracts, or 25, 50, 75, or 100 μg for the plants. In order to run each sample in two solvent systems, each cleaned-up extract was spotted on two 20 × 20-cm TLC plates (0.25 mm thickness, Merck Silica Gel 60 F-254, Brinkman Instruments Co.). Three digitoxin and two digitoxigenin standards (Sigma Chemical Co., St. Louis, Missouri) were spotted (20 μg) in channels along the edges and near the center of each plate along with six plant and six respective butterfly samples. All compounds were spotted 2 cm above the bottom of the plate and allowed to develop for 15 cm to a scored line. One plate was developed twice in ethyl acetate–methanol (97:3 by volume, the EAM system), and the other four times in chloroform–methanol–formamide (90:60:1 by volume, the CMF system), both in saturated, filter-paper-lined glass chambers. The plates were air dried after each development and then visualized by spraying first with a saturated solution of TNDP in benzene, and then, after air drying, with a 10% solution of KOH in 50% aqueous methanol (Neher, 1969). On separate plates, several plant and butterfly extracts were run side by side with standards of labriformin, labriformidin, and desglucosyrioside. Occurrence and quantitation of these compounds in individual pairs of *A. eriocarpa* plant–butterfly samples as well as pooled samples have been ascertained by high-performance liquid chromatography (Benson and Seiber, 1978). These standards, of greater than 90% purity, were previously isolated from *A. eriocarpa* (Seiber et al., 1978). Desglucosyrioside was referred to as "eriocarpin" by Benson and Seiber (1978) and Seiber et al. (1978). The structural identity of "eriocarpin" to desglucosyrioside has been established by Cheung et al., (1980).

The plates were photographed within 1 min of applying the KOH spray (the blue color of the TNDP–cardenolide complex rapidly fades, although this can be retarded by freezing the plates), using an Olympus OM-1 35 mm camera equipped with a Honeywell Max-O-Lite 7 ring flash and Kodachrome 25 film. Color enlargements (8 × 10 in.) were made from the slides and allowed easy comparison of the numerous samples.

We found that double or multiple development of the TLC plates gave better separation of the cardenolides. However, neither solvent system gives

complete resolution of the three major *A. eriocarpa* cardenolides, labriformin, labriformidin, and desglucosyrioside (Seiber et al., 1978; Nelson et al., 1981). By using both systems the absence of any of the three in the plant and/or butterfly samples can be ascertained.

Rationale for Selection of Plant and Butterfly Samples for TLC. Based on their gross cardenolide concentrations, 85 of the 172 butterflies and their respective plants which had been spectroassayed were chromatographed on 14 TLC plates in each of the two solvent systems. The method of selection of the butterflies and plants was based on both sexes of the butterflies, the range of cardenolide concentration in both plants and butterflies, on the six geographic areas from which the samples were collected, and for one of these (area 6) on early (June) and late (August) samples. For each geographic area, male (or female) butterflies at or near their highest, mean, and lowest cardenolide concentration were chosen and spotted adjacent to their corresponding plants on the same plate. Similarly, plants at or near their highest, mean, and lowest concentration upon which three additional males (or females) had fed were chosen. Thus, each plate, in addition to the three digitoxin and two digitoxigenin standards, was spotted with six butterflies of one sex and their corresponding plants.

TLC Quantitation of R *Digitoxin and Spot Intensity Values.* Since the chloroform–formamide–methanol system gives the best overall resolution of the milkweed cardenolides, the 14 plates developed in this system were subjected to extensive analysis to define a quantitatively based *Asclepias eriocarpa* fingerprint pattern.

In order to mark the center of each resolved cardenolide spot, each 8×10 in. enlarged color photograph was overlain with a clear plastic sheet and the spot center was dotted with a fine pen. The original 35 mm slide was then scrutinized to ascertain the accuracy of the marking. This is important for the plants because of green and pink pigments in parts of the channels.

The migration distances of all resolved cardenolides (from origin to spot center) were measured with a fine ruler to the nearest 0.2 mm. This distance was then divided by the corresponding migration distance of digitoxin, and each spot given an R digitoxin (R_d) value. Since the three digitoxin spots (two on the sides, and one on the center of the plate) migrated different distances, they were connected by a fine line drawn through their centers and the migration distances for digitoxin were separately determined as the baseline to digitoxin-line distance for each of the plant or butterfly channels. The two digitoxigenin R_d values were similarly determined for 13 of the 14 plates, and their mean and standard deviations are given in the results.

Spot intensities were scored visually by ranking them with unit values of 1 (small and/or just visible) to 5 (large and/or intensely developed). Upward adjustments were made arbitrarily for channels spotted with less than 100 and

75 μg amounts for plants and butterflies, respectively. This method of scoring the intensities, while somewhat qualitative, proved simple, fast, objective, and amenable to straight-forward statistical analyses.

Blue Jay Emetic-Dose Fifty (ED_{50}) Assay.

1. Plant and Butterfly Materials. Emetic-dose 50 determinations were made on butterfly and plant material during March and April 1977, according to the forced-feeding procedure developed previously with wild-captured blue jays as the bioassay animal (Brower et al., 1968; Brower and Moffitt, 1974).

Qualitative examination of the TLC patterns of the 85 butterflies and respective plants run in the CMF system indicated two major cardenolide groupings, which we designated as group I, heavy spot 4, and group II, heavy spot 8 (Figure 6). ED_{50}s were determined for pooled butterflies from each group, and the corresponding plants from group II only. Residual powders were combined for 4 males and 14 females in group I for a total of 1031 mg dry powder. Residual powders for group II butterflies included 6 males and 12 females for a total of 950 mg dry powder. Plant powders corresponding to each of the group II butterflies were combined in the same proportion but in 2× the amount, for a total of 1906 mg. After pooling, the material within each of the three categories was thoroughly mixed in a mortar and pestle and a standard spectroassay was run on each (Table 5).

The previously described bird-handling procedures were used in the sequential force-feeding experiments. The numbers of birds force fed for group I and group II butterflies were, respectively 18, and 13, and for the group II plants, 17. Of these, respectively, 15, 11, and 12 birds were used to estimate the actual ED_{50} values, according to the statistical method of Dixon and Massey (1957) as described in Brower et al., (1968).

2. A Single Cardenolide: Labriformin. A new procedure was developed to determine the ED_{50} of labriformin (mol wt = 617), the principal high R_f cardenolide of *Asclepias eriocarpa* (Seiber et al., 1978; Cheung et al., 1980; Nelson et al., 1981). Preliminary force-feeding tests with digitoxin absorbed on filter paper and administered in gelatin capsules produced ambiguous responses in the jays. We speculated that this resulted from very rapid absorption and extreme sickening in the birds which interfered with the emetic response.

To overcome this, the solution was absorbed on standard laboratory food (pigeon pellets, Ralston Purina Co.) which were ground in a mortar and pestle, passed through a fine screen, and 0.1 g weighed into a No. 2 gelatin capsule (Lilly Co.). A 9.724×10^{-5} M stock solution was prepared by weighing 0.0015 g of labriformin into a 25-ml volumetric flask and bringing it to volume at 20° C in 95% ethanol.

The individual dosages were prepared by pipetting the solution onto the food in each capsule which was dried at 60° C in a forced-draft oven. Initial

dosage levels were based on the micrograms of cardenolide per ED_{50} unit previously determined on the *A. eriocarpa* plant and butterfly material (Table 6). To meet the statistical conditions of the bioassay (Dixon and Massey, 1957), the dosage levels were logarithmically based, beginning with 549 μl of solution (ln = 6.3081) and using six log increments of 0.2, up to 1882 μl. If less than 1000 μl of solution were pipetted into the capsule, the difference was made up to 1000 μl with 95% ethanol. One or two 50-, 100-, 200-, 300-, 400-, 500-, and 1000-μl glass micropipets (Fisher Co.) were used in combination with a 50-μl Hamilton syringe (wire plunger, calibrated, continuous delivery) to add the desired quantities to each capsule. About 400 μl could be added at one time, so each aliquot was dried successively.

The ED_{50} tests were carried out between April 20 and May 11, 1977, using a total of 25 jays. The ED_{50} and confidence limits are based on 19 of these tests. The assay was judged valid by the criteria of Dixon and Massey (1957) but has a much larger variance than those using the plant and butterfly material (see Table 5, confidence limits). At the end of the assay, the stock solution was chromatographed to ascertain that the labriformin did not alter chemically over the testing period.

RESULTS

Gross Cardenolide Concentration (μg per 0.1 g Dry Weight).

Quantitative Variation in Plants. All 172 plants contained measurable amounts of cardenolide. The concentration range throughout all the geographic areas studied was 102–919 μg/0.1 g with a grand mean of 421.4 μg (Table 1). In terms of the percentage of the plants' dry weight, this cardenolide variation encompasses nearly one order of magnitude, i.e., from about 0.1 to 1.0%. The overall pattern of variation is plotted as a histogram (Figure 3). This figure also displays the expected normal distribution, calculated by the z statistic (Steel and Torrie, 1960). The observed distribution is slightly skewed and differs significantly from normality ($\chi^2_{11} = 29.0$, $0.005 > P > 0.001$). This does not seem attributable to sampling error (Table 1) and probably has some biological basis.

The variance of the cardenolide concentration in the plants upon which the males were reared does not differ significantly from those upon which the females were reared (F 79,91 = 1.085, $0.75 > P > 0.50$). The overall plant standard deviation is 170 μg (Table 1).

A two-way analysis of variance (ANOVA) test (Barthakur, 1971) was done to compare mean concentrations in the plants from the six geographic areas, the mean concentrations in the plants according to the sex of the butterflies which fed upon them, and the interaction between these two

TABLE 1. SUMMARY OF MEANS AND GRAND STANDARD DEVIATIONS OF CARDENOLIDE CONCENTRATIONS OF 172 WILD-COLLECTED INDIVIDUAL *Asclepias eriocarpa* PLANTS[a] AND MONARCH BUTTERFLIES REARED THERON[b]

	Plant material[c]			Butterfly material[d]		
	Males	Females	Means[e]	Males	Females	Means[e]
Area 1: North Coast Range (N = 14, 19)	299.0	350.2	324.6	239.9	339.2	289.6
Area 2: southern Cascades, montane (N = 9, 5)	488.0	335.2	411.6	262.7	305.4	284.0
Area 3: northern Sierra Nevada, foothills (N = 26, 13)	447.3	368.5	407.9	291.4	346.5	319.0
Area 4: southern Sierra Nevada, foothills (N = 13, 9)	491.6	528.4	510.0	297.8	395.0	346.4
Area 5: southern Sierra Nevada, montane (N = 16, 13)	374.6	433.0	403.8	348.8	406.5	377.6
Area 6: southern Sierra Nevada, King's River floodplain (N = 14, 21)	520.4	433.7	477.1	287.3	311.0	299.1
Grand means[f]	433.4	407.7	421.4	291.0	348.1	317.6
Grand standard deviations[f]	173.1	166.2	170.0	67.6	79.9	78.7
Grand Ns	92	80	172	92	80	172

[a]Plant numbers 72, 80, 318, 612, 685, 687, 706, and 709 were each fed on by two monarchs. The statistics for the plants therefore include 8 duplicate cardenolide determinations.
[b]Collections are from the six areas shown in Figure 1. Data are μg (equivalent to digitoxin) per 0.1 g dry wt of butterfly or plant material.
[c]The plant material corresponds to the butterflies according to the sex of the butterfly.
[d]The butterfly material represents the butterflies reared on their respective plants.
[e]These means are the mean male value added to the mean female value, divided by 2.
[f]Based on all 92 males, 80 females, and their respective plants.

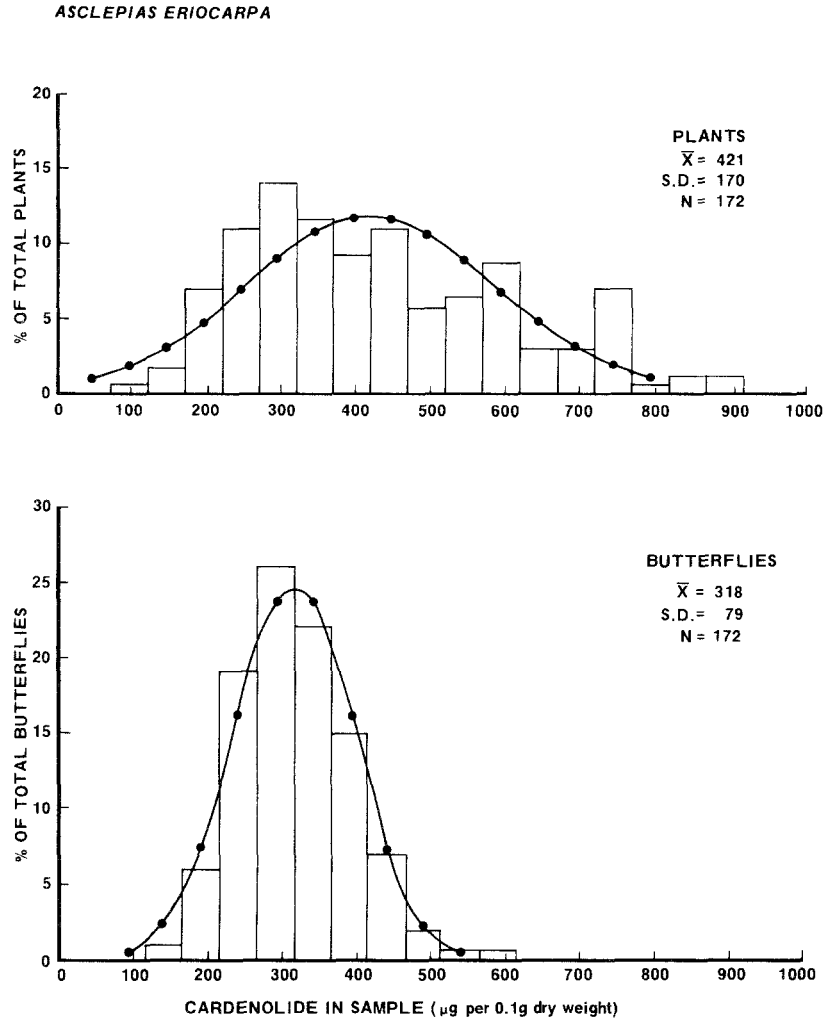


FIG. 3. Frequency distributions of the gross cardenolide content (as μg per 0.1 g dry weight, equivalent to digitoxin) of 172 *Asclepias eriocarpa* plant samples and 172 adult monarch butterflies reared thereon. All individual butterflies were collected as 5th instar larvae or chrysalids on the respective individual milkweed plants growing in their natural environments from the six geographic areas shown in Figure 1. The curves are the calculated normal distributions. The width of each bar represents 50 μg.

TABLE 2. TWO-WAY ANALYSES OF VARIANCE OF DATA IN TABLE 1.

Source of variation	SS	DF	MS	F	P
A. Plant material (N = 172)					
A (geographic area)	503260	5	100652	3.873	$0.005 > P > 0.001$
B (by sex of butterfly)	29611	1	29611	1.139	$0.75 > P > 0.50$
AB (interaction)	237898	5	47579	1.831	$0.90 > P > 0.75$
Within cell	4157648	160	25985	–	–
B. Butterfly material (N = 172)					
A. (geographic area)	160572	5	32114	7.152	$P < 0.001$
B (sex)	141563	1	141563	31.527	$P < 0.001$
AB (interaction)	27210	5	5442	1.212	$0.75 > P > 0.50$
Within cell	718432	160	4490	–	–

factors. Neither the sex category nor the interaction is significant (Table 2A, factors B and AB). However, the overall means for the six geographic areas range from 325 to 510 μg/0.1 g and, although not extensive, the differences are significant (factor A, $0.005 > P > 0.001$). Examination of the data suggests a north-to-south increase in concentration, but the manner in which we collected the data may have confounded this apparent geographic trend with a temporal one, since the sample with the highest concentration (area 4) was taken in June, and the one with the lowest concentration (area 1) was taken in September. Findings of a separate study on *A. eriocarpa* plants over a year at one locality support the temporal explanation (Nelson et al., 1981). It therefore appears that geographic origin contributes only a small part to the overall variation in the gross cardenolide content of *A. eriocarpa*.

Quantitative Variation in Butterflies. All 172 butterflies contained measurable amounts of cardenolide. The concentration range throughout all the geographic areas studied was 136–606 μg/0.1g with a grand mean of 317.6 μg/0.1g (Table 1). This range extends over one half an order of magnitude, i.e., approximately 0.14–0.61% of the butterfly's dry weight. The overall pattern of variation (Figure 3) is a good fit to a normal distribution ($x^2_7 = 4.9$, $0.75 > P > 0.50$).

The variance of the cardenolide concentration in the males is smaller than the females, but the difference is not statistically significant (F 79,91 = 1.399, $0.20 > P > 0.10$). The overall butterfly standard deviation is approximately 79 μg (Table 1), less than half of that of the plants on which they were reared (see below).

The geographic and/or temporal trend of higher concentrations southward found in the plants is also statistically significant for the butterflies reared on these plants ($P < 0.001$, Table 2B). As shown in previous studies (Brower et al., 1972; Brower and Moffitt, 1974; Brower and Glazier, 1975), the wild-captured females have a higher mean concentration than do the males.

The average for the 92 males in this study was 291 μg, or about 83% of that found in the 80 females, 352 μg. This difference is highly significant (Table 2B, factor B, $P < 0.001$) and is consistent in all six geographic areas (Table 2B, factor AB, $0.75 > P > 0.50$).

Cardenolide Content of Butterflies as a Function of Cardenolide Content of Plants. The mean cardenolide concentration in all butterflies is only 76% of that in their respective plants (Table 1), and the overall range is only about half (58%) that in the plants. Based on the above data analysis and comparison of the two histograms (Figure 3), it is evident that the butterflies normalize and reduce the variation in concentration found in the plants they fed upon as larvae. In terms of their respective variances (Table 1), this reduction is nearly fivefold and is highly significant: the ratio of the two variances, each with 171 degrees of freedom, gives an F value of 4.662 with $P < 0.001$. Based on the mean $\pm$ 2 SDs, 95% of the butterflies will have a restricted cardenolide concentration within the range of 160–476 μg/0.1g.

The lower values of both mean and variance parameters of the butterflies indicate that these insects do not passively reflect the overall cardenolide concentrations in the plants. To investigate the quantitative nature of this relationship, we plotted the data in which y = the concentration per 0.1 g in the butterflies and x = the concentration per 0.1 g in the respective plants (Figure 4). Linear regression analyses of these data (Barthakur, 1971, program 1316EB/ST3) indicated that, except for the males in which there is a weak positive correlation, neither the females alone nor both sexes together show a statistically significant regression (Table 3). These tests suggest a slight tendency for plants of higher concentration to produce butterflies of higher concentration, but more importantly, they indicate that the concentration in the butterflies is maintained within a narrow range despite wide variation of concentration in the plants.

Total Cardenolide per Butterfly

Total cardenolide content of the butterflies is a product of weight times cardenolide concentration. The average dry weight of the males was 0.207 g and the females 0.182 g (Table 4). This difference is highly significant (F_1, $169 = 24.8$, $P < 0.001$) and is consistent with the lighter weights of females from overwintering populations in California (Brower and Moffitt, 1974; Tuskes and Brower, 1978). The average total cardenolide content of the butterflies was 616 μg (Table 4). Difference between the sexes is not significant F_1, $169 = 2.06$, $0.25 > P > 0.10$) because the males are 14% heavier than the females and the product of weight times concentration in the two sexes therefore results in their having similar total cardenolide values.

Total cardenolide per butterfly as a function of its corresponding plant concentration per 0.1 g was also investigated by linear regression analysis with

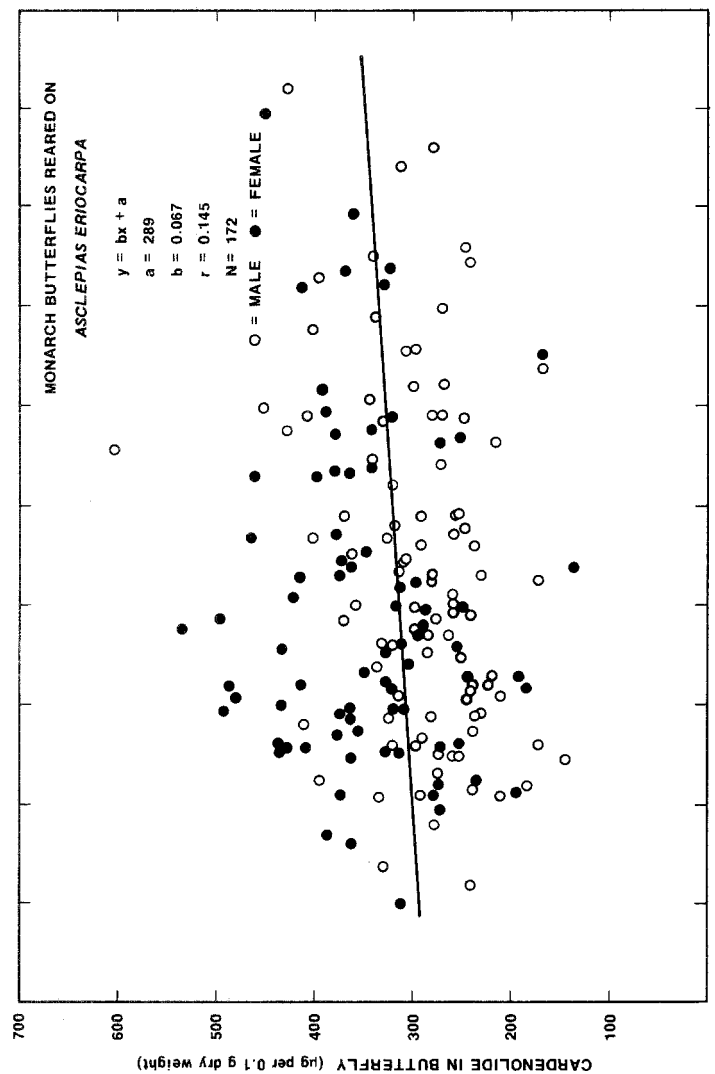


FIG. 4. Gross cardenolide concentrations of adult monarch butterflies (y axis) as a function of the gross cardenolide concentrations of their larval foodplants (x axis). Each of the 172 data points represents one corresponding individual butterfly–plant rearing experiment. Open circles are males and solid circles are females. The line corresponds to the regression equation $y = bx + a$.

TABLE 3. LINEAR REGRESSION ANALYSES OF CARDENOLIDES (μg/0.1 g DRY WT) IN BUTTERFLIES (y AXIS) VS CARDENOLIDES (μg/0.1 g DRY WT) IN THEIR RESPECTIVE PLANTS, ACCORDING TO THE FUNCTION $y = bx + a$

Values	Males only	Females only	Both sexes
Sum Xi	39876	32612	72488
Sum Yi	26773	27846	54619
Sum $(Xi)^2$	20011106	15477450	35488556
Sum $(Yi)^2$	8206731	10196988	18403719
Sum (Xi) (Yi)	11883621	11467569	23351190
No. of points	92	80	172
a (y intercept)	247	326	289
b (slope)	0.102	0.053	0.067
r (correlation coeff.)	0.262	0.111	0.145
Std. error	64.849	78.923	77.645
Significance of r[a]			
F, degrees of freedom	F 1,90 = 6.65	F 1,78 = 0.97	F 1,170 = 3.67
Prob. $b = 0$	$0.05 > P > 0.025$	$0.75 > P > 0.50$	$0.20 > P > 0.10$

[a]ANOVA test from Zar (1974), pp. 205-207. $F = MS$ regression $\div MS$ residual.

y, the total cardenolide per butterfly, as the dependent variable, and x, plant concentration, the independent variable. Values for the regression are: $N = 171$, $y = 0.145x + 554$, $r = 0.152$, $F_{1,169} = 4.03$, and $0.10 > P > 0.05$. Total cardenolide per butterfly is therefore effectively independent of their respective plant's concentration, at least within the plant concentration range found in *A. eriocarpa* (102–919 μg/0.1 g).

TABLE 4. SUMMARY OF DRY WEIGHTS AND TOTAL CARDENOLIDE CONTENT (EQUIVALENT TO DIGITOXIN) OF 171[a] ADULT MONARCH BUTTERFLIES REARED ON *A. eriocarpa* PLANTS FROM SIX GEOGRAPHIC AREAS IN TABLE 1

	Dry weights (g)			Total cardenolide (μg)		
Sample size	Means	SD	Range	Means	SD	Range
Males 92	0.207	0.037	0.058-0.298	599	159	270-998
Females 79	0.182	0.026	0.119-0.250	635	162	220-956
Both 171	0.196	0.035	0.058-0.298	616	161	220-998

[a]Discrepancy from Table 1 due to one butterfly which was not weighted.

TABLE 5. CARDENOLIDE CONCENTRATIONS AND EMETIC POTENCIES TO BLUE JAYS OF POOLED DRY, POWDERED *Asclepias eriocarpa*-REARED BUTTERFLIES, *A. eriocarpa* PLANTS THEY WERE REARED ON, AND LABRIFORMIN

Material tested	Absorbance[a]	Cardenolide concn. (μg/0.1 g dry wt)[b]	Emetic dose 50 data per 100 g Blue Jay: No. birds tested	ED_{50}	95% confidence limits	Blue jay emetic potencies[c]: Cardenolide per ED_{50} unit (μg)[b]	Mean ED_{50} units per butterfly	Mean ED_{50} units per mg cardenolide[d]
Butterfly group I	0.610	382.5	15	0.011 g	0.010–0.012 g	36	17	27.6
Butterfly group II	0.540	337.8	11	0.014 g	0.012–0.015 g	39	16	25.8
Plant group II	0.733	461.0	12	0.015 g	0.014–0.016 g	58	–	17.2
Labriformin	–	–	19	68 μg[e]	39–119 μg	57[e]	–	17.5

[a]Standard spectroassay, see text.
[b]For the butterfly and plant material, the μg of cardenolide are equivalent to μg of digitoxin (see text).
[c]Adjusted for the mean cardenolide content of this sample of 171 *A. eriocarpa*-reared butterflies (161 μg) and for the mean weight of 226 blue jays (84.25 g), from Brower and Moffitt (1974).
[d]As per Roeske et al. (1976), Table 6, adjusted to the mean blue jay weight of 84.25 g.
[e]Based on a solution of 0.0015 g of labriformin (mol wt = 617) in 25.0 ml of ethanol. The ED_{50} was determined as 1.133 ml of this solution (see text for details).

Blue Jay ED_{50} Bioassays

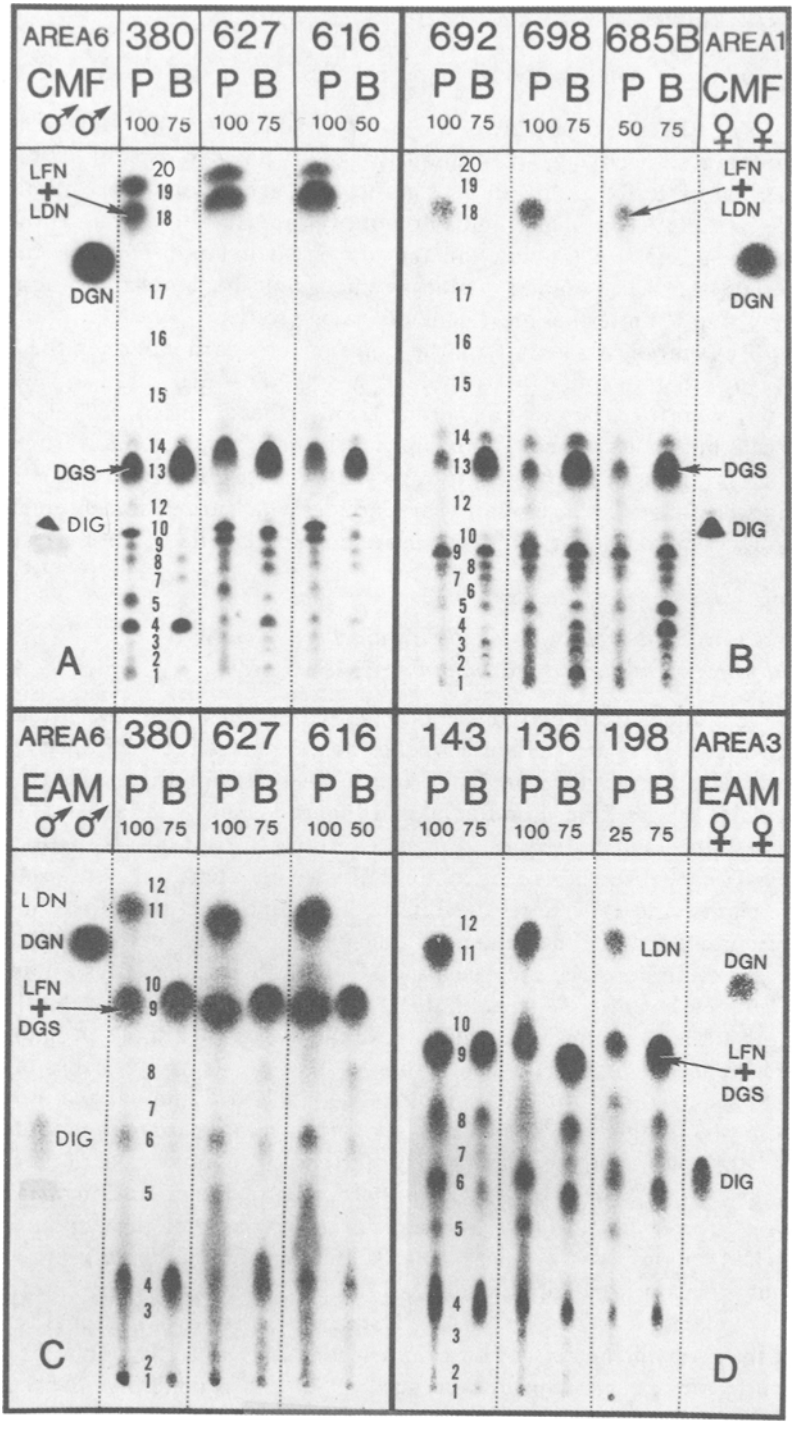
The ED_{50}s per 100 g jay of the group I and II butterflies and the group I plants are, respectively, 11, 14, and 15 mg of dry powdered material (Table 5). The two butterfly groups differ significantly; even though the difference is small, the 95% confidence limits do not overlap. Butterfly group II overlaps with the group II plant material, and so the two do not differ significantly at the 0.05 level. The numbers of blue jay emetic units per butterfly in the group I and group II butterflies are 17 and 16, respectively.

By combining the data on the range of total cardenolide in the 171 *A. eriocarpa* reared butterflies (Table 4, $\bar{X} \pm 2$ SD = 616 ± 322 μg) with the ED_{50} results for the group I and group II butterflies (Table 5), 95% of a sample of wild butterflies which had fed on *A. eriocarpa* would contain from 7.5 to 26.1 ED_{50} units per butterfly (average = 16.4). These results indicate that the milkweed *A. eriocarpa* contains cardenolides which are extremely emetic and produces monarch butterflies which are the most emetic of those investigated to date.

TLC Cardenolide Profiles of Plants and Butterflies: Chloroform–Methanol–Formamide (CMF) System

Examples from two of the 14 TLC plates run in the CMF system are reproduced in Figure 5. Figure 5A shows three plants and the respective male butterflies reared thereon from area 6 (August) in the southern Sierra Nevadas, Kings River floodplain, and Figure 5B shows three plants and the respective female butterflies from area 1 in the North Coast Range.

Twenty discernible cardenolides of varying intensities were resolved in the plants, and 16 were resolved in the butterflies. Not all of these spots are present in every channel, and one in the plants and three in the butterflies are of minor importance (see below). Means and standard deviations were calculated both for R digitoxin (R_d) and spot intensity (SI) values, for all spots in the 85 samples. In addition, we calculated each spot's probability of occurrence (PO), based on the proportion of plants or butterflies in which each occurred out of the 85 samples. These data are summarized in Table 6 from which Figure 6 was drawn showing the average cardenolide profiles for the CMF system in the plants and butterflies throughout most of the natural range of *A. eriocarpa* in California. In this figure, the migration distance for the digitoxin standard (mm above the origin) is based on three spots on each of the 14 plates ($N = 42$, mean $R_f = 31.19$ mm, SD = 2.30 mm). The mean R digitoxin value for digitoxigenin (2.67) is based on two spots per plate for 13 of the 14 plates ($N = 26$, SD = 0.15). The shapes and sizes of the spots are based on the plant and butterfly TLC channels for plant/butterfly pair 611. Spot 6 is omitted in the plant profile because of its low probability of occurrence (Table 6).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P B
100 75
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Plants.

1. Total Resolved Spots. In addition to spots 1–20, two plants had one additional cardenolide, one designated as 9a with an *R* digitoxin (R_d) of 0.87, and the other as 3a with an R_d of 0.29. A 21st spot also occurred occasionally, but was obscured by pigment and so was excluded from the analysis. Spot 6 is effectively absent in the plants ($PO = 0.01$) and spot 2 has a PO of only 0.18. Of the 18 remaining spots, numbers 11 and 16 are of uniformly low SI, leaving 16 spots which are particularly diagnostic. These are distributed in three distinct R_f regions: eight occur below the digitoxin R_f line, (1, 3–5, and 7–10), five occur between the digitoxin and digitoxigenin R_f lines (12–15, 17), and three occur above the digitoxigenin R_f line (18–20). Previous studies indicated that the two most prominent spots, 13 and 18 (Figure 6) are, respectively, desglucosyrioside and most likely a mixture of labriformin and labriformidin (Seiber et al., 1978; Cheung et al., 1980; Nelson et al., 1981). Overall, the butterflies are capable of storing cardenolides within approximately 62% of the R_d range of those found in the plants (Table 6).

2. *R* Digitoxin Values and Spot Intensity Values. Standard deviations of the R_d values of each spot for the entire sample of plants are relatively low, i.e., overall resolution is good (Table 6). Mean SI values in the three TLC regions are also in Table 6. Below digitoxin, six spots with a high $PO \geqslant 0.85$ can be expected to have mean SI values in excess of 2.00 (4, 5, 7–10) 50% of the time, and two of these (9 and 10) can be expected to exceed 3.00, 50% of the time. Between digitoxin and digitoxigenin three spots are intense (13–15) and have PO values of 0.99–1.00: spot 13 (desglucosyrioside) is the second most prominent in the plants and can be expected to have an intensity in excess of 4.0, 50% of the time, and spots 14 and 15 in excess of 2.0, 50% of the time. Spot

FIG. 5. Photographic reproduction of the thin-layer chromatographic profiles of the cardenolides stored by adult monarch butterflies from their *Asclepias eriocarpa* larval food plants in California. Each of the four sections of the plate (A–D) shows the TLC profiles of three pairs of plants and corresponding individual monarch butterflies reared on these plants as well as digitoxin (DIG) and digitoxigenin (DGN). Sections A and B were run in the chloroform–methanol–formamide system which separates 20 cardenolides in the plants and 16 in the butterflies (see generalized drawing in Figure 6). Section A shows 3 male butterflies reared on three plants from area 6 in the southern Sierra Nevada. Section B shows 3 female butterflies reared on 3 plants from area 1 in the North Coast Range. Sections C and D were run in the ethylacetate-methanol TLC system which separates 11 cardenolides in the plants and 8 in the butterflies. Spot 11 is labriformidin (LDN) and spot 9 is a mixture of desglucosyrioside (DGS) and labriformin (LFN). Section C shows the same 3 male butterflies and corresponding plants as in section A. Section D shows 3 female butterflies reared on 3 plants from area 3 in the northern Sierra Nevada. Note the lack of sexual differences, only minor geographic differences, and the overall constancy of the plant and butterfly profiles in both TLC systems.

TABLE 6. SUMMARY OF MEANS AND STANDARD DEVIATIONS FOR R DIGITOXIN VALUES AND SPOT INTENSITIES FOR 20 CARDENOLIDES AND THE PROBABILITY OF THEIR OCURRENCE IN PLANT OR BUTTERFLY[a]

Spot No.	Means				Standard deviations				Probability of spot		Subsample sizes	
	R digitoxin		Spot intensity		R digitoxin		Spot intensity					
	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly
20	3.43	–	1.40	–	0.114	–	0.680	–	0.24	0.00	20	–
19	3.19	–	2.89	–	0.171	–	1.280	–	0.93	0.00	79	–
18	3.00	–	4.74	–	0.166	–	0.559	–	1.00	0.00	85	–
17	2.40	–	1.62	–	0.118	–	0.726	–	0.99	0.00	84	–
16	2.13	2.02	1.03	1.00	0.111	0.085	0.157	0.000	0.94	0.06	80	5
15	1.87	1.80	2.12	1.99	0.097	0.093	0.730	0.893	1.00	1.00	85	85
14	1.56	1.51	2.15	2.64	0.074	0.069	0.799	0.687	0.99	1.00	84	85
13	1.35	1.34	4.26	4.98	0.072	0.066	0.657	0.152	1.00	1.00	85	85
12	1.13	1.10	1.49	1.33	0.054	0.011	0.618	0.577	0.74	0.04	63	3
11	0.98	0.97	1.24	1.49	0.036	0.025	0.488	0.668	0.48	0.62	41	53
10	0.90	0.93	3.46	1.70	0.031	0.026	1.002	0.891	0.92	0.54	78	46
9	0.83	0.84	3.05	3.20	0.025	0.024	0.825	0.783	0.87	1.00	74	85
8	0.75	0.75	2.26	3.04	0.021	0.024	0.731	0.662	0.85	1.00	72	85
7	0.68	0.65	2.12	2.35	0.024	0.029	0.747	0.868	0.89	1.00	76	85
6	0.61	0.61	1.00	1.33	–	0.015	–	0.500	0.01	0.11	1	9
5	0.51	0.48	2.67	2.48	0.029	0.020	0.822	0.765	1.00	1.00	85	85
4	0.34	0.35	2.95	3.35	0.022	0.020	1.231	1.173	0.89	0.98	76	83
3	0.24	0.25	1.42	1.51	0.029	0.025	0.556	0.639	0.78	0.92	66	78
2	0.15	0.18	1.13	1.34	0.021	0.022	0.351	0.539	0.18	0.41	15	35
1	0.07	0.07	1.34	1.83	0.018	0.010	0.599	0.691	0.73	0.99	62	84

[a]Data are based on chromatograms of 85 plants and corresponding butterflies reared thereon (chloroform–methanol–formamide system).

16, although it has a *PO* of 0.94, is less diagnostic because of its uniformly low *SI*. Spot 17 has a high *PO* (0.99), but has a relatively low and highly variable *SI*. Above digitoxigenin, spot 18 (a mixture of labriformin and labriformidin), with a *PO* of 1.00, is the most intense of all the plant spots with a value in excess of 4.7, 50% of the time. Spot 19 is also diagnostic with a *PO* of 0.93 and an intensity which, although variable, can be expected to exceed 2.8 more than 50% of the time. Spot 20, with a *PO* of 0.24, and a relatively low intensity and a high variability, is of lesser diagnostic value.

3. The *A. eriocarpa* Plant Cardenolide Profile. By combining these R_d, *PO*, and *SI* data, the overall *A. eriocarpa* plant profile emerges as shown in Figure 6. This profile of 20 cardenolides breaks into three easily discerned regions and has at least 11 cardenolides which are highly predictable, well resolved and of high intensity. These include six prominent cardenolides

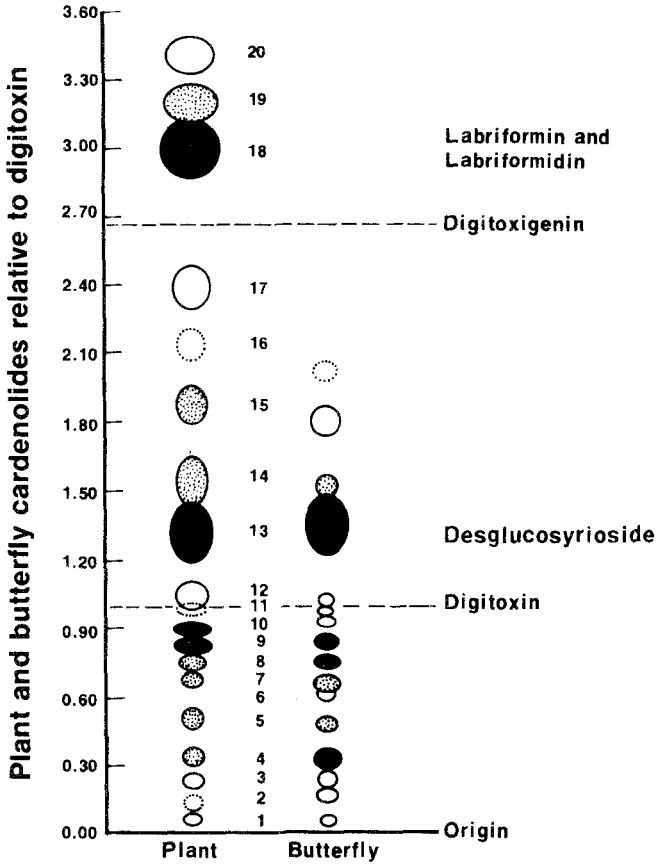

FIG. 6. The cardenolide fingerprint profile for freshly emerged monarch butterflies reared as larvae on *Asclepias eriocarpa* based on the chloroform–methanol–formamide TLC system. The diagram shows mean R digitoxin and spot intensity values for each of the 20 plant and 16 butterfly cardenolides and is based on the data in Table 6 for 85 plants and respective butterflies reared thereon. The mean migration distance for digitoxin was 31.19 mm (see text p. 599). Spot intensities are represented as follows: 1.00–1.25, dotted circle; 1.25–2.00, closed circle; 2.00–3.00, lightly stippled circle; 3.00–4.00, heavily stipled circle; 4.00–5.00, blackened circle. Eight of the cardenolides have a very high probability of occurrence, are highly correlated in the plant and butterfly, and are of high intensity (numbers 4, 5, 7, 8, 9, 13, 14, and 15). These eight cardenolides represent the most reliable plant–butterfly fingerprint profile of freshly emerged butterflies.

below digitoxin (4, 5, 7–10), three between digitoxin and digitoxigenin (13–15), and two above digitoxigenin (18 and 19).

Butterflies.

1. Total Resolved Spots. In addition to spots 1–16, seven butterflies each had one additional cardenolide designated as 2a (R_d values = 0.20, 0.21, 0.20,

and 0.19), 7a (R_d values = 0.77, 0.78), and 9a (R_d value = 0.84). Because of their low *PO* and *SI* values, they were excluded from the analysis. Spots 6, 12, and 16 occur infrequently with *PO* values ≤ 0.11 and are also usually resolved poorly with mean *SI* values of ≤ 1.33. This leaves 13 spots of diagnostic value and these, in contrast to the plants, are distributed in only two regions: none occurs above digitoxigenin. Ten occur below the digitoxin line (1–5, 7–11), and three between digitoxin and digitoxigenin (13–15).

2. *R* Digitoxin and Spot Intensity Values. The overall variation in the R_d values of these cardenolides is slightly lower than in the plants (Table 6), so that the resolution in the butterflies is better. The mean *SI* values in the two TLC regions are summarized in Table 6 and Figure 6. Below digitoxin, four spots are of moderate diagnostic value with *PO* values of 0.41–0.92 and *SI* values of 1.34–1.70 (spots 2, 3, 10, and 11). Six additional spots below digitoxin all have *PO* values ≥ 0.98 and can be expected to have *SI* values in excess of 1.80 more than 50% of the time (spots 1, 4, 5, and 7–9). Five of these will have *SI* values in excess of 2.30 (4, 5, and 7–9) and, of these, three (spots 4, 8, and 9) will exceed 3.00 more than 50% of the time. In the region above digitoxin, the three major spots (13–15) all have a *PO* of 1.00. Both spots 14 and 15 have *SI* values which can be expected to exceed 1.95 at least 95% of the time. Desglucosyrioside, spot 13 ($PO = 1.00$), is the most intense and can be expected to have an *SI* larger than 4.50 at least 99% of the time.

3. The Butterfly Cardenolide Profile. By combining these R_d, *PO*, and *SI* data, the overall *A. eriocarpa*-reared butterfly profile emerges as shown in Figure 6. This profile of 16 cardenolides breaks into two easily discernible regions and has nine spots which are highly predictable, well resolved, and of high intensity. These include six spots below digitoxin (1, 4, 5, and 7–9) and three between digitoxin and digitoxigenin (13–15). In contrast to the plants, no cardenolides occur above digitoxigenin.

Plant–Butterfly Relationships: Effects of Sex and Geography. If cardenolide fingerprinting is to be effective there must be a high degree of correlation between both R_d and *SI* values of the cardenolides in the plants and butterflies. The data were therefore subjected to linear regression analyses from which correlation coefficient values were determined with the butterflies as the dependent variable (y) and the plants as the independent variable (x). The significance of the correlations was tested by the method of Zar (1974), using a one-sided F statistic (Table 7). To be most effective, the relationship must be fairly uniform over the geographic range of the plants as well as unaffected by the sex of the butterfly. Therefore, sex, geographic effects, and their interactions were tested by two-way ANOVA.

A problem arose because of plate-to-plate variation in the R_d values. As a result, the interaction factor between sex and geography gave highly significant ($P < 0.005$) values for all but spots 1 and 2. In subsequent studies, both sexes should be run on each plate, so that a 3-way ANOVA can be used to

TABLE 7. LINEAR REGRESSION ANALYSES OF R DIGITOXIN (A) AND SPOT INTENSITY (B) VALUES OF 16 CARDENOLIDES VISUALIZED IN CHLOROFORM–METHANOL–FORMAMIDE TLC SYSTEM IN 85 SAMPLES OF PLANTS AND RESPECTIVE BUTTERFLIES REARED THEREON, ACCORDING TO THE FUNCTION $y = bx + a$

	(A) R digitoxin values	(B) Spot intensity values
Sum of Xi	744	2094
Sum of Yi	731	2284
Sum of $(Xi)^2$	908	6278
Sum of $(Yi)^2$	868	7422
Sum of (Xi) (Yi)	888	6427
No. of points	854	854
a (y intercept)	0.014	0.902
b (slope)	0.966	0.723
r (correlation coeff.)	0.998	0.675
Std. error	0.031	0.917
Significance of r[a]		
F 1, 852	>250,000	711
P	<<0.001	<<0.001

[a]ANOVA test from Zar, as in Table 4.

separate differences attributable to sex, geography, and plates more clearly.

The ANOVA results indicated that overall R_d differences attributable to sex and geography show no patterns: they are either very small and insignificant, or too small to be separated from the effect of plate-to-plate variation. The same analyses were run on the *SI* values without the difficulty of plate-to-plate variation encountered in analyzing the R_d values. The overall *SI* analyses show little variation that is attributable to sex, considerable unpatterned variation that can be attributed to the areas of collection, and perhaps more significantly, an increasing intensity of spot 4 southward in both the plants and the butterflies. This spot may prove useful in assessing a general north–south geographic origin of wild captured butterflies in future fingerprinting studies.

The A. eriocarpa *Fingerprint Profile.* As summarized in Figure 6 and Table 6, the butterflies contain 16 of the 20 cardenolides present in the *A. eriocarpa* plants that are resolved in this solvent system. Of these, ten (numbers 1, 3, 4, 5, 7, 8, 9, 13, 14, and 15) have highly correlated R_d values and eight (excepting 1 and 3) have highly correlated spot intensity values with means in excess of 1.99. Moreover these eight cardenolides have a probability of occurrence in excess of 85% and 98% in the plants and butterflies, respectively.

We thus define the cardenolide fingerprint profile of freshly emerged monarch butterflies which have fed on *A. eriocarpa* as shown in Figure 6. Eight of these cardenolides appear particularly diagnostic, i.e., numbers 4, 5, 7, 8, 9, 13 (desglucosyrioside), 14 (a mixture of labriformin and labriformidin), and 15.

TLC Cardenolide Profiles of Plants and Butterflies: Ethyl acetate-Methanol (EAM) System

Because of the greater streaking and because fewer cardenolides are resolved, we did not do as extensive an analysis on the 85 plants and respective butterflies as on the CMF system. Examples from two of the 14 TLC plates run in the EAM system are reproduced in Figure 5. Figure 5C shows three plants and respective male butterflies reared thereon from area 6 (August) in the southern Sierra Nevada, Kings' River floodplain. This is the same material as illustrated in Figure 5A. Figure 5D shows three plants and respective female butterflies from area 3, the northern Sierra Nevada foothills.

Eleven discernible cardenolides of varying intensity were resolved in the plants and seven of these (Nos. 1–4, 6, 8, and 9) plus one additional spot (No. 7) were resolved in the butterflies (see Figure 5D). Of these, spots 4, 6, and 8 are of intermediate intensity in both the plants and butterflies, while spot 9 (labriformin and desglucosyrioside) is extremely well developed in both. Spot 11 (labriformidin) is also highly developed but occurs only in the plants. Spots 5, 10, and 12 also occur only in the plants and are minor constituents.

As in the CMF system, these 12 cardenolides occur in three distinct regions: spots 1–6 occur below digitoxin, spots 7–10 occur between digitoxin and digitoxigenin, and spots 11 and 12 occur above digitoxigenin. More generally, the butterflies are capable of storing *A. eriocarpa* cardenolides of high and intermediate polarity, but cannot store those of low polarity, i.e., those with R_d *values* $\geqslant$ digitoxigenin. Overall in this TLC system, the butterflies can store cardenolides within approximately 78% of the range of those found in the plants.

As in the CMF system, no obvious differences were found in the TLC profiles for the two sexes in the 85 plants–butterfly pairs from all six geographic areas, with the exception of spot 4 (same number in both TLC systems). This cardenolide increases in relative concentration southward in both the plants and the butterflies.

Conclusions from Both TLC Systems

The cardenolide profiles which emerge in both TLC systems show a remarkable consistency in the plants, both sexes of the butterflies, and throughout a wide geographic range in California. Previous research (Seiber,

et al., 1978) indicated that spot 9 of the EAM system is a mixture of labriformin and desglucosyrioside, while spot 11 (EAM) is a labriformidin. Thus taking the two TLC systems together, we have proven that the monarch butterfly is capable of storing desglucosyrioside but cannot store either labriformidin or labriformin. We emphasize that the chemical identity of the cardenolides in the corresponding plant and butterfly spots, with the exceptions of labriformin, labriformidin, and desglucosyrioside, remains to be established.

In both TLC profiles, each spot represents at least one cardenolide. Since each cardenolide differs structurally, each probably varies in its physical-chemical characters, which in turn influence its chemical and biological stability. We emphasize that during aging of the adults some spots may disappear entirely from the fingerprint, others may partially degrade, and metabolism to new cardenolides or interconversion among existing cardenolides may occur. These possibilities may all affect the fingerprint, and further research is needed for their precise elucidation.

Relationship of Emetic Potency to Constituent Cardenolides of A. eriocarpa

The emetic potencies of butterfly groups I and II and plant group II are, respectively, 27.6, 25.8, and 17.2 blue jay ED_{50} units per mg of cardenolide (Table 5). Thus, relative to butterfly group I, butterfly group II is 93% as emetic, and plant group II is only 62% as emetic. The group I material is characterized by a relatively high concentration of cardenolide spot 4 and the group II material by a relatively high concentration of cardenolide spot 8 (see Figures 5A, B and 6). Moreover, since labriformin is one of the high R_f constituent cardenolides found in large amounts in the plants (spot 18), its similar emetic potency to the plant material (17.5 and 17.2 units, respectively) suggests that the higher R_f cardenolides in *A. eriocarpa* are less emetic to the jays.

DISCUSSION

Quantitative and Qualitative Variation in Cardenolides in Plants and Butterflies

Large variation occurs in the gross cardenolide content both of *Asclepias eriocarpa* plants (102–919 μg/0.1 g) and in the adult monarch butterflies reared on them (136–606 μg/0.1 g). In contrast, the cardenolide TLC profiles show relative constancy and include approximately 20 cardenolides in the plants, of which at least 16 correspond to those in the butterflies. Moreover, whereas the gross cardenolide content shows considerable geographic and/or temporal variation both in the plants and the butterflies, their TLC profiles are remarkably similar throughout California.

The consistency of the cardenolide profiles which contrasts with the variability in the concentration is further emphasized by a comparison of the sexes: TLC patterns of males and females show no differences, whereas the male concentrations are nearly 20% less than the females.

The uniformity of the cardenolide profile of *Asclepias eriocarpa* may be due to the absence of population races within this species of milkweed, which has a very limited distribution compared to many other *Asclepias* species (Woodson, 1954). In contrast, *A. syriaca* has a much more extensive distribution in North America and also occurs in Europe, where it spread eastward following its 16th-century introduction to the Paris botanical gardens. Correlated with this wider distribution is the fact that samples from different areas in the New and Old Worlds contain cardenolide races (or demes, or local populations) including some with none and others with up to 15 chromatographically defined cardenolides (review in Roeske et al., 1976; Brown et al., 1979; Rothschild et al., 1975, their Table 13).

The overall picture that emerges from this study is thus one of extensive quantitative variation in cardenolide concentrations, but relative constancy in the TLC cardenolide profiles of both the plants and the butterflies reared upon them. Because the TLC profile remains constant irrespective of variation in the total cardenolide content of the larval food plant, the *Asclepias eriocarpa* cardenolide fingerprint profile for freshly emerged adult monarch butterflies is established (Figure 6).

Sources of Variation in Cardenolide Content of Plants

Varibility of gross cardenolide concentration within the ranges reported in this paper has been published for small samples of seeds and leaves of *A. eriocarpa* by Roeske et al. (1976), Isman (1977), and Isman et al. (1977a,b). The latter authors (1977a) also found that wild-captured lygaeid bugs of two species collected on several different California milkweeds ranged in cardenolide content from 0 to 300 μg/individual insect and those found on *A. eriocarpa* ranged from less than 10 to more than 180 μg/insect. These authors did not correlate the cardenolide content of individual plants with the individual bugs found feeding on them, but their evidence did suggest that the variability was in part attributable to whether the bugs were feeding on the stems, flower heads, or pods, and in part to the season when the samples were obtained. Nelson et al. (1981) have followed the temporal change in cardenolides in various organs of a natural population of *A. eriocarpa* throughout one year, and their results show significant differences in the total cardenolide content of the organs as well as large changes through time which support the conclusions of Isman et al. (1977a).

Variation in the total cardenolide content of various asclepiadaceous plants as well as plants in other families has long been established (Stoll, 1940;

Abisch and Reichstein, 1962; Brower and Brower, 1964). Attempts to elucidate the sources of variation are numerous in the phytochemical and pharmacological literature. Thus Masler et al. (1961) estimated that the seeds and roots of *Asclepias syriaca* contained less cardenolide than the rest of the plant parts, whereas Rothschild et al. (1975) found the opposite. Karawya et al. (1973) reviewed the literature on *Nerium oleander* (Apocynaceae) and found reports of total cardenolide varying from 0.08 to 1.15% by weight. They then compared several different parts of a white and a red variety throughout the growing season, and showed differences attributable to color morph (presumed genetic difference), plant part, and time. Another study (Kuchokhidz et al., 1974) reported that the cardenolide content of the leaves of *Rhodea japonica* (Liliaceae) varies both seasonally and geographically. Evans and Cowley (1972) carried out a detailed investigation of *Digitalis purpurea* (Scrophulariaceae) during development from seedling to mature plants throughout the first and into the second year when flowering and seed set occurs. Total cardenolide increased for the first seven months, then decreased, and then increased again in the second growing season. Young leaves had more cardenolide than the older ones, and the basal rosette of leaves produced during the first year and present during the second had less than half the content of the young leaves. Hydrolysis of the cardenolide mixture, followed by quantitation of digitoxigenin and gitoxigenin, showed that the ratios of these two genins changed through development and were strikingly different in the various organs. Tahsler (1975) assayed lower, middle, and upper leaves of *Asclepias curassavica* and *A. nivea* for total cardenolide content and also found the highest amount in the young, actively growing leaves. TLC analyses, however, indicated no major differences in the cardenolide profiles of the three sets of leaves.

In terms of the developmental biology of cardenolide production, a paper by Hirotani and Furuya (1977) is of particular interest. Using *Digitalis purpurea* tissue cultures, they were able to show that root callus cells do not produce cardenolides, whereas leaflike tissues regenerated from the culture do.

Wichtl's (1975) review of the *Digitalis* literature provides a strong case for genetically determined control of both quantity and kinds of cardenolides in various races and varieties of *Digitalis purpurea.* However, Wichtl is extremely critical of the lack of systematic and carefully controlled experimentation found in many published studies purporting to demonstrate genetically based cardenolide races.

Variation in Other Secondary Plant Chemicals

Additional sources of variation in cardenolide production can be inferred from studies of other secondary plant chemicals. What follows is a brief

review of the literature selected to illustrate the varied causal bases of variation. Further references are available in Rosenthal and Janzen (1979).

McKey et al. (1978) found that the production of phenolic compounds in the leaves of several tropical rain forest trees was inversely related to soil nutrients. This was a valuable field confirmation of the findings of Koeppe et al. (1976). Both of these studies strongly support the hypothesis that nutrient stress is a factor which can result in an increase in protective chemicals. Moreover, herbicide treatment resulted in an increase in the alkaloid content of wild larkspur (*Delphinium*, Ranunculaceae) and other plants (Laycock, 1978). In other words, exogenous chemicals in the plant's environment can play an important inducing role, from which it can be predicted that allelochemics (Whittaker and Feeny, 1971) produced by adjacently growing plants may also influence secondary plant chemical production.

Temperature can also affect production. Parker and Williams (1974) showed that the amount of miserotoxin in *Astragalus miser* (Leguminosae) was reduced by growing the plants at low temperatures. Amounts also varied widely in plants from different areas and at different altitudes and, during development, decreased rapidly between pod formation and senescence.

Genetic polymorphisms of cyanogenic and acyanogenic forms of *Lotus corniculatus* (Leguminosae), *Tribolium repens* (Leguminosae), and bracken fern (*Pteridium aquilinum*) have been extensively investigated by Jones (1973); Ellis et al. (1977a); Jones et al. (1978); and Cooper-Driver and Swain (1976). In at least some populations, the frequencies of the two forms vary according to herbivore pressure and thus represent changes in gene frequencies due to differential selection by the herbivores. Dolinger et al. (1973) found parallel responses of Colorado *Lupinus* (Leguminosae) in which the number of alkaloids present in the inflorescences of different populations was directly proportional to the extent of larval feeding by lycaenid butterflies. A similar polymorphic response to bark beetle infestation has been reported for essential oil production in *Pinus ponderosa* populations in the northwestern U.S. (Sturgeon, 1979).

The selection of strains of human food crop species resistant to various invertebrate and fungal enemies is a major technology (e.g., Barney and Rock, 1975), from which genetic control both of polymorphic and continuous variation in the production of secondary plant chemicals is inferred. Additional genetic evidence is provided by the phenomenon of polyploidy. For example, alkaloid production is greatly increased by autopolyploid production in *Datura* (Solanaceae), *Cannabis* (Moraceae), and in many other plants (Levin, 1976a). Additional complexity is introduced to genetic determination by on/off responses of cyanogenesis to ambient temperatures (Ellis et al., 1977b).

Feeny's study (1970) of the increased titers of tannins in maturing oak

leaves and his explanation of the adaptive significance of this in reducing the general digestibility of the leaves to insects (1975) was a major contribution to our understanding of seasonal changes in phytochemical composition. Feeny (1977) elaborated on the concepts presented in his early tannin work in a paper on the defensive chemistry of mustard oil glucosides which are richly represented in the Cruciferae (Rodman and Chew, 1980). Feeny argues that by combining this chemical diversity with the ecological adaptation of being early successional weeds scattered in space and time, these herbaceous plants become highly unpredictable to their potential herbivores. This strategy is supported by a considerable body of evidence (Root, 1973; Cates and Orians, 1975; Rhodes and Cates, 1976; Feeny, 1976; Futuyma, 1976; Price and Willson, 1979).

Alkaloids are among the most numerous (Levin, 1976a cites more than 4000 structural types) and best studied of the secondary plant substances, the production of which is under genetic control but subject to numerous environmental influences. Sources of genetic variation are the existence of clones (Coulman et al., 1977), while environmental factors include the usual effects of locality, season, altitude, and plant age and part (Taylor, 1963; Levin, 1976a,b). Other factors can influence total plant content. Levin's (1976b) review indicates an enormous literature on induced production involving postinfectional increase of alkaloids as well as other secondary substances in plants attacked by fungi or bacteria. Experimental harvesting of the upper leaves on reed canary grass resulted in increased alkaloid content of the regrowth that occurred (Coulman et al., 1977), whereas the reverse happened when the duncecap larkspur (*Delphinium barbeyi*) was experimentally cropped (Laycock, 1975). These studies suggest that larval infestation could, depending upon the species of plant involved, either increase secondary plant substances or decrease them (see also Rothschild, 1973; Chew and Rodman, 1979; McKey, 1979).

Another source of variation is the time of day that the plant is harvested, which in turn is related to photosynthesis, 24-hr metabolic rhythms, and turnover in the plant (deWaal, 1942; Sinden et al., 1978).

There is thus no doubt that genetic factors in combination with selective herbivory in various natural populations result in an extraordinary heterogeneity of secondary chemical production in plants. Moreover, it is clear that variation is also a function of the developmental biology of plants, with the concomitant effects of temporal change and differences in the various organs and tissues. Finally a host of environmental factors affect secondary plant chemical production, including diel periodicity, temperature, altitude, competing plant species, nutrients, stress, and even herbivory itself. With this multiplicity of factors, it is perhaps not surprising that Swain (1977) concluded his review with the statement that we have little quantitative data

on variation in the concentrations of secondary compounds in plants. The study that we have presented here on cardenolides in *Asclepias eriocarpa* will perhaps begin to fill this most important lacuna in our knowledge of secondary plant chemicals.

Sources of Variation in Cardenolide Content of Butterflies

Genetically Controlled Variation. The ability of the monarch butterfly to sequester and utilize cardenolides in its chemical defense implies an underlying genetic control which originated in the past history of the evolution of danaine butterflies (Feeny, 1975; Duffey, 1980). Since the hereditary basis is undoubtedly polygenic, it must be subject to substantial variation. This hypothesis is supported by the fact that different species of danaids feeding upon the same species of milkweed foodplants are much poorer sequesterers than the monarch. For example, when raised on *Calotropis proceral,* the gross cardenolide concentration of adult *Danaus chrysippus* from west Africa was an average of only 30% that of *D. plexippus*, and on *A. curassavica*, only 44%. Similarly, the New World *D. gilippus* (which is closely related to *D. chrysippus*), when reared on *A. humistrata* Walt., contained only 77% as much cardenolide as *D. plexippus* (Brower et al., 1975). The lower storage ability of *D. chrysippus* compared to *D. plexippus* was confirmed by Rothschild et al. (1975). These authors (as previously noted by Rothschild et al., 1973) provided further evidence of genetic control in their report that geographic races of *D. chrysippus* from east and west Africa differ in their storage capabilities: however, their results are at variance with Brower et al. (1975). In another study, Rothschild and Marsh (1978) reported that the Argentine subspecies of the monarch is a less efficient storer than either the Trinidad or North American subspecies. While appropriate genetic crosses and careful control of the insect and plant propagation conditions are much to be desired, the combined findings on these species are reasonably compelling evidence that gross cardenolide uptake is subject to genetically controlled variation.

Further indication of genetic control is the storage by female monarchs of approximately 20% higher concentrations of *A. eriocarpa*-derived cardenolides than the males (Table 1). This sexual difference was consistently found in monarchs reared on other species of *Asclepias* (Brower et al., 1972, 1975; Brower and Moffitt, 1974; Brower and Glazier, 1975), did not occur in *D. gilippus*, and occurred inconsistently in *D. chrysippus* (Brower, et al., 1975, 1978). Higher storage levels also occurred in female *Oncopeltus* bugs fed upon *A. syriaca* seeds (Duffey and Scudder, 1974) and on the seeds of five species of California milkweeds (Isman, 1977). It is interesting that *Oncopeltus* has a life history pattern involving migration similar in several respects to that of the monarch (Dingle, 1978). We speculate that these sexual differences may be

adaptive in that the overwintering insects must survive for several months before remigration in the spring to distribute eggs and establish the new generation. The higher content of cardenolides in females in both insects may serve a protective function for the eggs into which they are passed during oogenesis (Duffey and Scudder, 1974; Thomashow, 1975).

Other species comparisons provide further evidence of genetic control. Rafaeli-Berstein and Mordue (1978) found that the grasshopper, *Zonocerus variegatus*, which is specialized to feed on cardenolide-rich and other toxic plants, is capable of a much higher rate of ouabain excretion than the less specialized *Locusta migratoria* grasshopper. Indeed, the same argument for genetic control applies to the 300-fold decrease in the sensitivity of sodium-potassium ATPase to ouabain found in monarch butterflies compared to two other species of non-cardenolide-feeding Lepidoptera (Vaughan and Jungreis, 1977).

Enrivonmentally Induced Variation. The overall cardenolide concentration of the butterflies in this study ranged from 136 to 606 μg/0.1g, which is considerably less than the variation in the cardenolide concentration of the plants. Our results, as summarized in Figure 3 and 4, thus agree with Dixon et al. (1978, p. 463), who concluded that the quantitative content of the plants is of minor importance in determining the quantity of cardenolide taken up by the monarch butterfly.

Several other factors either have been shown or are virtually certain to contribute to the variable pattern found in the butterflies. For example, the basic dietary requirements of the insect must be met, irrespective of an individual plant's cardenolide content. Monarch and other lepidopterous larvae consume and pass larger amounts of leaf material through their guts when the food is low in nutrients, nitrogen, or water (Boyd and Goodyear, 1971; Erickson, 1973; Schroeder, 1976; Fox and MacCauley, 1977; Scriber, 1977; Slansky and Feeny, 1977; Slansky and Slansky, 1977; Pandian et al., 1978; Scriber and Slansky, 1981). Under these conditions, more cardenolide would automatically be ingested and might well by digested, assimilated, and stored by the insect (see Roeske et al., 1976, Figure 11). An insect might also be forced to pass more food through its gut as a result of tannins, resins, and other protein digestion-reducing chemicals (Feeny, 1976; Rhoades and Cates, 1976; Cates and Rhoades, 1977). These and other allelochemics frequently increase during a plant's phenological development and thus could contribute to seasonal variation (Schweitzer, 1979). On the other hand, different secondary chemicals present in a particular milkweed may interfere with cardenolide incorporation (Duffey, 1980). More generally, nutrient and secondary plant chemicals interact not only with each other, but also with the gustatory, dietary, and detoxification physiology of the insect (House, 1969; Dadd, 1973; Brattsten et al., 1977), and these multiple interactions most certainly must affect the uptake of cardenolides.

Temperature may also affect the dynamics and amount of cardenolide incorporation for, as Mathavan and Pandian found (1975), total consumption of milkweed by *Danaus chrysippus* declined with higher temperature even though the rate of consumption increased (Mathavan et al., 1976). However, Dixon et al. (1978) argue that these two factors may cancel each other out so that there is no net change in cardenolide uptake.

Other components of the variance must include behavioral responses of the insects. For example, young leaves in general have a higher nutrient content (Barbosa and Greenblatt, 1979) and are preferred by ovipositing adult monarchs (Urquhart, 1960; Dixon et al., 1978) as well as by *Oncopeltus* and *Lygaeus* bugs prior to seed ripening (Duffey and Scudder, 1972). Since young *Asclepias nivea* and *A. curassavica* leaves (Tahsler, 1975) also have a higher cardenolide content than the older ones, as do young leaves and new shoots of *A. eriocarpa* (Nelson et al., 1981), the larvae de facto will ingest more cardenolide by feeding upon them. Moreover, the latex of several species of *Asclepias* has been reported to contain very high levels of cardenolide (Rothschild, and Ford, 1970; Rothschild et al., 1975), and *A. eriocarpa* latex contains an approximately ten times greater concentration of cardenolide per unit weight than any other part of the plant (Nelson et al., 1981). Therefore, if some larvae ingest considerable latex, either by preference (Rothschild, 1977; Dixon et al., 1978), or perhaps due to being forced to by competition with other larvae on the same plant, they could store large amounts of cardenolide. On the other hand, monarch larvae have also been reported chewing through the lactifers in the petioles of *A. curassavica* leaves. Since this behavior effectively stops the flow of latex to the leaf blade (Brewer, 1977), reduced cardenolide uptake could result. In comparison, milkweed bugs may be able to bypass or selectively sequester cardenolides, depending on whether their piercing and sucking mouthparts enter vascular or nonvascular tissue by either choice or chance (Botha et al., 1977).

Although of no effect in the present study because all the adults were killed before 12 hr old, aging of individuals in natural populations results in loss of cardenolide by metabolic degradation and/or excretion. Tuskes and Brower (1978) found that overwintering butterflies at Muir Beach, California, lost an average of 23% of their cardenolide over the course of 3½ months, and Brower et al. (1982a) found that overwintering butterflies in Mexico lost an average of 27% of their gross cardenolide content between January 15 and March 20, 1978. We do not yet know whether individual cardenolides are lost uniformly or preferentially during the adult life spans. Further indirect evidence of loss through aging comes from the work of Dixon et al. (1978), who found that the emetic potency to pigeons declines in aging butterflies. On the other hand, Isman et al. (1977a) reported that two species of milkweed bugs (*O. fasciatus* and *Lygaeus kalmii*) do not lose previously assimilated

cardenolides to any significant extent when maintained on a cardenolide-free diet over a 2-week period. However, in the same paper they present evidence that loss in *Oncopeltus* over longer time periods may occur, because spring remigrants in California all contained very low cardenolide levels compared to samples collected on the same species of plants later in the season. Further experimentation is needed to determine both the nature and extent of quantitative and qualitative losses of cardenolides during the natural overwintering, diapause and in more active periods in the life histories of all these insects.

Other environmental factors could result in increased or decreased cardenolide content and/or changes in their molecular structures. These include water balance during the overwintering season in which monarchs imbibe large amounts of water, and the maintenance of lipid levels through nectar feeding at many species of flowers (Brower et al. (1982b). Although no cardenolides have been found in nectar, the nectar of certain species of flowers contains various other glycosides (Baker and Baker, 1973). The fact that several species of danaine butterflies as adults ingest pyrrolizidine alkaloids from plants in the wild and utilize them in their sexual behavior (Edgar et al., 1974; Boppré et al., 1978) and probably also in chemical defense (Edgar et al., 1976; 1979; Rothschild and Edgar, 1978; Eisner, personal communication) proves that secondary plant chemical sequestration is not limited to the larval stage in these insects. It is possible that these and other secondary chemicals sequestered by adult monarchs may interact with the cardenolides.

In conclusion, the possible contribution to the variance in gross cardenolide content of the monarch includes a multiplicity of potential factors, both genetic and environmental. It is therefore all the more remarkable that the gross cardenolide concentration in the butterflies varies to only one fifth the extent of that of their foodplants in addition to there being a stable and relatively invariant cardenolide profile. These facts imply regulation which will now be discussed.

Regulation of Cardenolide Uptake

Qualitative Aspects Based on TLC. Our results have shown that the monarch butterfly incorporates the majority of cardenolides found in the *A. eriocarpa* plants and that the TLC profiles of the butterflies are remarkably uniform, even though they represent both sexes and were determined on plants gathered over a 2-month period from widely distributed localities in California. These findings indicate a basic underlying and stable system controlling the absorption, distribution, excretion, and storage of cardenolides during the physiologically complex growth and metamorphosis cycles of the monarch butterfly. Further indication of this was provided by Thoma-

show's (1975) TLC analysis of *Asclepias curassavica*-reared monarchs which showed that eggs, the five larval stages, chrysalids, and adult male and female butterflies all contain virtually identical arrays of cardenolides.

Our results also show that four of the 20 *A. eriocarpa* cardenolides visualized in the CMF solvent system (spots 17–20, Figure 6) are not stored by the adult monarchs. Two of these (Figure 5C,D; Figure 6, spot 18) occur in high concentrations and previously were characterized as labriformin and labriformidin (Seiber et al., 1978; Cheung et al., 1980). Although several possibilities exist for the exclusion from storage of some of the ingested cardenolides (Roeske et al., 1976), the explanation we favor for labriformin and labriformidin is that during larval feeding they are rapidly metabolized and in the process are converted to desglucosyrioside (Figure 6, spot 13), a cardenolide which is storable and in fact stored. Our evidence is derived from the following: the structures of labriformin, labriformidin, and desglucosyrioside are chemically analogous to uscharin, uscharidin, and the calactin–calotropin isomer mixture from *Calotropis procera* and *Asclepias curassavica* (Figure 7). Thus the thiazoline ring attached at C-3′ of the sugar moiety in labriformin can be cleaved by hydrolysis yielding the C-3′ ketone (labriformidin) which in turn may be reduced at C-3′ producing the secondary alcohol desglucosyrioside. Analogous hydrolysis of uscharin yields uscharidin and, by reduction, calactin–calotropin. These transformations have been established in vitro through purely chemical reactions (Seiber et al., 1978; Brüschweiler et al., 1969; Hesse and Ludwig, 1960; and references therein). Furthermore, the reduction of uscharidin to calactin and calotropin was recently shown to occur in vivo by administering uscharidin to 4th instar monarch larvae (Seiber et al., 1980). We presume that uscharin is also converted during larval feeding to calactin and calotropin via the reaction scheme in Figure 7 since uscharin, like uscharidin, is not stored in monarchs reared on *Asclepias curassavica* (Reichstein et al., 1968; Roeske et al., 1976). However, this remains to be demonstrated by means of the experimental administration of pure uscharin to monarch larvae.

Preliminary results of another study in our laboratory have indicated that a single administration of either labriformin or labriformidin to 4th instar monarch larvae results in their metabolism principally to desglucosyrioside which in turn is stored in the adult stage. These findings are analogous with the more extensively studied *A. curassavica* cardenolides and with the results in this paper. Thus we have strong evidence that selective storage from among labriformin, labriformidin, and desglucosyrioside in *A. eriocarpa*-reared monarches is due principally to metabolism of the first two cardenolides to the third during larval feeding.

It is noteworthy that similar metabolism and storage mechanisms apparently exist for the two structurally distinct groups of cardenolides shown in Figure 7. Previous evidence indicates that still a third group of cardenolides

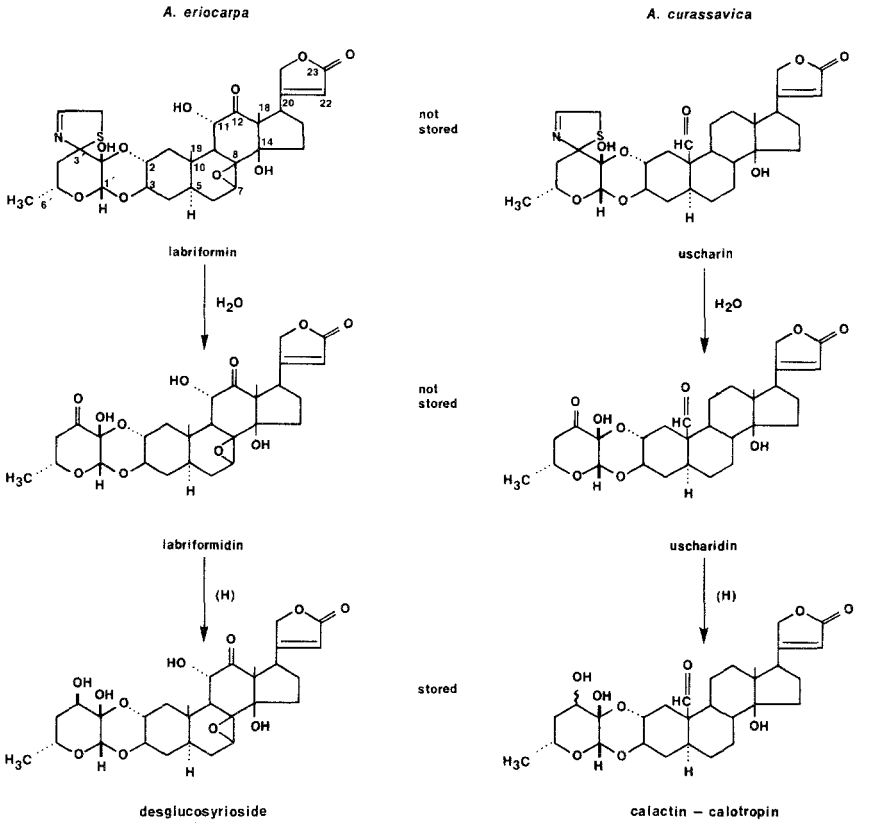

FIG. 7. Structures and relationships of three major cardenolides in *Asclepias eriocarpa* and *A. curassavica* plants. The *A. eriocarpa* cardenolides labriformin and labriformidin do not completely separate in the chloroform–methanol–formamide TLC system (spot 18, Figure 5A and B, Figure 6). Neither of these cardenolides is stored by the monarch butterfly. However, desglucosyrioside is stored by the monarch (spot 13, same figures). In the ethyl acetate-methanol TLC system (Figure 5C and D) labriformin cochromatographs with desglucosyrioside (spot 9) while labriformidin is separated (spot 11, same figures). The three *A. curassavica* cardenolides are parallel in their behavior in both TLC systems and in how they are metabolized by the monarch to a form in which they can be stored. Note that in the first reaction the thiazoline group is removed by hydrolysis from the C-3′ atom of the sugar portion of the cardenolide and is replaced by the keto group. In the second reaction this keto group is reduced to a hydroxyl group. It thus appears that the cardenolides are not directly stored by the monarch with either the thiazoline or the keto configuration at C-3′ of the sugar and that a hydroxyl group must occur at this position before storage is possible.

present in *Gomphocarpus physocarpus* from East Africa may be similarly processed, since with this plant it was again the higher R_f cardenolides in the leaves which were absent from monarchs reared upon it (Roeske et al., 1976). Moreover, the same results were found in rearing *Danaus chrysippus* on this plant (Ferm, 1977). [The identification and nomenclature of this species of *Gomphocarpus* (=*G. physocarpus* E. Mey) was verified by Dr. David Spellman of the Missouri Botanical Garden on herbarium specimens grown at Amherst College from seeds collected by L.P. Brower on August 23, 1975, from D.F. Owen's original site in Kenya. This site is along the verge of the east side of highway A-104 near Molo, 44.8 km northwest of the Stags Head Hotel in Nakuru, Kenya; see also Agnew, 1974].

The collective findings on the cardenolides presented in Figure 7 place the sugar moiety, specifically through functionality at C-3′, in an important role in determining which cardenolides are to be metabolized and which can be stored directly by the monarch. Furthermore, the grasshopper *Poekilocerus bufonius* Klug also stores principally calactin–calotropin when fed either *Calotropis procera* or *Asclepias curassavica* (von Euw et al., 1976), so this chemical conversion and sorting process may be a general one for insect herbivores of cardenolide-bearing plants.

Our present results indicate that, in addition to desglucosyrioside, several more polar but as yet unidentified cardenolides among spots 1–12 (CMF system, Figures 5 and 6) may also be stored directly from *A. eriocarpa* without metabolic alteration. These compounds apparently possess the requisite physicochemical properties necessary for successful storage. It has also been shown that a number of the leaf cardenolides of *A. curassavica* are found in *A. curassavica*-reared monarchs in addition to calactin–calotropin (Reichstein et al., 1968). All of the identified compounds found to be stored have one or more free hydroxy groups in addition to the one at C-14: calactin and calotropin at C-2′ and C-3′, calotoxin at C-2′, C-3′, and C-4′ of the sugar group, and calotropagenin at C-2 and C-3 of steroid ring A. Analogously, hydroxylated cardenolides may account for some of the more polar stored cardenolides from *A. eriocarpa*. Considering the structures of known stored cardenolides from these two plant species, some of which are shown in Figure 7, leads us to speculate that it is tissue or exoskeletal binding which involves one or more OH groups that allows for storage of these cardenolides in the monarch, although the nature of the binding process has not yet been determined.

Quantitative Aspects of Regulation. This study has established that monarch butterflies reared on *Asclepias eriocarpa* incorporate a relatively constant quantity of total cardenolide, independent of the cardenolide concentration in the larval food plant. Butterflies incorporate a higher percentage of cardenolide relative to that in the plant when fed on plants having low gross cardenolide concentrations and vice versa. This consistency

of incorporation indicates that a capacity for *A. eriocarpa* cardenolides exists in the butterflies, and that a mechanism exists for achieving this capacity over a wide range of plant cardenolide concentrations. This mechanism represents regulation in that it involves an adjustment of cardenolide content within the butterfly to a norm existing for *A. eriocarpa* cardenolides.

This concentration norm for *A. eriocarpa*-reared butterflies (318 μg/0.1 g) is similar to that of monarchs reared on *Asclepias curassavica* (319 μg/0.1 g) (Roeske et al., 1976), a species for which cardenolide incorporation by butterflies also appears to be independent of plant concentration (Dixon et al., 1978). This is striking in that the major cardenolides of these two plant species differ substantially in the structure of the steroid moiety (Fig. 7). That dependence of quantity of cardenolide incorporated upon structure can exist is clearly illustrated by the facts that monarchs both concentrate and store substantially less cardenolide when reared on *Gomphocarpus physocarpus* than on either *Asclepias curassavica* or *A. eriocarpa.* Comparative average concentration values for these three plants were 331, 386, and 421 μg/0.1 g, and for the respective butterflies were 102, 319, and 318 μg/0.1 g. The average total cardenolides per butterfly reared on the three plants, respectively, were 210, 670, and 616 μg. (The comparative data are from Roeske et al., 1976.) Futhermore, controlled feeding of several individual cardenolides to *D. plexippus* also gave rise to differences in incorporation efficiency which extended over a range of doses (Seiber et al., 1980).

There may exist other species of *Asclepias* for which incorporation to the capacity attained for *A. eriocarpa* and *A. curassavica* is not achieved. Our preliminary experiments indicated that *A. speciosa*, *A. cordifolia*, and *A. fascicularis* may fall into this group, although it is not yet clear whether the lower incorporation of cardenolide from these species derives from an unusually low leaf concentration, structural features that render the cardenolides of these species inherently less storable, or a combination of these factors.

With regard to the mechanism underlying the control or regulation of cardenolide incorporation, Roeske et al. (1976) used Waldbauer's (1968) indices of consumption and utilization to compare total storage of cardenolide from *Gomphocarpus physocarpus* and *Asclepias curassavica.* Their results showed that the butterflies stored 3 and 13% of the respective total cardenolide contents and passed 49 and 42% into the larval feces. Of the balances unaccounted for (48 and 45%), part was possibly metabolized to noncardenolide, but substantial amounts were lost into the 5th instar larval and pupal skins which are shed and discarded during metamorphosis (Thomashow, 1975), and also into the meconium excreted at adult hatching (unpublished data). Because of the large losses into the larval feces, it is possible that regulation is a function of rate limitation on the amount of cardenolide that passes through the wall of the gut. Alternatively or in addition to this possible mechanism, the gut may pass all the ingested

cardenolide into the hemolymph but then dump the excess via malphigian excretion back into the hindgut from which it is voided in the feces. This latter mechanism has been shown to occur by Rafaeli-Bernstein and Mordue (1978) in ouabain feeding experiments with the grasshopper *Zonocerus variegatus*.

The qualitative and quantitative regulation we have found in this study and in previous laboratory experiments (Seiber et al., 1980) suggests a dynamic control of uptake, metabolic alteration, storage, and excretion. The underlying physical-chemical basis of this control remains to be elucidated, although we have evidence that available OH groups on the sugar or on steroid ring A may be necessary for successful storage.

Cardenolide Uptake and Storage in Monarch Butterflies Compared to Oncopeltus Bugs.

It seems likely that the ability of these two phylogenetically distinct (Lepidoptera vs. Hemiptera) and developmentally disparate (complete vs. incomplete metamorphosis) insects to store milkweed cardenolides has evolved independently. Supporting this contention is the fact that many species of milkweed-feeding insects (including not only other Hemiptera and Lepidoptera, but also Orthoptera and Coleoptera) either lack the ability to store cardenolides or do so only in small amounts (Rothschild, 1973; Isman et al., 1977b). It is therefore reasonable to expect differences in the underlying processes of digestion, transport, metabolism, storage, and excretion of the cardenolides in the two groups of insects.

The physiochemical basis of cardenolide storage has been studied most intensively in the bugs. Duffey and Scudder (1974) showed that adult *Oncopeltus* bugs concentrate cardenolides from *Asclepias syriaca* seeds and store them in a series of specialized glands, especially in the so-called dorsolateral glands. They also found that 60–95% of the total cardenolide in adult bugs is in these glands but that low concentrations also occur in the hemolymph and elsewhere. Utilizing TLC, they found both polar and nonpolar constituents from the *A. syriaca* seeds in the hemolymph, but the dorsolateral glands stored only the more polar ones. Hypothesizing that metabolism of nonpolar to polar glycosides was occurring, they impregnated sunflower seeds either with the highly polar ouabain or the less polar digitoxin. Chromatography indicated that the bugs stored ouabain without alteration and in high concentrations, whereas digitoxin was converted to two polar metabolites and then stored by the bugs. Yoder et al. (1976) measured uptake of tritiated digitoxin and ouabain by the cells of incubated *Oncopeltus* gut tissue. They discovered that both apparently diffuse across the cell membranes in direct proportion to their concentrations, but that ouabain's rate was only about 10% that of digitoxin, presumably because of its more polar and therefore less lipophilic properties. Duffey et al. (1978) found that, once in the hemolymph, ouabain is rapidly sequestered into the dorsolateral

glands where its concentration was 75 times that of the hemolymph. In contrast, the less polar digitoxin gave a concentration ratio in the dorsolateral-space fluid to hemolymph of less than 5. For both cardenolides, uptake in the dorsolateral space fluid increased linearly with dose administered in the feed (seeds of *A. syriaca*). The authors provided evidence that the ouabain concentration involves a physical emulsion-phase transfer process (see also Duffey, 1980) which transports ouabain to the dorsolateral gland so rapidly that its concentration in the hemolymph is low at all times. Digitoxin, on the other hand, must diffuse into the dorsolateral-space fluid and does not interact with the emulsion-phase sequestration system as does ouabain.

Thus storage in the dorsolateral-space fluid of the bugs appears to be a function of the affinity of the sequestration system for polar cardenolides. The results of Isman (1977) and Duffey et al. (1978) provided no evidence for a limiting capacity to cardenolide storage by the bugs: uptake increased directly with cardenolide dose when bugs were (1) reared on seeds of ten species of *Asclepias* varying by more than a factor of 10 in cardenolide content, (2) when cardenolide was injected into the hemolymph (Duffey, 1977), and (3) when increasing doses of ouabain or digitoxin were mixed with the feed. However, Vaughan (1979) reported that individual bugs fed on *A. syriaca* seeds of different cardenolide concentrations did not store a constant proportion of that cardenolide in their bodies. Instead those bugs eating seeds low in cardenolide concentration stored a greater percentage of cardenolide than did bugs fed on seeds of high cardenolide concentration—a finding more in agreement with our present results for *D. plexippus* on *A. eriocarpa.*

In making comparisons between the two insects, it is well to remember that several experiments with *Oncopeltus* used ouabain and digitoxin as chemical probes both because of their contrasting polarity and because of their availability. Neither of these cardenolides has been found in *Asclepias* species, and, in fact, both differ significantly from the *Asclepias* cardenolides in the configuration at the A/B steroid ring juncture Seiber et al. (1980) found that this structural feature affected storage in *D. plexippus* larvae in that uzarigenin (derived from *Asclepias* and possessing a *trans* A/B ring juncture) was stored partially as the parent genin while digitoxigenin (the isomer of uzarigenin possessing a *cis* A/B ring juncture) was stored only as a polar metabolite. Comparisons in the ability of the two insects to sequester and store cardenolides could clearly benefit from administration of milkweed-derived cardenolides to each under similar experimental conditions. It may well be that arguments previously advanced for the prominent role of polarity in the disposition of cardenolides by the bug are premature, that *Oncopeltus* may in fact be capable of storing cardenolides over the broader polarity range typical of monarchs, and that the bugs may be capable of a quantitative regulation of cardenolide uptake from some milkweed species comparable to that for monarchs on *A. eriocarpa* and *A. curassavica.*

Cardenolides, Emesis, and Other Defensive Chemicals.

In this paper we have again emphasized the emetic properties of cardenolides derived from milkweed food plants as providing the monarch butterfly with its principal chemical defense against potential vertebrate predators, particularly birds. We do not agree with Duffey (1977, p. 339) that emesis may be "an inadequate index of the impact of these chemicals in a natural predator population." Evidence now exists that cardenolides induce emesis in 12 species of birds belonging to 9 families (Fink and Brower, 1981). Exceptions to the general rule are expected and do exist: quail (Rothschild and Kellett, 1972) and two species of finches (grosbeaks, Fink and Brower, 1981; towhees, Brower et al., 1982c) do not vomit when given high oral doses of cardenolides, and gulls (Chaney and Kare, 1966) exhibit emetic insensitivity to intravenous lanatoside-C. Emesis is an objective criterion of unpalatability, is statistically quantifiable by means of the blue jay ED_{50} assay and is the best measure presently available for assessing the impact of these chemicals. Cardenolides are ecologically significant because their strong emetic action and bitterness together, through delayed reinforcement learning, elicit long-term visual and/or gustatory avoidance of the poisonous food following one or more initial encounters by a sensitive predator (reviews in Brower and Glazier, 1975; Garcia and Hankins, 1975; Garcia, 1980). Other noxious effects of cardenolides on the birds also occur (Parsons, 1965; Brower et al., 1967), but their importance in negative reinforcement and potentiation has not yet been established.

In emphasizing the importance of cardenolides in the defense of the monarch butterfly, we do not wish to minimize the possible roles that other secondary plant chemicals may play. Following an earlier suggestion of Rothschild and Marsh (1978), Edgar et al. (1979) speculate that pyrrolizidine alkaloids which are sequestered by adults of many danaine butterflies throughout the world may be more important as vertebrate deterrents than cardenolides. Indeed, Boppré (1978) even argues that cardenolides are only a recent addition to the chemical arsenal of the Danaidae. Rothschild et al. (1975) contend that several African danaine species which feed on *Asclepias* lacking cardenolides must be chemically protected either by other secondary plant substances or by compounds they have synthesized. This theme is often repeated (e.g., Roeske et al., 1976) and is well expressed in the following quote from Dixon et al. (1978, p. 462): "Up to now Monarch investigators have been so hypnotized by the discovery of cardnolide storage thay they have failed to look for alkaloids, gymnemic acids, ... in the adult butterflies and have also underestimated the importance of deterrent tastes and smells of the imagines, also probably acquired from their foodplant." We might add to this list the noncardenolide substances which are cardioactive (March et al., 1977; Dixon et al., 1978) and other noncardenolides which are emetic (Bowers, 1980). We therefore challenge our colleagues to select an alternative group of

purportedly defensive chemicals and to develop a bioassay which is repeatable, statistically quantifiable, allows comparisons within and between species, and which is ecologically relevant. We submit that cardenolide-induced emesis eminently fulfills these criteria.

Prior to the feeding experiments presented in this paper, the evidence that cardenolides per se are the emetic compounds, although substantial, has been indirect, as correctly pointed out by Duffey (1977, p. 343). We have now proven that labriformin isolated from *Asclepias eriocarpa* is emetic to blue jays. By inference from the feeding of pulverized plant material and the butterflies reared thereon, the case is made even stronger, although the emetic potencies of desglucosyrioside and the other cardenolides alone and in mixtures still need determination. Evidence from TLC R_{fS} and emeticity data indicate that the polarity and emeticity of these cardenolides are at least in part directly related. Further work is needed to prove this, however (Duffey, 1977).

Coevolutionary Triad: Milkweeds, Monarchs, and Birds.

We here define coevolution as a process involving reciprocal natural selection in which evolutionary changes accumulate as a result of positive genetic feedback between two or more interacting species (see also Ehrlich and Raven, 1965, 1973; Ehrlich, 1970; Janzen, 1979; Heinrich, 1979; Price, et al., 1980). More generally, plants and animals are considered to have interacted in a positive feedback system in which the secondary chemistry of the plants (Fraenkel, 1959) is intimately involved with the animals' evolution and vice versa. Levin (1976b, p. 146) has stated this concept eloquently: "The diversity of secondary compounds is an evolutionary product, indefinite and indeterminate and subject to self-augmentation through time. . . . Plant species are subject to predictable, systematic, and directional pressures from pathogens and herbivores which participate with them in an incessant evolutionary dance."

In the case of the monarch butterfly, coevolution has involved three levels in the food chain: the milkweed plants, the monarch larvae which eat the milkweeds, and the birds, which in eating the adult monarch butterfly become poisoned indirectly by the milkweed. Several lines of evidence in our triad support coevolution: The monarch possesses an Na^+-K^+-ATPase system which is comparatively insensitive to cardenolides. Moreover, the monarch is capable of quantitative regulation in the uptake of the milkweed cardenolides which increases the likelihood of acquiring an emetic dosage when the larvae feed on low concentration plants and may prevent overdosage to monarchs feeding on plants with high cardenolide concentrations. In addition, the monarch appears capable of qualitative regulation: it converts some nonpolar cardenolides to those of intermediate polarity which are stored by the butterfly. It also appears that cardenolides of intermediate polarity (e.g., desglucosyrioside, calactin–calotropin) may be more emetic, so that the

capacity to convert less polar cardenolides to these may facilitate predator deterrency. The plant may also retain a line of defense to overgrazing by the monarch as indicated by the fact that uzarigenin when dosed to larvae causes melanism (Seiber et al., 1980). This particular cardenolide from *A. eriocarpa* and *A. curassavica* (Nelson et al., 1981) is found only in the stems of these plants, and its presence may deter monarchs from feeding on the stems, thus preserving the plants' ability to sustain vegetative regrowth. The same melanistic response occurs in monarchs when they are forced, through overcrowding in culture, to eat the stems and thereby kill the plant (Brower, personal observation).

The fact that grosbeaks are able to eat monarch butterflies containing up to 16 ED_{50} units of *A. curassavica* cardenolides (Fink and Brower, 1981) suggests that they may have evolved insensitivity to the milkweed cardenolides. This may be the basis by which these birds are able to prey extensively upon the butterflies at the Mexican overwintering sites (Calvert et al., 1979) with the result that fewer butterflies will survive to reproduce upon the milkweeds. Finally (same references) orioles by their behavior are able to eat the monarch without being poisoned so that they, too, will contribute to reduction in the monarchs' pressure upon the milkweeds. For milkweeds, cardenolides toxic to all herbivores but not to the predators of the herbivores would provide optimal protection: for butterflies, cardenolides easily metabolized and nontoxic to them but extremely toxic to the predators would provide them the greatest advantage.

Further study of this system involving monarchs, milkweed bugs, and other milkweed herbivores should go far in laying to rest the evolutionary skepticism surrounding many authors' views of secondary plant chemicals. For example, Mothes recently stated (1976, p. 387): "In the evolution of life, Nature has prepared a great abundance of substances without any intention and without the probability of any one of them fulfilling a task in further evolution." We believe that what we are witnessing with the monarch as a central actor is but one stopped frame in a continuum of time involving a dynamic biochemical interaction among the partners of a fascinating coevolutionary triad.

SUMMARY

A large-scale field and laboratory study was carried out to relate natural variation in the gross cardenolide content of more than 150 individual *Asclepias eriocarpa* plants to the variation found in 172 individual monarch butterflies found as 5th instar larvae or pupae on these plants. The plant and respective butterfly samples were gathered during the summer of 1975 from six geographically separate and ecologically different areas in California.

We present new procedures for sample cleanup and thin-layer chromo-

tography (TLC) in two solvent systems allowing separation of the cardenolides found in the butterflies and plants (Figure 5A–D). Cardenolide TLC profiles for 85 of the plant–butterfly pairs and statistical analyses of variation in the profiles attributable to the geographic localities and sex of the butterflies were determined.

Emetic potencies of the cardenolides present in two groups of plants, one group of butterflies, and in one of the major cardenolide constituents of *A. eriocarpa* (labriformin) were determined by means of the blue jay emetic-dose fifty (ED_{50}) assay.

Sources of variation in cardenolides in both the plants and butterflies were discussed in relation to the literature on secondary plant chemical variation. The cardenolide-sequestering system in the monarch butterfly, including the regulation of storage, was compared and contrasted with that of the lygaeid bug, *Oncopeltus fasciatus*.

The gross cardenolide content of the plants exhibited normal variation with a large variance, ranging from 102 to 919 μg of cardenolide per 0.1 g of dry powder with a mean of 421 μg/0.1 g. Geographic variation was not a major contributor to this variation. The butterflies reared on this plant reduced the variation by more than 50% and ranged from 136 to 606 μg/0.1 g dry weight with a mean of 318 μ/0.1 g. The mean cardenolide concentration in the males was 83% of that in the females, in confirmation of previous findings. The butterflies have a clear-cut ability to regulate the concentration of cardenolides from *A. eriocarpa*: butterflies reared on plants low in cardenolide increased their concentrations relative to the plants, whereas those reared on plants high in cardenolides did the reverse.

Males reared on *A. eriocarpa* weigh more than the females, so the resultant total cardenolide in the two sexes does not differ significantly; the mean for both sexes was 616 μg per butterfly, with a range of 220–998 μg.

Based on two slightly different TLC profiles, we formed two groups of plants and respective butterfly material by pooling the leftover powders. Group I contained TLC profiles with a slightly higher amount of low R_f cardenolides, and the butterflies proved slightly more emetic when reared on this group of plants. Ninety-five percent of the butterflies contained between 7.5 and 26.1 ED_{50} units with an average of 16.4 ED_{50} units per butterfly. These are the most emetic monarch butterflies studied to date.

The results of the chloroform–methanol–formamide TLC system were analyzed in detail. Twenty discernible cardenolides of varying intensity were resolved in the plants and 16 were resolved in the butterflies. This allowed us to define the *A. eriocarpa* cardenolide fingerprint profile for freshly emerged monarch butterflies reared on this plant (Figures 5A and B, 6).

The overall picture is one of broad quantitative variation in cardenolide concentrations, but relative constancy in the TLC profiles both of the plants and of the butterflies reared upon them.

This study, combined with previous work in our laboratory as well as that of others, indicates that the relatively nonpolar labriformin and labriformidin in the plant are not stored by the monarch but are metabolized in vivo to desglucosyrioside which the butterfly does store (Figures 5–7). This metabolism and storage of desglucosyrioside is chemically analogous to the manner in which monarchs and grasshoppers handle another series of milkweed cardenolides, those found in *Asclepias curassavica*. For the cardenolides with sugars of the type found in labriformin, labriformidin, and desglucosyrioside, it appears that the sugar moiety through functionality at C-3′ determines which cardenolides are to be metabolized and which can be stored directly by the monarch. The monarch also appears able to store many of the lower R_f cardenolides from *A. eriocarpa* without altering them. The combined qualitative and quantitative picture of cardenolide handling by the monarch suggests a dynamic control of uptake, metabolic alteration, storage, and excretion.

Differences in the sequestering process in the monarch butterfly in comparison to milkweed bugs (*Oncopeltus fasciatus*) may be less than previously emphasized in the literature. However, the monarch does seem better able to regulate storage of the cardenolides from milkweeds and also appears able to sequester cardenolides across a greater polarity range than *Oncopeltus*.

This study provides additional evidence in support of the hypothesis that the monarch butterfly is a central organism involved in a coevolutionary triad, simultaneously affecting and affected both by its avian predators and by the secondary chemistry of the milkweed plants with which it is intimately related.

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EXOCRINE GLANDS OF *Polyrhachis simplex*: Chemistry and Function

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Abstract—The mandibular glands of the Israeli weaver ant, *Polyrhachis simplex*, contain a mixture of 4-heptanone, 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol; its Dufour's gland secretion consists mainly of *n*-tridecane. The significance of these glandular secretions in the biology of the weaver ant is discussed.

Key Words—*Polyrhachis simplex*, weaver ant, Hymenoptera, Formicidae, mandibular gland, Dufour's gland secretions, 4-heptanone, 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, *n*-tridecane.

INTRODUCTION

The weaver ant, *Polyrhachis simplex*, an oriental formicine ant, is found in Israel in oases along the Dead Sea basin which marks its westernmost distribution line (Bytinski-Salz, 1953). These insects are renowned for weaving their nests with silk originating from the larval salivary glands. Ofer (1970) conducted extensive investgations of these ants both in the field and in the laboratory where colonies were reared successfully. His field studies were made in the Arugot Stream region which has a large population of *P. simplex*, and there he observed their intriguing vertical migration from the high banks to the stream in late spring and return in the winter. However, the chemical nature of the secretions which may elicit certain communicative responses in the ants has not been investigated previously. In this paper we present the gas chromatographic–mass spectrometric analyses of the secretions of the mandibular and Dufour's glands and report some observations pertaining to their role in the biology of the ant colony.

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METHODS AND MATERIALS

Insects. Colonies of *P. simplex* were collected at Hatzevah in the Rift valley. They were transferred to the laboratory and placed in a glass-walled artificial nesting site at the corner of a 3 × 3-m foraging table. The only entrance to the nest was level with the table surface. The ants were fed daily on honey and dead insects and had access to a water supply. Soon after their transfer the ants started weaving and arranging brood cells containing newly laid eggs.

Chemical Analyses. The mandibular and Dufour's glands were excised from chilled living workers and stored in pentane. Gas chromatographic–mass spectrometric analyses of the extracts were conducted on a LKB-9000 mass spectrometer using a 3.7-m 10% SP-1000 column programed from 55 to 200° C at 8° / min and a 1.8-m OV-1 column programed from 60 to 320° C at 10° / min. The components of the extracts were identified by their mass spectra which were compared to spectra of authentic samples. As a further check, retention times of unknowns and authentic standards were compared on polar (AT 1000) and nonpolar (SE-30) columns using a Perkin-Elmer gas chromatograph. The relative concentrations of the different constituents were estimated using a Hewlett-Packard peak integrator coupled to the gas chromatograph. Standards and extracts were also coinjected on these columns to confirm retention times.

Behavioral Tests. The response of the ants to crushed mandibular glands and to specific compounds identified in the glandular extracts was studied in the laboratory. The products were applied in a suitable solvent to small pieces of cardboard (5 × 5 mm) which were placed randomly on the foraging table after the solvent had evaporated. The reactions of the ants were recorded.

Field studies were conducted in the Arugot Stream area during 1979–1980.

RESULTS

Chemistry. Gas chromatographic analyses of the volatiles of the mandibular gland extracts (Figure 1) revealed the presence of three components representing approximately 37, 51, and 10.5% of the volatiles along with traces of other constituents. The compound eluting first had a molecular ion at m/z 114, a base peak at m/z 43, and an intense fragment ion (95%) at m/z 71. Tentative identification of the unknown as 4-heptanone was confirmed by comparison of its MS and its GC retention time with those of an authentic sample and by coinjection. On the basis of their mass spectra, the second and third components were identified as 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol, respectively. Comparison with authentic samples of this ketone and corresponding alcohol likewise confirmed their structures.

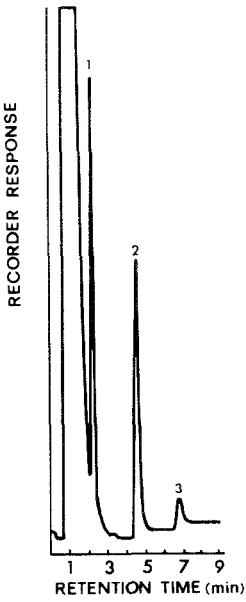

FIG. 1. Gas chromatogram of the mandibular gland extracts of *Polyrhachis simplex*. Extracts were run on a 1.8-m 10% AT 1000 column at 120°C. Peak 1 is 4-heptanone, peak 2 is 6-methyl-5-hepten-2-one, and peak 3 is 6-methyl-5-hepten-2-ol.

GC-MS analysis of the Dufour's gland extract showed that it consisted of straight-chain hydrocarbons with over 90% of the secretion tridecane and minor amounts of undecane, dodecane, pentadecane and heptadecane.

Behavior. P. simplex ants exhibited a complex behavior in response to conspecific crushed mandibular glands. Their first reaction was excitation and rapid random movement for about 10 sec, then, in a matter of 30–50 sec, all the ants on the foraging table assumed a typical defensive posture with open mandibles and abdomen bent toward the emitting source. They remained motionless approximately 5 min, then they approached the odor source and quickly removed in from the table. Similar behavior was observed in the field, where ants in a radius of 30 cm assumed a defensive posture when they were exposed to crushed worker heads or mandibular gland extracts. This behavior was distinct from the response to formic acid (a component of the poison gland); the latter consisted of excitation, rapid movement toward the odor source with open mandibles, followed by fierce biting of the source.

When the ant colonies were first established in the laboratory, many of the ants were seen in a characteristic defensive posture without any apparent cause of disturbance. Incidents of mutual carriage were common, a behavior typical of ants in unknown terrain. Similar bahavior was observed in the field during new nest establishment or a few days after a flash flood when the nest area was drastically disturbed.

Each of the two ketones, the major components of the mandibular gland extract, elicited the same response on the part of the ants as the crushed heads or mandibular glands. On the other hand, the ants did not respond to tridecane, the Dufour's gland component, except for a short period of excitement.

DISCUSSION

One of the major compounds found in the mandibular gland secretion of *P. simplex*, 4-heptanone, is not a common insect product. Olubago et al. (1980) identified it recently as the major volatile component in the mandibular glands of the myrmicine ant *Zacryptocerus varians*. In this ant, it is accompanied by 4-heptanol which does not seem to be present in *P. simplex*.

The other ketone, 6-methyl-5-hepten-2-one, occurs widely in ants. It is the major volatile in the mandibular glands of several *Formica* species in the subgenera *Neoformica* and *Proformica* (Duffield et al., 1977), and it is abundant in the secretion of an Australian species of *Calomyrmex* ant (Brown and Moore, 1979); in other formicine ants, *Lasius fuliginosus* (Bernardi et al., 1967) and *L. carniolicus* (Bergström and Löfqvist, 1970), it appears as a trace component. This ketone is also commonly found, not in the mandibular glands, but in the anal glands of dolichoderine ants; it has been detected in *Tapinoma* (Trave and Pavan, 1956), *Iridomyrmex* (Cavill et al., 1956), and *Conomyrma* (McGurk et al., 1968).

The corresponding alcohol, 6-methyl-5-hepten-2-ol, is also found in the *Calomyrmex* mandibular secretion (Brown and Moore, 1979) but appears only as a minor component in *Formica* species (Duffield et al., 1977). The alcohol may be a biosynthetic precursor of the ketone or perhaps it is itself an aggregation pheromone as in the case of scolytid beetle *Gnathotrichus sulcatus* (Byrne et al., 1974).

The mandibular gland secretion of *P. simplex* is much less complex than that of the African weaver ant, *Oecophylla longinoda* (Bradshaw et al., 1979); however, it is still not possible to assign it a definite biological role. Although in large concentrations it definitely appears to act as an alarm pheromone, it may also have a function in the seasonal establishment of new nest locations. Additional behavioral observations will be needed to reach a more conclusive answer.

The secretion of the Dufour's gland of most formicine ants is usually quite complex. The major components, *n*-undecane and *n*-tridecane, are commonly accompanied by other normal and branched alkanes, alkenes, alcohols, ketones, and other oxygenated compounds (Blum and Hermann, 1978). In contrast, the secretion of *P. simplex* consists almost entirely of *n*-tridecane, and the function of this compound is somewhat puzzling. It does not appear to provoke any sustained alarm behavior in these ants, as, for

example, undecane does in *Acanthomyops claviger* (Regnier and Wilson, 1968) or in *Lasius niger* (Bergström and Löfqvist, 1970). It may be that it serves only as a wetting and potentiating agent for the formic acid of the venom gland.

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SEASONAL VARIATION IN LABORATORY RESPONSE TO BEHAVIORAL CHEMICALS OF THE SOUTHERN PINE BEETLE[1]

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Abstract—The response of *Dendroctonus frontalis* to an attractant mixture (frontalin, *trans*-verbenol, and loblolly pine turpentine) was measured in the laboratory over a four-year period. Beetle response was highest in late winter and early spring, and lowest in midsummer and early fall. Males consistently responded higher than females. Female beetles displayed significantly higher responses in early morning and late afternoon than in the middle of the day. Analysis of beetle pronotal width and fat content revealed a high degree of correlation between these two parameters in female beetles, but there was no correlation of response with either fat content or pronotal width for either sex. There was no evident relationship between mean monthly beetle response and total amounts of frontalin and *trans*-verbenol found in hindgut extracts. Daily temperature in months both during which beetles were bioassayed and immediately prior to bioassay was highly correlated to response to the attractant.

Key Words—*Dendroctonus frontalis*, Coleoptera, Scolytidae, seasonal variation, pheromone content, environmental parameters.

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INTRODUCTION

Several sources of nontreatment variation influenced field bioassays of behavioral chemicals of the southern pine beetle, *Dendroctonus frontalis* Zimmerman (Payne et al., 1978). One of these sources, seasonal variation, has been well documented for a number of scolytids in both field and laboratory bioassays (Borden, 1977). *Ips confusus* (= *paraconfusus* Lanier) response to male-produced frass and frass extract declined from September to April (Wood and Bushing, 1963; Borden, 1967). Diapausing *I. pini* were unresponsive to frass or frass extracts (Birch, 1974) as were diapausing *D. pseudotsugae* (Borden, 1977). *D. brevicomis* were unresponsive to the attractant *exo*-brevicomin (7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane) in the autumn (Silverstein et al., 1968). Coster et al. (1977) reported seasonal variations in the mass attack behavior of the southern pine beetle. Seasonal variation in both average fat content and in pronotal width of newly emerged *D. frontalis* has been described (Hedden and Billings, 1977). Thatcher and Pickard (1964) reported on the seasonal variation in behavior, development, and brood mortality of *D. frontalis* in east Texas. However, no intensive study has been undertaken to evaluate seasonal variation in the response of *D. frontalis* to synthetic chemical attractants.

In April 1975 we initiated a laboratory study on the seasonal variation in responses of both sexes of *D. frontalis* to an attractant mixture and to a pentane control. Examination of seasonal variation in fat content, pronotal width, and pheromone components from beetle hindguts began in May 1976.

METHODS AND MATERIALS

Bioassay. Beetles were assayed for their response to an attractant mixture of frontalin (Kinzer et al., 1969), *trans*-verbenol (Renwick, 1967), and loblolly pine turpentine in a 1:1:12 ratio. Pentane was used as a control and as a solvent for dilution of the attractant mixture.

Newly emerged *D. frontalis* were collected from infested bark, which was gathered periodically in east Texas and placed into an emergence chamber (Browne, 1972). Insects were collected daily, sexed twice, screened for damage, and placed individually into gelatin capsules where they remained at room temperature until assayed. Beetles were used only on the day they were collected, and each beetle was tested only once.

The tests were made using the bioassay described by Payne et al. (1976). Airflow in the system was 1 liter/min. The attractant mixture was eluted at 14 μg/min. For each assay, five beetles of the same sex were released on the center of a clean paper on the assay table, and their behavior was observed. A positive response was recorded when any beetle walked to within 1 cm of the

chemical source. Beetles were allowed up to 5 min to respond. Each assay of five insects was considered one observation and recorded as responders versus nonresponders. Beetles were tested over the entire working day and time was recorded for each assay. The bioassay room was held at ca. 20° C.

Pheromone Components. Pheromones were extracted from emergent southern pine beetle hindguts (1976) and whole abdomens (1977–1978) in pentane (100–120 beetles/0.5 ml pentane). Aliquots of 6 μl (1.4 beetle equivalents) were subjected to GLC analyses. Duplicate runs were made on each sample, and peak heights of each compound were measured and averaged for both runs. Quantities of each compound are expressed as μg/beetle.

GLC analyses were made on a Varian Aerograph 2100 equipped with a flame ionization detector. The flow rate for the nitrogen carrier gas was 12 cc/min. Integrator and detector were set at 150° C. A 6.1-m × 0.635-cm OD glass silylated column packed with 5% Carbowax, 20 m on Chromosorb-W AW-DMCS, was used. The oven temperature at the injection of samples was 100° C. The temperature increased to 135° C at 30 min and increased further to 180° C at 60 min (burn-off at end of run).

Samples of standard compounds were used to establish standard curves. The standards were run before and after each daily series of runs on samples.

Environmental Parameters. Hourly barometric pressure readings and daily temperature data for College Station were obtained from Easterwood Airport in College Station, Texas. Monthly rainfall and temperature values for both College Station and Conroe, Texas (the closest reporting station to the location of field collections), were obtained from National Oceanic and Atmospheric Administration climatological publications.

Because environmental conditions present during beetle development could possibly influence subsequent behavior of adult insects, we performed analyses on environmental parameters for those months in which beetles were assayed and the month immediately prior to testing of each group of beetles. This latter time period accounts for the time during which the majority of development occurred for those beetles subsequently assayed.

Pronotal Width and Fat Content. Measurements of fat content and pronotal width of bioassayed beetles were made during eight months of the four-year period (May, 1976; and July, 1976, through January, 1977) using the techniques described by Hedden and Billings (1977). Crude fat content, reported as a percentage of dry weight, was removed by extraction with petroleum ether and was calculated for each group of 20 beetles/sex/month. Pronotal width measurements were taken with a stereomicroscope and ocular micrometer from 20 beetles/sex/month.

Analysis. Data were analyzed using parametric and nonparametric tests. These included Duncan's multiple-range test, *t* test, Spearman rank correla-

tion, Pearson product-moment correlation, and multiple regression procedures. The data were subjected to arcsin transformation prior to testing with parametric methods.

RESULTS

Seasonal Variation. Seasonal variation in the responses of both sexes of *D. frontalis* and respective controls are presented in Figure 1 where the four-year period was pooled by month. Responses of males were consistently higher than those of females, but the general pattern was the same for both sexes.

On a seasonal basis, highest responses typically occurred in late winter and spring (February/March to May/June) while lowest responses were found in midsummer to early fall (July/August to October/November). One exception is the high response of males in September.

Pheromone Components. Comparison of $\bar{X}$ monthly beetle response to the attractant mixture with the $\bar{X}$ monthly content of two pheromones present in the hindgut extracts, frontalin and *trans*-verbenol, showed no relation between the two as determined by Spearman rank correlation and multiple-regression procedures (highest $R^2 = 0.37$).

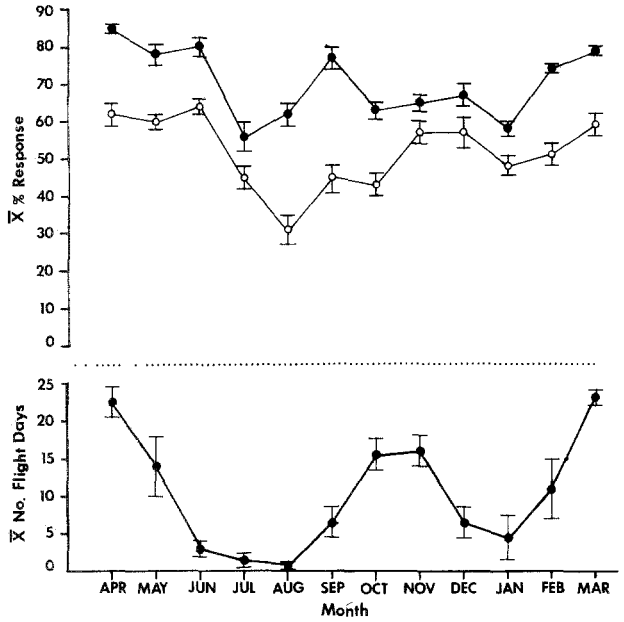

FIG. 1. Response ($\bar{X}\% \pm$ SE) of *D. frontalis* to an attractant mixture for the period of April 1975 to March 1979. Data were pooled for month. Solid circle = males; open circle = females.

Environmental Parameters. Spearman rank correlation analyses indicated no correlation between mean monthly response and mean monthly rainfall either in Conroe (where brood was collected) or College Station (where beetles were assayed). Analyses also showed no correlation between mean monthly response and mean monthly temperature at Conroe (*t* test indicated no difference in temperature at Conroe and College Station). Neither was there any correlation between mean daily response and maximum, minimum, or difference in temperature ($T°_{max} - T°_{min}$). Analysis between mean hourly response and both absolute barometric pressure and relative barometric pressure (rising, steady, or falling) showed no correlation. Lack of correlation in all of the above held true for both sexes.

In addition to examining the data on a monthly basis, we grouped the data into categories based on temperature characteristics. White and Franklin (1976) reported beetle flight activity to be very strong between 22 and 29° C. On this basis we tested response level as a function of two temperature regimes. Days on which beetles were bioassayed were grouped on the basis of the maximum temperature in that day. Days in which the maximum temperature ($T°_{max}$) was between 22° and 29° C (inclusive) comprised one category, while all other days made up the other category. The *t* tests indicated that response of males and females was significantly higher ($P > 0.001$) for that period in which the daily maximum temperature was between 22° and 29° C inclusive. Mean percent response for the period $22° C \leqslant T°_{max} \leqslant 29° C$ was 56.4 for females and 78.5 for males, while response for $22° C > T°_{max} > 29° C$ was 48.4 for females and 69.3 for males. Using this information, we defined an optimal temperature day (OTD) as one in which the maximum temperature was between 22° and 29° C inclusive. Four groupings were made on the basis of the number of OTDs per month (0–6, 7–13, 14–20, and 21–27 OTDs per month) (Table 1). No month had more than 27 OTDs. These groupings were made for OTDs in months in which beetles were assayed and those months immediately prior to that time.

Figures 2 and 3 depict mean percent response of male and female beetles when grouped as described above for months current with testing (Figure 2) and months prior to assay (Figure 3). In both cases, male and female response was highest for those months with the most OTDs (group 4). Response of males in group 4 was significantly higher (Duncan's multiple-range test, $P < 0.05$) among all groups, whereas response of females in group 4 was significantly higher than group 1.

Time. D. frontalis has higher flight activity in the morning and late afternoon (Coster et al., 1977; Vité et al., 1964). Based on this information, we divided the data into two time periods. Beetles assayed before 11:00 AM and after 4:00 PM comprised one period, while beetles tested between these times represented another period. Overall, there was no significant difference in the response of males due to time, but female *D. frontalis* responded significantly

TABLE 1. GROUPS BASED ON NUMBER OF OPTIMAL TEMPERATURE DAYS[a] PER MONTH

Month	Group 1 $0 \leqslant OTD \leqslant 6$	Group 2 $7 \leqslant OTD \leqslant 13$	Group 3 $14 \leqslant OTD \leqslant 20$	Group 4 $21 \leqslant OTD \leqslant 27$
A. Current group[b]				
January	3	1	0	0
February	1	2	0	1
March	0	0	0	4
April	0	0	1	3
May	1	1	1	1
June	4	0	0	0
July	4	0	0	0
August	4	0	0	0
September	2	2	0	0
October	0	1	3	0
November	0	1	3	0
December	1	3	0	0
B. Prior group[b]				
January	1	3	0	0
February	3	1	0	0
March	1	2	0	1
April	0	0	1	3
May	0	0	1	3
June	1	1	1	1
July	4	0	0	0
August	4	0	0	0
September	4	0	0	0
October	2	2	0	0
November	0	1	3	0
December	0	1	3	0

[a]Optimal temperature day occurs when maximum temperature is between 22° and 29°C, inclusive.

[b]A, current with *D. frontalis* assay; B, immediately prior to assay. Values are the number of times (over four years) each month occurred in that group.

higher (t test, $P < 0.001$) in early morning and late afternoon than in the middle of the day. Tests within each of the four groups, based on OTDs per month, indicated that responses of female beetles were significantly higher (t test, $P > 0.001$) during early morning and late afternoon periods for groups 1 and 2 based on current month groupings, and in groups 1 and 3 based on previous month groupings. Again, male response did not differ with time for any group.

Pronotal Width and Fat Content. There were seasonal differences in pronotal width and fat content for both male and female beetles (Table 2). Fat

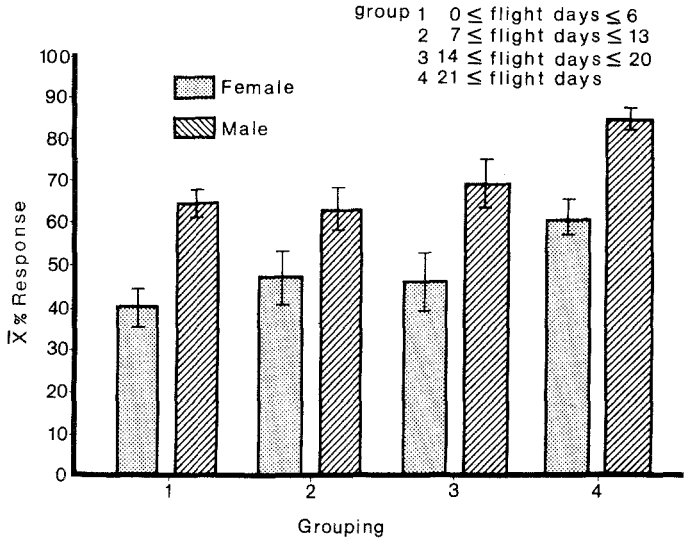

FIG. 2. Response ($\bar{X}\% \pm$ SE) of *D. frontalis* to an attractant mixture based on groupings of months by number of optimal flight days ($22°C \leqslant T°_{max} \leqslant 29°C$) in month current with testing (April 1975–March 1979).

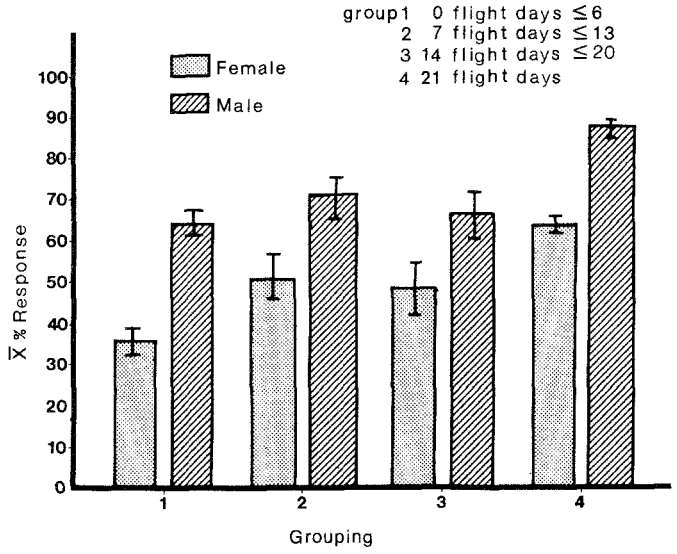

FIG. 3. Response ($\bar{X}\% \pm$ SE) of *D. frontalis* to an attractant mixture based on groupings of months by number of optimal flight days ($22°C \leqslant T°_{max} \leqslant 29°C$) in month previous to testing (April 1975–March 1979).

TABLE 2. MEAN PRONOTAL WIDTH (MM) ± SD AND FAT CONTENT (% DRY WEIGHT) FOR MALE AND FEMALE *D. frontalis* (MAY 1976 THROUGH JANUARY 1977)

		Female				Male			
Year	Month[a]	X ± SE width[b]			Fat content[b]	X ± SE width[b]			Fat content[b]
1976	May	1.20	0.06	A	24.1	1.21	0.09	A	22.3
	July	1.14	0.10	B	22.6	1.13	0.08	CB	19.1
	August	1.06	1.10	C	20.9	1.06	0.09	D	19.4
	September	1.10	0.08	BC	21.3	1.11	0.07	C	17.1
	October	1.14	0.06	B	26.1	1.12	0.07	C	23.1
	November	1.12	0.11	B	20.8	1.12	0.06	C	20.6
	December	1.09	0.08	BC	22.9	1.14	0.08	CB	21.6
1977	January	1.20	0.09	A	30.5	1.18	0.09	AB	20.9

[a]Means followed by same letter are not significantly different (Duncan's multiple-range test).
[b]20 beetles/month/sex, except 19 females in January.

content and pronotal width of females displayed a high degree of correlation (Pearson product-moment coefficient = 0.73), but there was no correlation of response with either fat content or pronotal width for either sex.

DISCUSSION

Seasonal Variation. Seasonal variation in response to attractants in the laboratory has been reported for a number of bark beetles. *Ips confusus* (= *paraconfusus* Lanier) female response to male-produced frass was lowest in January and February, and highest in May and June, while response to extracts of male-produced frass fell during winter and autumn (Borden, 1967). Response of females was higher than that of males, but the seasonal trends were similar (Borden, 1967). Birch (1974) attributed such variation to seasonal changes in beetle physiology, possibly due to the condition of the host tree.

In our study, we found *D. frontalis* exhibited lowest responses in July and August and highest responses from late February through June. Moser and Dell (1979a) reported a similar pattern of southern pine beetle attraction to frontalure- (frontalin plus alpha-pinene) baited traps in the field. It is during mid- and late summer, when ambient temperatures are the highest, that *D. frontalis* populations tend to show slow infestation growth. In fact, *D. frontalis* has been found to be adversely affected by summer level temperatures (Coulson et al., 1979). Thatcher and Pickard (1964) reported ratios of increase of beetle populations of 2–3:1 for each generation during late fall through

early spring, whereas during the period from June to September, ratios of increase were less than 1:1. Also, the number and size of new infestations increased most rapidly during April, May, and June, the period when beetle response in the laboratory was highest (Figure 1). Low brood activity and production also were characteristic of the midsummer period when the rate of potential evapotranspiration from host trees was highest (Moser and Dell, 1979b).

It is of adaptive significance for *D. frontalis* to exhibit relatively low response capability during mid- and late summer compared to fall, winter, and spring. In this way a greater percentage of the population would remain in trees within infestations and thus be insulated from the desiccating high temperatures. In the fall, winter, and spring, when temperatures are less threatening, *D. frontalis* displays increased response capability as evidenced by the proliferation and expansion of infestations (Billings, 1979).

Pheromone Components. Birch (1974) attributed the seasonal decline in response of female *I. pini* to male frass to both a decline in responsiveness and seasonal changes in pheromone production. Whereas Brich was working with frass as an attractant source, we utilized a synthetic mixture at constant concentrations, thus all seasonality in response was due to changes in responsiveness rather than changes in the concentration of attractant. One might reason that since pheromone concentration does vary seasonally, beetle responsiveness might change in a compensatory manner. This does not appear to be the case with *D. frontalis* since the hindgut concentrations of frontalin and *trans*-verbenol showed no correlation with response.

Environmental Parameters. The number of optimal temperature days per month appeared to have the most effect on beetle response. Since there was some correlation between the number of OTDs per month (Figures 2 and 3) and response of beetles in the following month, environmental conditions present during brood development quite possibly influenced subsequent response to pheromone. The temperature in the bioassay room was relatively constant, thus a relationship between response under controlled conditions and outside environmental conditions indicates that beetles may be genetically programed to respond optimally under certain environmental conditions. The affect of temperature on the developing brood may also be of significance, as well as other as-yet-unrecognized factors.

Temperature is one of the environmental factors implicated in controlling bark beetle activity. Moser and Dell (1979b) state that maximum temperature during the colder part of the year is the most obvious influence on beetle flight activity. Coster et al. (1978) examined the influence of precipitation, relative wind speed, temperature, percentage of cloud cover, and percentage of relative humidity on mass aggregation of *D. frontalis*. They found that, considering all days and all times of day, only wind speed correlated with

beetle numbers, but in the morning (9:00 AM) all variables were significant predictors of southern pine beetle numbers.

Barometric pressure has been found to affect scolytid behavior. Bennett and Borden (1971) found that *D. pseudotsugae* and *Trypodendrum lineatum* flew erratically and for short periods of time under laboratory conditions when there was cloud cover and stated that cloud cover may be associated with changes in pressure and humidity. Lanier and Burns (1978) observed that *Scolytus multistriatus* and *Ips pini* responded subnormally in unstable weather. They found that, although there was no relation between response level and pressure reading, artificial fluctuations of 25 mm Hg depressed beetle responsiveness for both these species. They also found that ambient pressure changes as small as 0.99 mm Hg significantly affected response of *S. multistriatus* to female-infested bolts. Our lack of correlation of response with absolute barometric pressure supports Lanier and Burns' (1978) findings of no relationship between pressure reading and response. However, the lack of correlation in our data between response and relative hourly changes in pressure (rising, falling, or steady) somewhat contradict their findings that small pressure changes alter response. This very likely may be due to the fact that changes in pressure in our study were not large enough to effect changes in response, since our measurements were on an hourly basis.

Time. The fact that female *D. frontalis* had high responses in early morning and late afternoon may be related to weather factors. It is interesting to note that this time-based difference occurred during those periods when flight conditions (based on OTDs per month) were suboptimal (Figure 2, groups 1 and 2; Figure 3, groups 1 and 3). High flight activity (thus response activity?) in early morning and late afternoon would appear logical in hotter months, where high midday temperatures may discourage flight. However, colder months also were contained in the groups in which time was a factor (Table 1). In these months, one would expect higher beetle activity during the warmest part of the day. Coster et al. (1977) reported higher trap catch of *D. frontalis* in early morning and late afternoon, but their study was performed only in spring and summer. We have no explanation for the fact that males showed no diurnal fluctuations in response.

Pronotal Width and Fat Content. The lack of correlation between either fat content or pronotal width with response in the laboratory tends to discount any significant relationship between these factors. Our data generally agree with those of Hedden and Billings (1977) and support the idea that beetle size and fat content are related to beetle population dynamics and infestation structure, and not to response to pheromone. Atkins (1969) did find a correlation between lipid content and attraction to the host in *D. pseudotsugae*. He demonstrated that as lipid reserves were depleted during flight, the beetles became increasingly attracted to the host.

In conclusion, it can be said that although there are marked seasonal fluctuations in the response of both sexes of *D. frontalis,* the direct causes of these fluctuations are difficult to pinpoint. Definite seasonal patterns do emerge, and these correlate well with some aspects of beetle population characteristics, such as infestation growth in number and size (Billings 1979).

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SEX PHEROMONE COMMUNICATION IN THE ODD BEETLE, *Thylodrias contractus* MOTSCHULSKY (COLEOPTERA: DERMESTIDAE)[1]

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Abstract—Adult virgin females of the odd beetle, *Thylodrias contractus*, produce a volatile sex pheromone that influences the behavior of conspecific males and attracts them in laboratory olfactometer tests. The active substance can be collected by allowing virgin females to walk on absorbent paper disks. Disks exposed to mated females do not elicit a response from males. The male odd beetle repeatedly rubs the antennae, head, and thorax of a female with a setate glandular area on his second abdominal sternum during courtship, probably secreting a reciprocally active aphrodisiac substance.

Key Words—Sex pheromone, aphrodisiac, odd beetle, *Thylodrias contractus* Motschulsky, Coleoptera, Dermestidae, bioassay, behavior, exocrine gland.

INTRODUCTION

The presence and activity of female-produced sex pheromones in dermestid beetles was first demonstrated for members of the genera *Attagenus* and *Trogoderma* (Burkholder and Dicke, 1966). Adult females of some *Anthrenus* and *Dermestes* species also emit sex pheromones (Burkholder et al., 1974; Abdel-Kader and Barak, 1979; Shaaya, 1980), and there is little reason to doubt that other members of this destructive family of beetles also use olfactory communication between the sexes.

During a study (Mertins, 1981) of the life history, biology, and behavior of the odd beetle, *Thylodrias contractus* Motschulsky, I observed that, when

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a virgin female was placed into a container with one or more males, there was an almost immediate increase in male activity, followed by rapid orientation to and location of the female. This paper is a report on several experiments and observations of the mating behavior of *T. contractus* supporting the presence of sex pheromone communication in this species.

METHODS AND MATERIALS

The insects used were from a laboratory culture maintained since 1973 under ambient room temperature, light, and humidity contiditions in tight-fitting plastic rearing containers. The larval diet consisted of dried insect cadavers, primarily adult greater wax moths, *Galleria mellonella* (L.), or bits of cooked, dried beef liver. Insects for observation were removed from the cultures in the pupal stage and isolated individually in No. 3 gelatin capsules to ensure virginity. They were observed daily until adult emergence.

Activities and mating behavior of individual pairs of odd beetles were observed with a dissecting microscope in an arena consisting of the bottom of a 5-cm (OD) disposable plastic Petri dish (Falcon Plastics, Oxnard, California) lined with a suitably sized disk of Whatman No. 1 filter paper. Individual virgin females were removed from gelatin capsules immediately or shortly after eclosion and held for various periods (12–120 hr) in 15-mm (OD) × 45-mm polyethylene-capped glass vials, one female per vial, with highly absorbent paper disks[2] on the bottoms. Control vials without insects and a series containing virgin males were likewise instituted and handled at the same time. For another series of tests, a group of mated females were similarly treated immediately after copulation.

I investigated the presence and activity of the female sex pheromone primarily with the glass desiccator olfactometer and a bioassay similar to that described in detail by Burkholder and Dicke (1966). Briefly, the olfactometer consisted of a standard 125-mm-diameter glass desiccator, with the top replaced by a round glass plate, 6.5 mm thick. A 20-mm diameter hole in the center of the plate allowed introduction of test insects through a small inverted glass funnel arrangement. Three other holes, each 35 mm from the center of the plate and each 15 mm in diameter, were drilled equidistant from each other. These allowed insertion of the test disks by means of glass-rod-and-insect-pin holders. The test arena within the desiccator was a 10-cm-diam circle of Whatman No. 1 filter paper held in a glass-walled ring. Absorbent paper disks exposed to virgin females, mated females, or males were tested against individual virgin males in the olfactometer by comparison with control disks. The exposure of an individual contaminated disk (along with its two uncontaminated controls) to an individual male constituted one test.

[2]No. 740-E antibacterial assay disks, 12.7-mm, Carl Schleicher and Schuell Co., Keene, New Hampshire.

The response of the male was recorded five times at 1-min intervals after the beginning of the test. The response was designated positive if the male was within the one third of the test area containing the contaminated disk and negative if he was within the other two thirds. The test was considered positive if three or more of the five interval observations were positive and the male showed definite orientation to the contaminated disk. Tests were conducted during the months of February through June under ambient laboratory environmental conditions (21 ± 1°C, 40 ± 20% relative humidity) between 0900 and 1700 CST.

Observations of the abdominal sternal gland on the male were made using standard histological techniques, including fixation in modified Carnoy's solution, staining with Delafield's hematoxylin and eosin, and microscopic examination at 1000× (Mertins and Coppel, 1971).

RESULTS AND DISCUSSION

A solitary male odd beetle in a plastic Petri dish usually is quiescent most of the time, resting with the body near and parallel to the filter paper substrate. The legs are spread out widely, and the antennae are positioned back against the thorax but elbowed forward at the 7th joint. Within 1–10 sec after addition of a virgin female to the dish, the male typically rouses from lethargy, straightens his antennae, and actively moves about the arena, sometimes rapidly and directly toward the female. The anterior part of the insect is elevated at ca. 30°, and the antennae are spread forward and upward, slowly waving from side to side.

When a female was introduced to the arena at the maximum possible distance from the resident male ($N = 34$ pairs), the average elapsed time between introduction and first contact by the male was 70.4 ± 67.7 (SE) sec with a range of 3–300 sec. Orientation of the male to the female is primarily by chemical stimuli evidently through an olfactory sense in the antennae. This was easily demonstrated in a preliminary experiment in which a female-contaminated paper disk was introduced to a Petri dish arena containing 50 virgin males (Figure 1); all but three moribund males were on the disk within 20 sec. Males frequently attempted to copulate with the disk and with each other.

Another preliminary experiment tested the response of males in the olfactometer to paper disks exposed to unmated females for successive 12-hr periods during the first 60 hr after eclosion (Table 1). Male response to the disks was minimal until the female was at least 36 hr old. Therefore, subsequent tests used only disks exposed to females for at least 48 hr after emergence.

A total of 45 different virgin females was tested against 35 different males in 71 separate olfactometer tests. The male response was considered positive

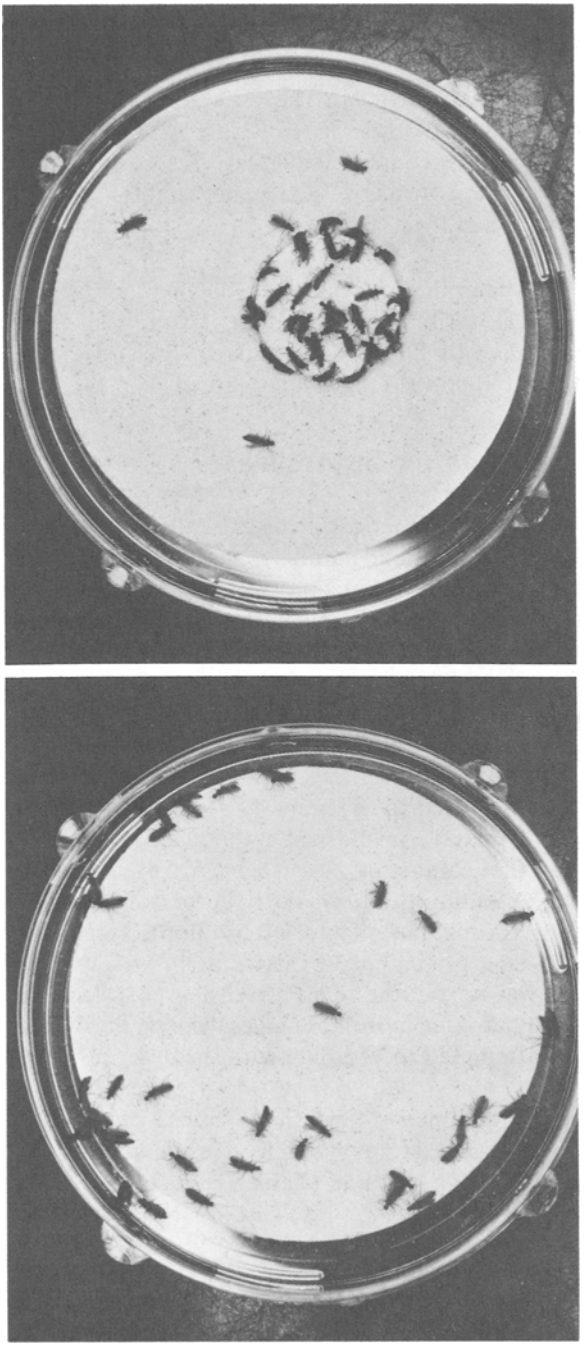

FIG. 1. Fifty male *Thylodrias contractus* in a 5-cm Petri dish arena before (left) and 20 sec after (right) addition of a paper disk exposed for 24 hr to a 48-hr-old virgin female.

TABLE 1. MALE RESPONSE IN OLFACTOMETER TESTS OF PAPER DISKS EXPOSED TO UNMATED FEMALE ODD BEETLES DURING SUCCESSIVE 12-hr PERIODS

	Virgin female held on disk during designated hours after eclosion				
	0–12	12–24	24–36	36–48	48
No. ♂♂ showing + resonse	0	1	3	13	10
No. ♂♂ showing – response	6	7	10	4	2

in 55 tests and negative in 16. Response was unaffected by time of the day. By chi-square analysis, with $P = 0.05$ as the limit of significance, the response of males to female-contaminated disks was significantly different from the controls ($\chi^2 = 62.1$, $df = 2$, $P < 0.001$), indicating a very strong attraction to the female pheromone. Nine of the 16 negative tests involved females that later proved to be unthrifty and short-lived, infertile, or both. In the olfactometer, males usually responded to the active disk with behavior much like that in Petri dishes previously described, concentrating their activities directly under the disk. However, because the disk was suspended out of reach 5 mm above their substrate, males inclined their bodies at a greater angle from the horizontal, extended their antennae vertically, and frequently reached toward the disk with their prothoracic legs. Concurrently, males often tried to copulate with the substrate under the disk and, when strongly stimulated, sometimes fell over backwards in the attempt. Activity of the pheromone-contaminated disks was persistent when they were held in the polyethylene-capped vials. One such disk showed no loss of activity when tested 57 days after female contamination; another still retained some activity after 16 months.

An experiment similar to the preceding series used eight different mated females and eight different males in 17 separate olfactometer tests. The male response was considered weakly positive in two tests and negative in 15, suggesting that male orientation to the female-contaminated disks was not significantly different from the controls ($\chi^2 = 3.6$, $df = 2$, $0.05 < P < 0.20$). Evidently, female pheromone activity disappears at the time of mating. This observation is supported further by the fact that males failed to respond to mated females. A series of tests involving male-contaminated disks found no positive male responses when compared with control disks.

Blanchard (in Slosson, 1908) was the first to note and illustrate the "lobe-like pubescent tumidity" posteromedially on the second abdominal sternum of the male odd beetle. The structure is mentioned also by Hinton (1945) and Robert (1947) without further comment. This medial transverse swelling of the integument is more lightly colored than the surrounding sternite and bears

a 0.3-mm-wide, dense, brushlike tuft of setae that project posteroventrally (Figures 2 and 3). My unpublished observations of the courtship and mating behavior of *T. contractus* suggested the possibility that this structure might be involved in such activities, perhaps as a source of a chemical pheromone. During courtship the male mounts the female dorsally and orients his body in the same direction as hers. He then moves anteriorly until the setate prominence of his second abdominal sternum is above the head and uplifted antennae of the female. There he proceeds to rub the brush back and forth and occasionally sideways on the female, for ca. 1 min, sometimes moving the activity backward onto her thorax before attempting copulation.

Histological examination of the structure (Figure 4) disclosed a discrete closely packed mass of presumably secretory cells beneath the setate cuticle. The cells are vacuolate, dark-staining, and contain large round nuclei. The cuticle of the second sternite is thick and sclerotized except in the area immediately over the glandular tissue, where it is thinner and without color.

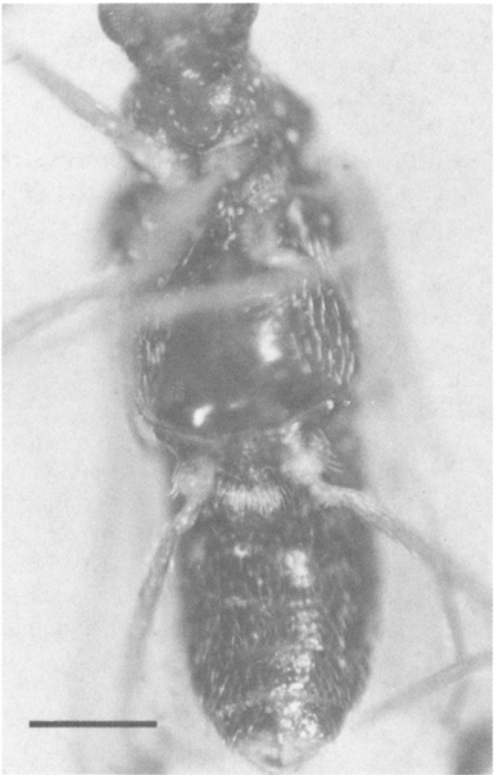

FIG. 2. Adult male *Thylodrias contractus* (ventral view) showing the setate glandular structure on the 2nd abdominal sternum (bar = 0.5 mm).

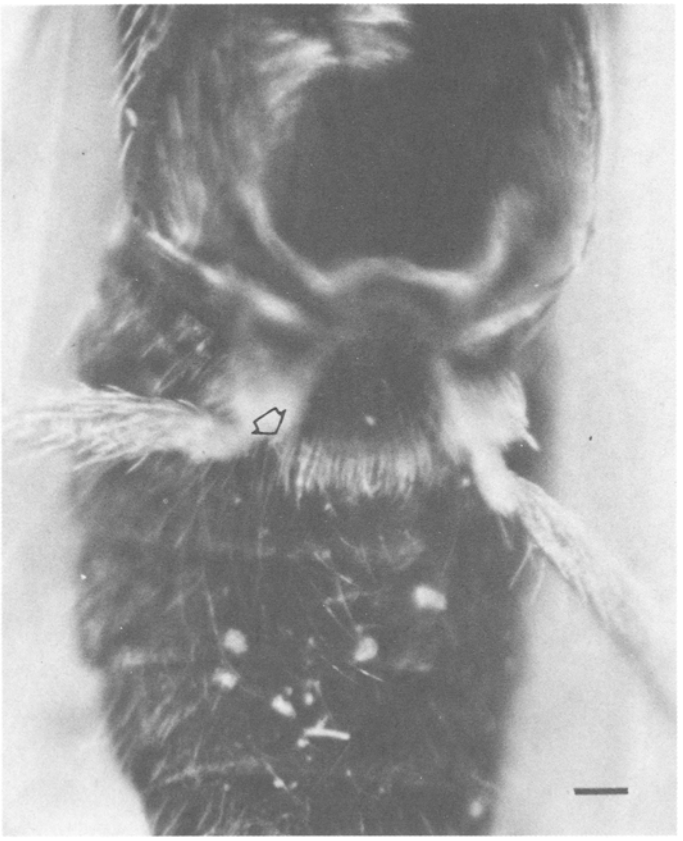

FIG. 3. Adult male *Thylodrias contractus* (ventral view) showing more closely the setate glandular structure on the 2nd abdominal sternum (bar = 0.1 mm).

Numerous setae ca. 0.06 mm long occur densely in this area and fully penetrate the cuticle basally. Delicate filamentous structures seem to penetrate further into the cell mass of the gland from the bases of the setae.

The results of these observations and experiments closely parallel those cited by Burkholder and Dicke (1966) and by Burkholder et al. (1974) as evidence of female sex pheromones in three other genera of dermestids. Sex-pheromone-releasing or calling behavior such as that reported for *Trogoderma*, *Attagenus*, *Anthrenus*, and *Dermestes* (Hammack et al., 1976; Cross et al., 1977; Barak and Burkholder, 1977; Burkholder et al., 1974; Abdel-Kader and Barak, 1979) was not observed in female *Thylodrias*. Behavioral and anatomical evidence does exist, however, for the reciprocal production of a pheromone by the male odd beetle. It is possible that this chemical may serve as a recognition signal or aphrodisiac to the female. Alternatively, deposition

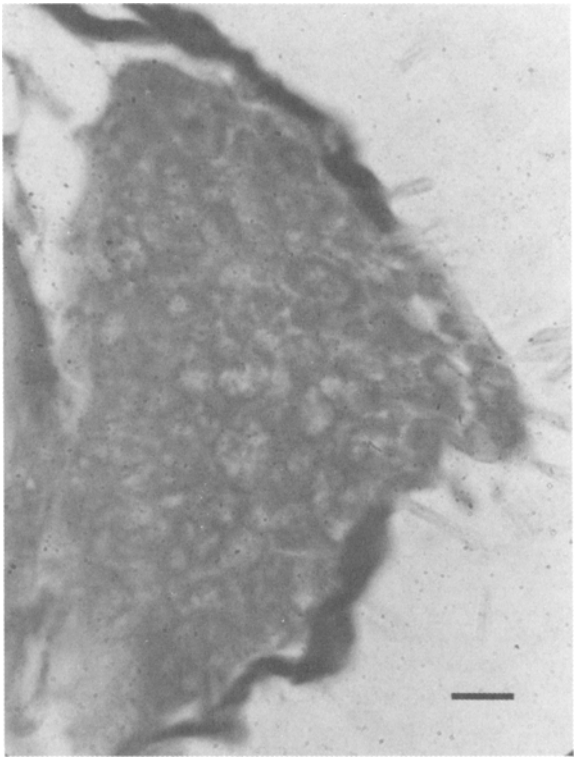

FIG. 4. Sagittal section (8 μm) through the ventral abdominal gland of a male *Thylodrias contractus* (bar = 10 μm).

of such a substance could serve to mark a female as already inseminated or to mask the female-produced sex pheromone, thus eliminating interference from superfluous males during her oviposition period. Sexually dimorphic setate structures that seem to be analogous have been described in members of several beetle families (Halstead, 1963), including Dermestidae (*Dermestes* spp.). Some of these have been associated with pheromone production (Levinson et al., 1978, 1980; Faustini, et al., 1981).

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SEX PHEROMONE OF *Pandemis heparana* (Den. and Schiff.) (Lepidoptera:Tortricidae)

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Abstract—90% (*Z*)-11-tetradecen-1-yl acetate, 5% (*Z*)-9-tetradecen-1-yl acetate, 5% (*Z*)-11-tetradecen-1-ol, dodecyl acetate (<1%), and tetradecyl acetate (<1%) were identified from gland extracts of *Pandemis heparana* females by gas chromatography and gas chromatography–mass spectrometry analysis (including mass fragmentography studies), chemical characterization, total synthesis, laboratory and field bioassays. In the field, a mixture of (*Z*)-11-tetradecen-1-yl acetate and (*Z*)-9-tetradecen-1-yl acetate (95:5) was found to be essential for attractiveness of *P. heparana* males.

Key Words—*Pandemis heparana*, sex pheromone, Lepidoptera, Tortricidae, gas chromatography, mass spectrometry, insect behavior, (*Z*)-11-tetradecen-1-yl acetate, (*Z*)-9-tetradecen-1-yl acetate, (*Z*)-11-tetradecen-1-ol, tetradecyl acetate, dodecyl acetate.

INTRODUCTION

Pandemis heparana (Den. and Schiff.) (Lepidoptera, Tortricidae, Tortricinae) is widely distributed in Europe and Asia where it is a most destructive pest of apple orchards. In France *P. heparana* has been observed in most commercial orchards.

Since the introduction of specific control methods against the codling moth (*Cydia pomonella L.*), *P. heparana* has become more difficult to control and has thus increased in economic importance. Previous work (Bassino et al., 1978) has shown that virgin-female-baited traps can attract males; hence the identification of the sex pheromone would facilitate the use of traps for monitoring purposes and would be the first step in the development of a possible control method.

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METHODS AND MATERIALS

Insects used in this study were collected as larvae from orchards in the Rhône Valley and reared on semisynthetic diet (Frerot, 1980) at 20–23°C (70–80% relative humidity) on a 16:8 light–dark cycle. The sexes were segregated as pupae and maintained separately thereafter.

The pheromone was collected by extraction (in hexane for 24 hr) of the carefully excised glandular region of the female abdominal tip. Only females 2–3 days postemergence were used, and the extraction procedure carried out when pheromone production was at a maximum, as predetermined by studies of calling behavior and gas chromatography analysis. The extract was decanted through prewashed glass wool and concentrated in an argon stream to 100 μl. Alternatively, the excised glandular area was sealed in aluminum capsules which were stored in liquid nitrogen until analyzed by gas chromatography (Descoins et al., 1979).

Gas chromatography was carried out on (1) an Intersmat IGC 120 (packed columns with nitrogen as carrier gas), (2) a Girdel 300 (glass capillary columns with helium as carrier gas), and (3) a Perkin-Elmer 3920 (packed columns with nitrogen as carrier gas). All instruments were fitted with flame ionization detectors; the Perkin-Elmer instrument was also fitted with an effluent splitter and the MS-41 solid sampler probe. The columns used were: (A) 2.3 mm × 2 m stainless-steel column, 5% SE30 on 100–120 mesh Gas Chrom Q; (B) 2.3 mm × 2 m stainless-steel column, 5% ECNSS-M on 100–120 mesh Gas Chrom Q; (C) 2.3 mm × 3 m stainless-steel column, 3% FFAP on 100–120 mesh Chromosorb GHP; (D) 2.3 mm × 5 m stainless-steel column, 10% SP 2340 on 60–80 mesh Chromosorb WAW; (E) 0.5 mm × 60 m WCOT Glass column, Carbowax 20M; (F) 0.5 mm × 50 m WCOT Glass column, stabilized DEGS.

Mass spectral and fragmentographic analyses were carried out on a gas chromatograph–mass spectrometer under computer control (Ribermag R-10-10) at 70 eV. Chemical ionization spectra were obtained using methane or ammonia as reagent gas.

The chemicals used in this study were synthesized in our laboratory by one of us (M.L.), using standard procedures, and purified by high-pressure liquid chromatography. The *Z* and *E* isomers were obtained pure. Microozonolyses were conducted in hexane at −70°C according to the procedure of Beroza and Bierl (1966, 1967).

Microepoxydations were performed at room temperature with excess-*m*-chloroperbenzoic acid in predistilled methylene chloride. After 1 hr the reaction mixtures were analyzed by gas chromatography or gas chromatography–mass spectrometry. Microacetylations were carried out at room temperature using 2–3 drops of acetyl chloride over 15 min. After evaporation to dryness, without heating, the residue was taken up in hexane for analysis.

The laboratory bioassay technique used was that developed by Sower et al. (1973) and modified by Lalanne-Cassou (1977), In this bioassay 60-cm × 3-cm ID glass tubes in which applicators are held at 50 cm from the males were used. The males' (6–10 per set) sexual behavior was analyzed during a 5-min exposure as follows: upwind buzzing flight (+ response), clasper extension and copulation attempts with other males or the applicator (++ response). Each test was replicated 5 times and compared to blanks.

Field tests were carried out between May and October 1979 in three orchards and a deciduous forest near Paris and Avignon. Delta traps (INRA) suspended at 1.5 m from the ground and 50 m from each other were used and rotated randomly at weekly intervals. The synthetic chemicals (1 mg) were dispensed from rubber septa which were changed every six weeks.

RESULTS AND DISCUSSION

GC Preliminary Results. The GC analyses of a 500-gland extract on columns A, B, C, D, and E (Figure 1) exhibited peaks whose retention times corresponded to those of 14-carbon monounsaturated alcohols and the corresponding acetates. Micropreparative chromatography (column C) yielded four fractions which were collected and bioassayed. On column E at 180°C the alcohol fraction yielded only one peak at 36.2 min, consistent with (*Z*)-11-tetradecen-1-ol (*Z*-11-TDol). The acetate fraction showed two peaks at 27.6 min (minor) and 29.1 min (major) in a ratio of 95:5. The major peak had the same retention time as (*Z*)-11-tetradecen-1-yl acetate (*Z*-11-TDA), while the minor component had a retention time similar to that of (*Z*)-9- and (*E*)-10-tetradecen-1-yl acetate. Using column F at 160°C, the retention time

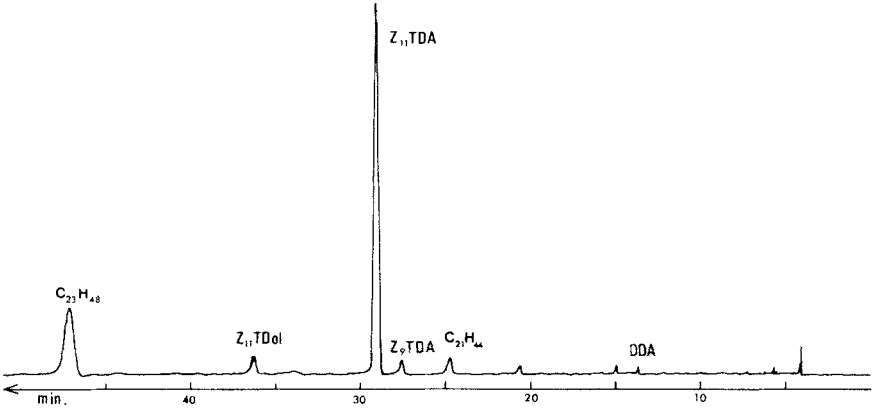

FIG. 1. GC analysis of a *P. heparana* extract (500 ♀) on a 60-m WCOT glass column, Carbowax 20 M, 180°C.

of the minor component (14.4 min) corresponded only to that of (*Z*)-9-tetradecen-1-yl acetate (*Z*-9-TDA).

The GC analysis of glands (microcapsule method) of 2- to 3-day-old females over a 24-hr period led us to ascertain that the maximum pheromone production appeared around the beginning of the dark period (±1 hr).

This was done by evaluating the amount of the major component of the pheromonal secretion [(*Z*)-11-tetradecen-1-yl acetate]. A secondary production peak appeared at the beginning of the light period, but this peak did not relate to any male or female sexual activity. The same phenomenon was noticed by Nagata (1972) with *Adoxophyes fasciata* (Walls).

The three components, *Z*-11-TDA, *Z*-9-TDA, and *Z*-11-TDol, were further characterized by GC-MS analysis and microderivatizations.

GC-MS Studies. The GC-MS analysis (EI and CI–ammonia or –methane) of the 500-gland extract confirmed the presence of 14-carbon monounsaturated alcohols and acetates. It also showed the following saturated hydrocarbons: $C_{21}H_{44}$ (M = 296), $C_{22}H_{46}$ (M = 310), and $C_{23}H_{48}$ (M = 324). The CI–ammonia analysis of the extract indicated the possible presence of dodecyl and tetradecyl acetates as minor components (<1%). Further evidence for the identification of these saturated acetates was given by EI fragmentographic studies (three diagnostic peaks: M-60, M-60-28, and *m/e* 116).

Some crude extract was epoxidized and the derivatives subjected to GC-MS on column C (190–220° C at 15° C/min after an initial hold of 24 min). The electron impact mass spectrum of the major acetate exhibited in the high mass region a unique mass fragment at *m/e* 241 consistent with (CH—CH—$(CH_2)_{10}$—$OAc)^+$, indicative of an 11,12 epoxide (Bierl-Leonhardt et al., 1980). Its retention time was the same as that of standard (*Z*)-11,12-epoxytetradecyl acetate, confirming the structure of the major component of the *P. heparana* pheromone as *Z*-11-TDA.

The insufficient amount of the epoxide of the minor acetate necessitated the use of mass fragmentographic analysis for its identification. When EI fragmentography was carried out on the epoxidized acetates using the following diagnostic peaks: *m/e* 241 (epoxide at 11, 12), *m/e* 227 (epoxide at 10,11), and *m/e* 213 (epoxide at 9,10) (see Figure 2), only two peaks were detected, one at R_t = 33 min for *m/e* 241 and the second at R_t = 32 min for *m/e* 213, indicating the presence of a 9,10 epoxide. Although a small response in the *m/e* 241 fragmentogram could be seen at R_t = 32 min, the relative intensities of the *m/e* 213 and 241 peaks were unequivocally in favor of the 9,10 epoxide derivative. Coincidence of the retention time of this minor component with that obtained using standard (*Z*)-9,10-epoxytetradecyl acetate finalized the evidence that *Z*-9-TDA is the minor unsaturated acetate component in *P. heparana*.

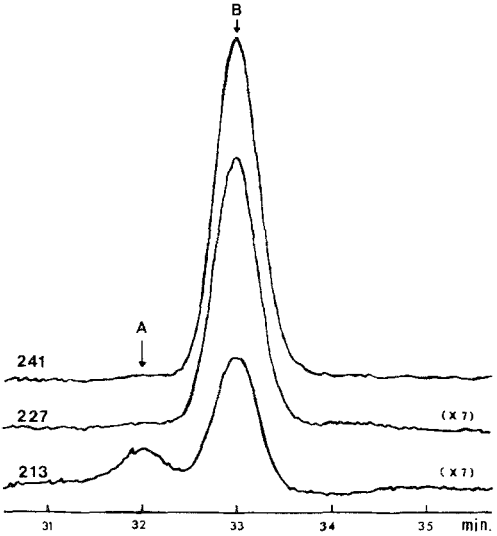

FIG. 2. Double bond localization in the minor acetate of *P. heparana* secretion. EI mass fragmentograms of the epoxidized crude extract: *m/e* 213, 227, and 241 are, respectively, the diagnostic peaks of 9,10. 10,11 and 11,12 epoxide derivatives. (A) epoxidized *Z*-9-TDA; (B) epoxidized *Z*-11-TDA. Chromatographic conditions: 3% FFAP on 100–120 mesh Chromosorb GHP, 3 m; 190°C (24 min) to 220°C (15°C; min).

Microozonolysis of the acetates (obtained by micropreparative GC of the gland extract on column C) gave two aldehyde-acetates whose CI-ammonia mass fragmentographic data and retention times were in accordance with the above conclusions.

Confirmation of the presence of an alcohol was obtained using a subtraction loop (Bierl et al., 1969) and by acetylation of the alcohol fraction. The product gave the same EI and CI-ammonia mass spectra consistent with a 14-carbon monounsaturated acetate. The location and geometry of the double bond were determined as described above for the acetates. Data indicated that the *P. heparana* alcohol is *Z*-11-TDol.

Laboratory Bioassays. The four fractions obtained from micropreparative GC on column C were bioassayed in comparison with a 10^{-2} FE extract. The acetate fraction when tested alone elicited a + response; the three other fractions when tested separately were inactive. The maximum response was observed when the acetate and alcohol fractions were mixed (++ response).

Further bioassays were conducted with synthetic compounds and indicated that *Z*-11-TDA (2.5 ng) was the only single compound which would elicit a male response. Since *E*-11-TDA is a potent inhibitor, the *Z*-TDA must be isomerically pure, a 99.5:0.5 mixture of the *Z*/*E* isomers elicits no male

TABLE 1. ATTRACTION OF *Pandemis heparana* MALES TO MIXTURES OF COMPOUNDS IDENTIFIED FROM *P. heparana* FEMALES (FIELD BIOASSAYS)

	Treatment	Total ♂ catches	Mean[a] ♂ catches per trap/week	
Site 1				
1000 μg	*Z*-11-TDA	10	0.7	a
950 μg	*Z*-11-TDA + 50 μg *Z*-9-TDA	304	23.4	b
850 μg	*Z*-11-TDA + 50 μg *Z*-9-TDA + 100 μg *Z*-11-TDol	342	26.3	b
Site 2				
1000 μg	*Z*-11-TDA	0	0	c
900 μg	*Z*-11-TDA + 100 μg *Z*-11-TDol	2	0.2	c
950 μg	*Z*-11-TDA + 50 μg *Z*-9-TDA	219	18.2	d
850 μg	*Z*-11-TDA + 50 μg *Z*-9-TDA + 100 μg *Z*-11-TDol	652	54.3	d

[a]Means followed by the same letter are not significantly different from each other according to Friedmann's test at the 5% level (the data for each site are analyzed separately).

response. *Z*-9-TDA and *Z*-11-TDol, when tested separately, had no effect but precopulatory excitation was at a maximum with a mixture of *Z*-11-TDA (2.5 ng)–*Z*-9-TDA–*Z*-11-TDol (90:5:5). This composition is identical to that found in the insect extract.

Field Bioassays. Field bioassays were carried out on four different sites. On all of them, mixtures of *Z*-11-TDA, *Z*-9-TDA, and *Z*-11-TDol (90:5:5) were very attractive for *P. heparana* males.

On two sites, near Paris, different formulations were bioassayed (see Table 1; site 1, an apple orchard; site 2, a deciduous forest). These experiments showed that catches of *P. heparana* males with *Z*-11-TDA alone or combined with *Z*-11-TDol were very low and nonspecific (catches of *Archips rosanus*, *Tortrix viridana* as the main species). But the two mixtures containing *Z*-9-TDA were found to be very attractive, indicating a determinant role of this compound. Nevertheless we have to improve the specificity of these synthetic mixtures, due to the attraction of a great number of *Cacaecimorpha pronubana* (Hubner) males, especially in southeast France, and also of *Clepsis spectrana* (Treischke) males. Furthermore, although the hind wings of *C. pronubana* are orange and those of *P. heparana* grey, it may be difficult to discriminate between the two species on the sticky traps.

Z-11-TDA is the main component for *C. pronubana* (*Z*-11-TDol and *E*-11-TDA are minor components), but *Z*-9-TDA (not found in the secretion) does not produce any inhibitory effect (Lalanne-Cassou, 1982).

In the case of *Clepsis spectrana*, a mixture of Z-11-TDA and Z-9-TDA (75:25) (Minks et al., 1973) is used as the sex pheromone.

Since we did not observe any significant difference in the catches between the mixtures of Z-11/Z-9-TDA and Z-11/Z-9-TDA + Z-11-TDol, we intend to obtain more information about the role of Z-11-TDol in further field experiments. A possible role of Z-9-TDol previously suggested (Voerman, 1979) but not detected in our extracts will also be investigated.

We can conclude that *P. heparana* females utilize a mixture of Z-11-TDA Z-9-TDA, Z-11-TDol (95:5:5) and minor (<1%) saturated acetates (DDA, TDA) in their sex pheromone secretion. Bioassay results indicate that a mixture of Z-11 and Z-9-TDA is essential for eliciting sex attraction responses from *P. heparana* males. Similar Z-11 and Z-9-TDA blends have been reported for two North American *Pandemis* species: *P. pyrusana* (Rob.) (Z-11/Z-9-TDA, 94:6) and *P. limitata* (Rob.) (Z-11/Z-9-TDA, 91:9) (Roelofs et al., 1976, 1977).

In addition, our finding concerning *P. heparana* secretion can be of immediate use in surveys or in mating disruption techniques.

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Oscillatoria Sp. (CYANOPHYTA) MAT METABOLITES IMPLICATED IN HABITAT SELECTION IN *Bembidion obtusidens* (COLEOPTERA: CARABIDAE)[1]

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Abstract—In laboratory bioassays *Bembidion obtusidens* Fall adults were attracted to volatile metabolites (methyl esters of hexadecanoic and 9-octadecenoic acid) of mat-forming, filamentous blue-green algae (*Oscillatoria* sp.; Cyanophyta) growing in the microhabitat of these beetles on the shores of saline lakes. Commercial preparations of these metabolites also were attractive, suggesting that *Oscillatoria* metabolites are token stimuli which serve as habitat cues for *Bembidion* adults.

Key Words—*Bembidion obtusidens*, Coleoptera, Carabidae, *Oscillatoria animalis*, *Oscillatoria subbrevis*, Cyanophyta, metabolites, volatiles, ecophenes, methylhexadecanoate, methyloctadecenoate, attractant, habitat selection.

INTRODUCTION

Animals select and remain with their habitats by responding to environmental stimuli that serve as habitat cues. The sensory modalities demonstrated in insects are adaptations for perceiving a broad spectrum of stimuli providing them with information about local environmental conditions. Studies of host (=habitat) selection in phytophagous insects have concentrated on chemosensory processes but, because beetles of the family Carabidae are either predatory or not directly associated with specific plants and because of the correlation between their distributions and microclimatic factors (Thiele, 1977), work on this group has focused on responses to physical stimuli. Thus, with an estimated 40,000 species of carabids (Thiele, 1977), making it one of

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the largest insect families, remarkably little ecological or behavioral work using current principles of chemical ecology has been done on this group of insects. My observations of microhabitat specificity in intertidal (Evans, 1980) and saline lakeshore carabids, coupled with the overwhelming evidence of the chemosensory capabilities of insects in general, has led to the hypothesis that chemical stimuli are implicated in habitat selection by carabids.

Many species of the carabid genus *Bembidion* inhabit areas adjacent to water, with some, such as *B. obtusidens* Fall, *B. scudderi* LeConte, *B. henshawi* Hayward, and *B. consimile* Hayward, restricted to saline lake beach habitats in prairie areas of Canada and the drier regions of the United States (Lindroth, 1963). Adults disperse by flight, since most species have fully developed hind wings (Lindroth, 1963), and habitats are chosen from a diverse array of littoral sites varying in physical, chemical, and biological characteristics of both soil and water.

METHODS AND MATERIALS

B. obtusidens adults were collected at Wells Lake, near Marsden, west central Saskatchewan, either in their overwintering sites in beach grass tussocks or as they ran on the dry, salt-encrusted surface of the beach. The chemistry and biota of Wells Lake is similar to that of Manito Lake (they are less than 0.2 km apart), which was reported in extensive studies of Saskatchewan saline lakes by Rawson and Moore (1944), Hammer (1978), and Haynes and Hammer (1978). Manito Lake is a sodium sulfate type lake with a salinity of 23.88 per thousand (Hammer, 1978), hence beach soil would be expected to have high concentrations of this salt. In the upper third of the beach on the north side of Wells Lake numerous shallow depressions contain polygonal shrinkage cracks in the soil from which *Bembidion* adults emerge when disturbed. The polygons, ranging in size from 4 to 7 cm in diameter, consist of a 0.5- to 1.5-cm-thick matrix of soil particles and dense growths of *Oscillatoria animalis* Agardh [=*Porphyrosiphon animalis* (Agardh) Drouet, 1968 comb. nov.] *and O. subbrevis* Schmidle [=*Microcoleus lyngbyaceus* (Keutzing) Crouan.] forming a distinct mat. When dry these mats are easily collected and, after storage in closed containers for several hours, emit a strong odor. Diatoms, brine flies, springtails, mites, and other microorganisms as well as *B. obtusidens* larvae and adults live in this microhabitat. To collect volatiles for analysis, dry samples of *Oscillatoria* mat were loosely placed in each of 4 Plexiglas cylinders, 31 cm long × 5 cm diameter. The end of each cylinder was connected to the end of another and laboratory air was aspirated through this system at 1100 ml/min after first going through a 400-cc gas purifier (Cole-Parmer Instrument Co.) containing type 5A, 4–8 mesh molecular sieves adsorbent. After flowing through the four cylinders the

air and volatiles from the mats flowed through a 85 cm × 8-mm-diameter glass tubing containing 18 g of the above adsorbent. After an 18-hr extraction the molecular sieves with the adsorbed volatiles were placed in 10 ml analytical grade carbon tetrachloride (0.0002% residue after evaporation) for 30 min, then filtered twice through a 0.2-μm pore membrane filter (Nucleopore). For chemical analysis, the carbon tetrachloride extracts were concentrated to about 0.1 ml by evaporating the solvent in a nitrogen gas stream. Infrared spectra were obtained by applying the concentrated extract in 5-μl aliquots to the center of a 25-mm-diameter KBr disk, allowing them to evaporate, and analyzing the residue with a Nicolet model 7199 FTIR spectrophotometer. A Varian 1400 gas chromatograph and an Associated Electric Industries MS 12 mass spectrometer was used on other concentrates to obtain mass spectra of major peaks. Final identification of the volatiles was made with a Varian 3700 gas chromatograph and a 10-m nonpolar liquid phase, 0.25 mm inside diameter, glass capillary column (Quadrex OV101, Milton Row Co. Applied Science Div.) using a nitrogen carrier (1.5 ml/min) and 2.5-μl samples of the concentrate for injection into the column port.

Bioassays were conducted with the use of choice chambers (Figure 1). During tests beetles were placed on the screen surface and constrained there by the glass cylinder. The screen prevented tarsal or antennal contact with the filter paper. Carbon tetrachloride was used as a control while unconcentrated carbon tetrachloride extracts of the volatiles or carbon tetrachloride solutions of identified components of the volatiles constituted the treatments. These materials were evenly applied with a 1-cc glass syringe to 5.5-cm-diameter Whatman No. 2 filter paper halves suspended on four evenly spaced 0.9-mm-diameter glass capillary rods resting on the edges of a circular dish so as to allow the solvent to evaporate without losing much of the solute on the small supporting surface area of the rods. For each test 10 or less adult *B. obtusidens* were placed in each of three choice chambers kept in separate darkened containers; 5 counts were taken at 2-min intervals with the insects activated by fluorescent light and by shaking after each count to redistribute them. The data were analyzed using a goodness-of-fit test for observed ratios (G test; Sokal and Rohlf, 1969).

RESULTS

Identification of Volatiles. The IR spectrum of the *Oscillatoria* mat volatiles showed a mixture of long-chain fatty-acid esters, and this was subsequently confirmed when the GC mass spectra indicated molecular weights of 270 and 296 for two major constituents in the volatiles. Capillary column gas chromatography finally showed the volatiles to be a 2:1 mixture of methyl esters of hexadecanoic (palmitic) acid and 9-octadecenoic (oleic)

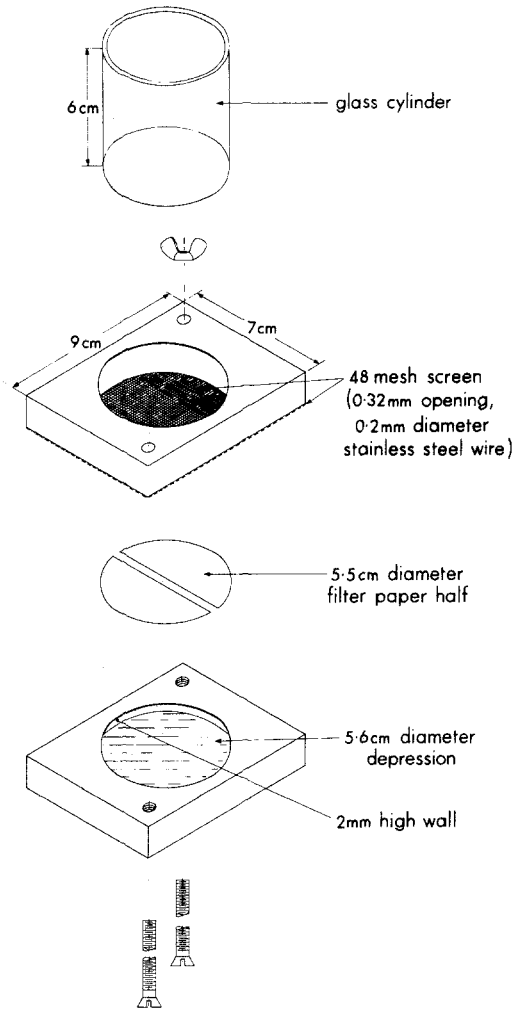

FIG. 1. Plexiglas choice chamber for testing olfactory responses of *Bembidion obtusidens* adults.

acid, respectively, with retention times of 17.51 min and 19.48 min. (Figure 2). Standards of these esters showed similar retention times. Some minor constituents were also present but these were not investigated.

The ratios of the concentrations of the two esters cannot be expected to remain static because of the inherent instability of the methyloctadecenoate double bond. For example, a ratio of 1:3.5 of the hexadecanoate to octadeceneoate was obtained from *Oscillatoria* mats kept in airtight con-

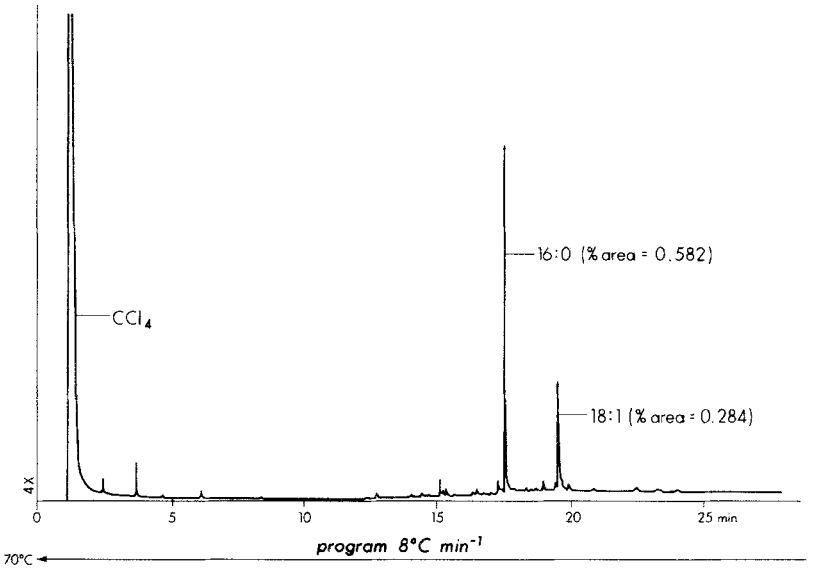

FIG. 2. Gas chromatogram of volatile metabolites of *Oscillatoria* sp. mat showing methyl esters of hexadecanoic acid (16:0) and 9-octadecenoic acid (18:1).

tainers in the dark at 10°C for several months. Reducing oxidation increased the concentration of the methyloctadecenoate, although the conditions could also have changed the growth form (and metabolic products) of the algae (see below). The concentration of esters in the volatiles result from initial concentrations in the algal matrix, their respective vapor pressures, and the extent of oxidation during laboratory extraction and handling. The ratios obtained probably differ from those in the field, so a specific ratio is not required to obtain a response from *B. obtusidens*, although an optimum one presumably exists.

Bioassays. Heterogeneity was high in the choice tests, including the controls, because these beetles are somewhat gregarious, but this factor does not prevent testing the null hypothesis of a 1:1 ratio in the distribution of the insects on the treated and control filter paper halves.

Both natural volatiles and a similar mixture of methylhexadecanoate and methyloctadecenoate were attractive to adult *B. obtusidens* (Table 1) while individual esters were not. However, the natural volatiles gave greater departures from the expected 1:1 ratios than those of the commercial preparations either because optimum concentrations of the latter were not tested or, more likely, because the minor constituents contributing to the bouquet of the natural volatiles were not present.

TABLE 1. OLFACTORY RESPONSES OF *Bembidion obtusidens* IN CHOICE TESTS TO VARIOUS TREATMENTS OF HALF FILTER PAPER DISKS

Tests (0.021 ml solution/cm^2)	N	Numbers/ side	Ratio	$G_{heterogeneity}$*	G_{pooled}*
CCl_4 extract of volatiles of *Oscillatoria* mat		1245			
vs.	13		1.80:1	25.93, $P < 0.05$	161.94, $P < 0.005$
CCl_4		696			
0.0049 M methyl palmitate and 0.0018 M methyl oleate in CCl_4		1520			
vs.	16		1.62:1	41.25, $P < 0.005$	140.78, $P < 0.005$
CCl_4		935			
0.0049 M methyl palmitate in CCl_4		287			
vs.	4		1:1.03	14.76, $P < 0.005$	0.146, NS
CCl_4		278			
0.0018 M methyl oleate in CCl_4		221			
vs.	4		1:1.14	4.30, NS	2.29, NS
CCl_4		254			
CCl_4		396			
vs.	8		1:1.02	24.78, $P < 0.005$	0.084, NS
CCl_4		404			

*Goodness of fit test for observed ratios

DISCUSSION

Since an algal mat consists of a matrix of sediment and algal filaments, it is assumed that the methyl esters originated in the *Oscillatoria* as extracellular metabolites. Such compounds are commonly produced by algae, both procaryote and eucaryote (Hellebust, 1974); volatile esters, acids, aldehydes, ketones, and alcohols are common, but esters of long-chain fatty acids have not been previously reported. However, various strains (ecophenes?) of *Oscillatoria* had an average ratio of C16:0 to C18:1 fatty acids of 2:1 (Kenyon et al., 1974), although pathways from fatty acids to the corresponding methyl esters, if they exist, have not been considered in this study.

The attraction of *B. obtusidens* adults to volatile metabolites of *Oscillatoria* mats in the laboratory suggests that these compounds are used by the beetles as olfactory cues in habitat selection. Preliminary tests with other carabids from saline habitats (*Bembidion scudderi*) and from leaf litter (*Agonum sp.*) also showed that adults of these species were attracted to volatiles extracted from algae or from decaying leaves so chemosensory responses to algal, fungal or bacterial metabolites may be a fundamental habitat selection mechanism in the Carabidae. Thus a new dimension, involving reliance on microorganisms, has been added to physical (temperature, humidity, light intensity, soil texture and moisture, etc.) habitat selection processes in these insects. In littoral areas filamentous blue-green algae are particularly well adapted for living in soil, in water, and on rock and plant surfaces and for surviving alternating periods of flooding and desiccation and wide ranges of salinity (Brock, 1973). Littoral carabids may, therefore, have an obligatory relationship with these organisms which show great diversity in ecological growth forms (ecophenes). The collection records for *O. subbrevis*, for example, indicate an incredible range of habitats. According to Drouet (1968), this species "is found in all regions of the earth in marine, brackish, and fresh waters, and in habitats influenced by these waters." Mat-forming individuals, such as those found at Wells Lake, must certainly differ biochemically as well as morphologically from planktonic individuals that form massive blooms in freshwater lakes. Because of this extreme ecophenotypy, Drouet (1968) has reduced more than 2400 subspecies and species of the Oscillatoriaceae to 25. Rippka et al. (1979) have gone further by ignoring specific epithets of blue-green algae (referred to by them as Cyanobacteria); instead they consider generic distinctions only, based on biochemical and morphological characters. Prevailing environmental conditions of a site can be recognized if biochemical and morphological characters of algal ecophenes are known through laboratory analysis. *Bembidion* adults and probably other carabids find their habitats by recognizing the odor of the resident ecophenes. This finding has significant application to studies of habitat partitioning, species distributions, and behavior of members of the

Carabidae as well as to physiological and biochemical studies of cyanophytes, especially in relation to the characterization of ecophenes. Research on manipulation of insect predators for biological control of insect pests (Tassan et al., 1979) may also benefit from this study.

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AGGREGATION PHEROMONE OF THE MALE GRANARY WEEVIL, *Sitophilus granarius* (L.)[1-3]

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Abstract—Evidence for a male-produced aggregation pheromone in *Sitophilus granarius* is reported. Hexane extracts of Tenax®-trapped volatiles from males held on wheat were attractive to both sexes in a multiple-choice olfactometer and pitfall bioassay chamber. A quantitative relationship existed between days of insect exposure on disks and degree of responsiveness. The maximum response was to 35 insect-day-equivalents. Diel-related activity showed both sexes responsive during photophase and nonresponsive during scotophase.

Key Words—*Sitophilus granarius, Coleoptera, Curculionidae, aggregation pheromone, granary weevil.*

INTRODUCTION

The cosmopolitan granary weevil, *Sitophilus granarius* (L.), has become a major pest of stored products. Both adults and larvae feed on a variety of grains such as wheat, rice, and maize.

Control of the granary weevil is largely through the application of contact insecticides and fumigants which has resulted in the development of insecticide-resistant strains (Monro et al., 1972; Lloyd, 1973). Recent concern for environmental safety has caused a shift in pest control toward the utilization of naturally occurring biological attractants such as pheromones.

[1]Coleoptera: Curculionidae.
[2]Contribution to Regional Research Project NC151, "Marketing and Delivery of Quality Cereals and Oilseeds in Domestic and Foreign Markets."
[3]Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.
[4]Present address: Philip Morris Research Center, P.O. Box 26583, Richmond, Virginia 23261.

This offers distinct advantages in efficiency and safety as compared to strategies based solely on pesticides.

Little is known about granary weevil chemical communication. Surtees (1964) showed a lack of intraspecific attraction in *S. granarius*. Donat (1970) demonstrated that frass and whole-body extracts, both undifferentiated as to sex, were attractive to both sexes. Recently, Phillips and Burkholder (1981) found evidence of a male-produced aggregation pheromone in the rice weevil, *Sitophilus oryzae* (L.). This study similarly presents evidence for a granary weevil male-produced aggregation pheromone attractive to both sexes.

METHODS AND MATERIALS

Tests Insects. Weevils were of a laboratory strain originating from field-collected specimens from Wisconsin. Cultures were started in 1-liter glass canning jars on 500 ml of "ERA" spring wheat with 13% moisture content. Adult weevils ($N = 200$) were allowed to feed, mate, and oviposit on the grain substrate for 1 week. Adults were then sifted from the grain, and the progeny allowed to emerge. Newly emerged weevils were sexed by rostrum characteristics (Halstead, 1963) and the sexes isolated in separate culture containers. Weevil cultures and virgin insects were maintained at $27 \pm 2°C$ and $60 \pm 10\%$ relative humidity with a 16:8 light–dark photoperiod regime. Grain-infesting weevils live in a darkened or subdued light environment, thus all bioassays were conducted in a darkened room regardless of light phase.

Bioassay. In initial experiments a 5-choice olfactometer (Burkholder, 1970) was used. Briefly, head-space vapor attractant choices were offered to the test insects in flasks at one or more of 5 alternatives equidistant from the point of insect release. Bioassays were performed between 1300 and 1900 hr in a room maintained at standard rearing room conditions. Gelman® flowmeters controlled air flow for each choice at 0.25 liters/min. Twenty-five 4- to 8-day old virgin males or females were released onto the chamber floor, and their distribution recorded after 6 hr, a period that was shown to allow optimal insect acclimation and dispersal in preliminary tests. Only those insects in the collecting flasks were counted. Four replicates were completed for each test and analyzed by the *t*-test for dependent paired data. Statistical significance was accepted at $P < 0.01$.

The dual-choice pitfall bioassay chamber (Phillips and Burkholder, 1981) consists of a 4×8-cm glass crystallizing dish resting on a 9.5-cm glass base. Two 1-cm-diam holes were drilled in the bottom of glass base 2 cm from its center and 4 cm apart. The entire chamber rests on two open 5-dram vials directly below the holes in the base. Ten 4-day-old weevils of known sex were introduced to the system through a small hole in the top of the crystallizing dish. A 30-min period of dark conditioning followed. While illuminated for a

brief period under a safelamp (Wratten series OC light amber filter), the chambers were lifted off the paired vials.

The concentration of volatiles collected during disk exposure was expressed quantitatively in terms of insect day equivalents (IDE) where 1 IDE = the volatiles from one weevil absorbed on a single disk for 1 day (Phillips and Burkholder, 1981). A 1.27-cm-diam antibacterial assay disk (Schleicher J. Schuell, Inc., Keene, New Hampshire) treated with 5 μl (14 IDE) of test material in hexane and a control disk treated with 5 μl (14 DE) of wheat kernel food extract in hexane were placed in opposite vials. The chambers were then set back on the vials, covered with a light-proof box, and the room was darkened. After 30 min, the number of insects in each vial was recorded. Each test was repeated 10 times with new weevils. Attraction was expressed as a percentage (P) as shown by Suzuki and Sugawara (1979):

$$P = \frac{(T - C)}{N} \times 100$$

where T is the number of weevils in the treated vial, C is the number in the control vial, and N is the number of test weevils released per replicate into the pitfall chamber.

Photoperiod Response. The dual-choice pitfall bioassay system was used for the photoperiod experiment. As the weevils are extremely photophobic, all bioassays were conducted in a room darkened for 30 min, regardless of light phase. Bioassay replicates were carried out on successive days. The percentage response of 4-day-old males and females to male odors (20 IDE) was recorded throughout a 24-hr period. Each sex was exposed to male odors for a 30-min period every 4 hr from 0400 to 2400 hr.

Pheromone Dose–Response. Virgin weevils were sexed as they emerged and placed singly in culture containers. To collect weevil volatiles, males held singly were isolated in 2.0 × 8.5-cm shell vials, each of which contained an assay disk (Burkholder and Dicke, 1966) and a single cracked wheat kernel. Control vials contained only wheat kernels and assay disks. Each vial was capped, placed in a rack, and maintained at the standard rearing conditions. Every 2 days the vials were aerated briefly for 15 sec. For bioassays, weevils and kernels were removed from the vials and discarded. Assay disks within the vials were saved and set aside for dose–response bioassay studies using the dual-choice pitfall system and virgin weevils.

Pheromone Isolation. The aeration chamber used for the collection of insect volatiles was modified slightly from that of Cross et al. (1976). The system consisted of a glass chamber with a brass screen inserted to support approximately 500 ml of wheat and 250 male *S. granarius* adults. Air was drawn over the insects by vacuum applied downstream from a Tenax® (2,6-diphenyl-*p*-phenylene oxide, Applied Science Lab, Pennsylvania) holder. The

air was filtered through charcoal upstream from the aeration chamber. Aeration was at 0.5 liters/min for 7 days with the chamber kept at 27 ± 2°C on a 16:8 light–dark photoperiod. After aeration, the Tenax was extracted in three successive 50-ml rinses of redistilled hexane. Rotary evaporation of the solvent left approximately 0.1 ml of a yellow, oily residue.

The control was an identical aeration chamber containing only wheat and extracted in the same manner as the treatment. Pitfall bioassays were conducted between 1300 and 1600 hr under standard rearing conditions.

RESULTS AND DISCUSSION

The responses of both males and females in each type of olfactometer indicate that *S. granarius* males produce an aggregation pheromone. Male and female responses to head-space vapors in the multiple-choice olfactometer (Figure 1) show that virgin females have a greater response to male culture head-space vapors (53%) than did virgin males (35%). Few males or females responded to the female or grain head-space vapors (control). Both sexes respond to male odors over a distance.

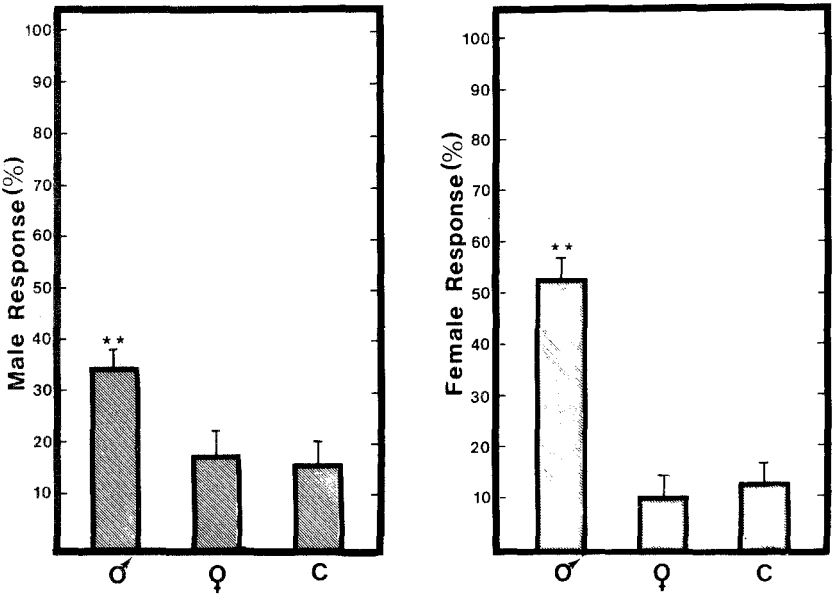

FIG. 1. Response of virgin 4 to 8-day-old *Sitophilus granarius* males and females to head-space vapors in a multiple-choice olfactometer. Percentage calculated from the response of 25 males or females after 6 hr; average of four replicates. ($P < 0.01$**; vertical lines denote $S_{\bar{x}}$).

TABLE 1. RESPONSE OF *Sitophilus granarius* MALES AND FEMALES TO TENAX COLUMN ELUATES FROM EACH SEX WHEN TESTED IN PITFALL BIOASSAY[a]

Tenax eluate from (experimental stimulus)[b]	Sex tested[c]	% Response (± SE) Experimental stimulus	% Response (± SE) Control stimulus	Attractiveness[d] (%)
Male + wheat	male	65 ± 1.6	11 ± 1.3	+54.0 **
	female	73 ± 1.1	13 ± 1.0	+60.0 **
Female + wheat	male	15 ± 1.6	17 ± 1.4	- 1.7 NS
	female	23 ± 2.5	38 ± 2.8	-15.0 NS
Male	male	12 ± 1.3	11 ± 1.1	+ 1.0 NS
	female	13 ± 1.2	12 ± 1.3	+ 1.0 NS
Female	male	10 ± 1.1	12 ± 1.4	- 2.0 NS
	female	11 ± 2.4	12 ± 1.0	- 1.0 NS
Food	male	38 ± 1.4	28 ± 1.0	+10.0 NS
	female	42 ± 1.0	28 ± 1.3	+14.0 NS

[a]From Phillips and Burkholder (1981).
[b]14 IDE/disk (IDE: insect day equivalent) or 14 day equivalents for wheat volatiles.
[c]Ten unmated weevils (4 days old) were used per replicate, ten replicates/treatment.
[d]Significantly greater response to experimental stimulus than to control indicated by: $^{**}P < 0.01$, NS = not significant.

The dual-choice pitfall bioassay confirmed that males and females responded to extracts of male-aerated Tenax (Table 1). The female response to 14 male IDE was slightly greater than that of males (60 ± SE 1.1% vs. 54 ± SE 1.6% attractiveness, respectively). Neither sex responded to the Tenax-trapped volatiles from females or grain. Furthermore, few males or females responded to male volatile extracts collected in the absence of food (1 ± SE 1.3% vs. 1 ± SE 1.2%, respectively). In view of the fact that approximately 66–70% of all possible responding weevils were attracted to dual wheat controls, the possibility of wheat volatiles synergistic with male-produced pheromone cannot be discounted. Evidence for a male-produced aggregation pheromone in *S. granarius* gives rise to the question of whether similarities in chemical communication exist with other weevil species. The boll weevil, *Anthonomus grandis* Boheman, produces two attractants: the 4-component male sex pheromone grandlure (Tumlinson et al., 1971); and a weak female sex pheromone composed of the two terpenoid alcohol components of grandlure plus two cotton bud hydrocarbons (Hedin et al., 1979). Unlike grandlure, the male granary weevil aggregation pheromone attracts males equally as well as females in these experiments. However, grandlure in combination with certain cotton bud hydrocarbons attracts

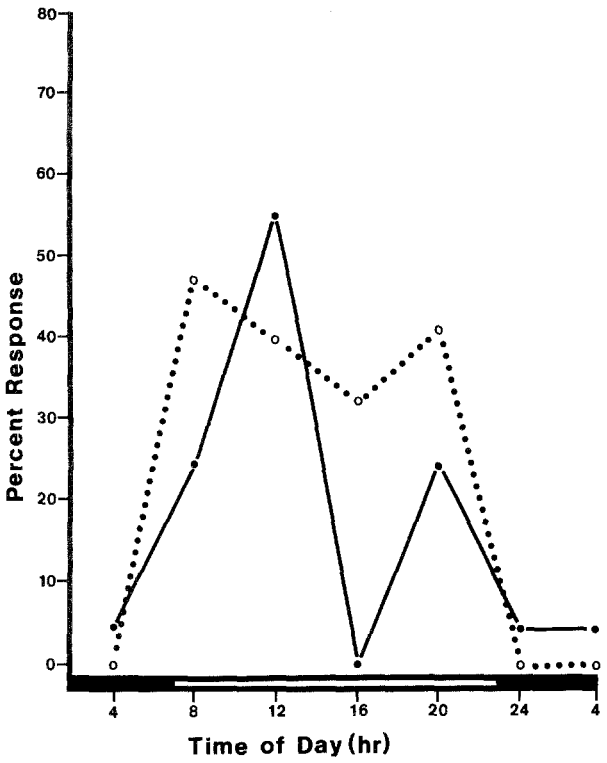

FIG. 2. Effect of time of day on *Sitophilus granarius* attraction expressed as % male response (•—•) and female response (o· · ·o) to male extracts. Dark bars represent scotophase.

males and females in equal numbers in the laboratory (Hedin et al., 1979). Phillips and Burkholder (1981) found evidence for a male-produced aggregation pheromone that showed nearly equal levels of attractance to both sexes in *S. oryzae* in the presence of wheat.

The increased response to male extracts exhibited by both sexes during early photophase is of particular interest (Figure 2). For males, response was largely restricted to a 4-hr interval centered toward early photophase (0800–1200 hr), whereas female response fluctuated at elevated levels throughout photophase (0800–2000 hr). There is a bimodal response exhibited by males that may relate to peak response at midday and early evening under natural conditions. No evidence for attraction of either sex to male odor was observed during scotophase. Furthermore, neither sex was attracted to female extracts during any of the photophase intervals examined.

Our results clearly indicate that time of day influences responsiveness of the sexes to the male-produced pheromone in *S. granarius*. This is particularly

evident in the lack of response during scotophase. Photoperiodic rhythms are common in pheromone systems (Shorey, 1974). Hammack and Burkholder (1976, 1981) found a relationship between calling behavior and sex pheromone release in females of *Trogoderma glabrum* (Herbst) and *T. granarium* Everts.

Four 4-day-old weevils of both sexes responded to disks exposed to male odors from 1 to 42 days (Figure 3). The minimum detectable level per disk needed to elicit aggregation behavior was 1 IDE. However, the response was greater by the females (56%) than by males (35%). The highest response recorded for both sexes was at 35 IDE (88 ± SE 1.2% and 77 ± SE 1.3%, females and males, respectively), indicating that volatiles collected over a longer period of time are cumulative and, thus, more attractive.

The male-produced attractant appears to serve as a population

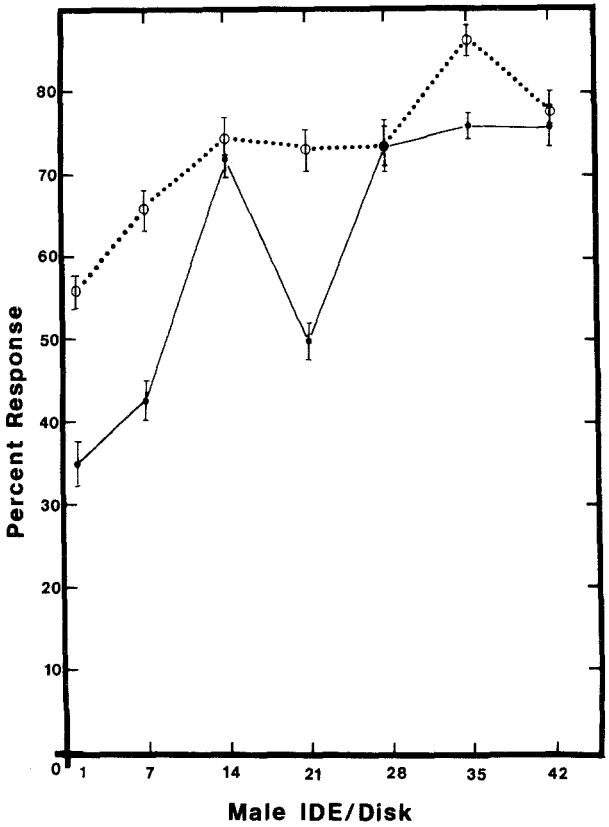

FIG. 3. Response of *Sitophilus granarius* 4-day-old males (•—•) and females (o· · ·o) to varying male pheromone dose (IDE). Percentage calculated from the response of 10 males or females after 30 min; average of 10 replicates; vertical lines denote $S_{\bar{x}}$).

aggregation pheromone that brings the sexes together in the presence of food. There is a selective advantage of a male to signal a female for mating, feeding, and oviposition sites. There is also a selective advantage for other males to aggregate for food and mates. The synthesis and use of the pheromone as a tool to monitor or control populations in stored grain ecosystems would certainly prove advantageous.

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ISOLATION, IDENTIFICATION, SYNTHESIS, AND BIOASSAY OF THE PHEROMONE OF THE COMSTOCK MEALYBUG AND SOME ANALOGS

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Abstract—The sex pheromone of the Comstock mealybug, *Pseudococcus comstocki* (Kuwana) was isolated from volatiles trapped from air passing over virgin female insects. Combined gas chromatography, mass and infrared spectroscopy, and microreactions indicated that the structure was 2,6-dimethyl-1,5-heptadien-3-ol acetate. This was confirmed by synthesis; several analogs were also prepared. The natural and synthetic pheromone caused similar trap capture of male insects. Synthetic analogs were significantly less attractive in field tests and the addition of minor amounts of the corresponding alcohol to the pheromone appeared to enhance trap capture, but the results were not statistically conclusive.

Key Words—Sex pheromone, Comstock mealybug, *Pseudococcus comstocki*, 2,6-dimethyl-1,5-heptadien-3-ol acetate, mass spectrometry, trap capture, synthetic analogs, Homoptera, Pseudococcidae.

INTRODUCTION

The Comstock mealybug, *Pseudococcus comstocki* (Kuwana) is a small homopteran insect that is a worldwide pest principally of catalpa, stone fruits, mulberry, and pome fruits. The most serious losses have been reported among the pome fruits (Bartlett and Clancy, 1972). Since the 1920s the insect has been sporadically reported as a serious pest on apples, pears, peaches, and some ornamentals in the eastern United States (Hough, 1925; Cox, 1940; Cutright,

1951) and in the Niagara Peninsula of Ontario, Canada (Phillips, 1961). In 1967, the insect was introduced into California where it has become a pest of 65 different host plants, including citrus fruits, pomegranate, and quince. Economic losses result from the severe smutting that occurs generally on all host plants. Copious quantities of honeydew excreted by all life stages of the mealybug favor development of a sooty mold fungus, *Papnodium citri* Beyk (Pratt, 1958). The resultant fungal growth or smut is very difficult to remove from the fruit by the conventional washing and waxing process used by the packers, and the consequent cosmetic damage entails considerable economic loss. Also, the black coating produced by the fungus prevents light from reaching the surface of the leaves, thus interfering with their function. It may also retard growth and cause light blooming and fruiting. Detection, survey, and monitoring of the pest's populations are therefore vital to the economic survival of growers and to the prevention of further spread of this insect.

The female insect of this species emits a pheromone which attracts the male for the purpose of mating; virgin females in traps have been used to detect the presence of male insects in cities, nurseries, and orchards since 1974 (Anonymous, 1974; Meyerdirk and Newell, 1979). However, the possibility that females may escape from a trap and spread an infestation has restricted the use of such monitor traps in certain areas. The identification of the pheromone and its synthesis were therefore urgently needed to provide an effective tool for the detection of infestations and also to monitor control efforts. We earlier reported (Bierl-Leonhardt et al., 1980) the identity and synthesis of this pheromone as 2,6-dimethyl-1,5-heptadien-3-ol acetate; this structure was confirmed independently by a predominantly synthetic approach (Negishi et al., 1980). Our paper details the microanalytical procedures used for identification and reports the biological activity of synthetic analogs and mixtures of the pheromone with its corresponding alcohol. This is the first pheromone to be identified for the family. Pseudococcidae (order Homoptera).

METHODS AND MATERIALS

Pheromone Collection and Bioassay. The pheromone was absorbed from a stream of air that had passed over virgin female insects. Initially, insects were sexed by hand sorting in the late third instar or third molt stage and the females were then reared on sprouted potatoes, *Solanum tuberosum* L., to an age of ca. 30 days. Later for mass rearing, colonies of mealybugs in the first molt stage were dipped in or sprayed with an emulsion containing 0.01 ppm of the juvenile hormone analog epofenonane, 2-ethyl-3-[3-ethyl-5-(4-ethylphenoxy)pentyl]-2-methyloxirane. This treatment allowed the female insects to mature but inhibited the development of the males (Moreno, unpublished observations), and thus large numbers of virgin females could be

reared for use in pheromone collections. Negishi et al. (1980) reported the use of double-folded tissue paper to separate virgin female from male insects by their behavior. The virgin female mealybugs feeding on potato sprouts or Japanese pumpkin, *Cucurbita moschata* (Duch.) Duch. ex Poir., were then placed in a 45-liter glass jar through which charcoal-purified air was passed at 700–4000 ml/min (Moreno, unpublished observations). The pheromone emitted from each jar of females was trapped on a 2.5 × 30-cm column of Porapak Q®, 100/120 mesh, for a period of 14 days (the column of Porapak was changed every 7 days); 200 ml of pentane was used to elute the collected volatiles from the Porapak before a new jar of females was processed. Over a 2 to 3 year period, a total of about 5×10^6 female-day-equivalents (FDE) were trapped and approximately 30 mg of pheromone was obtained. Although average yield was initially about 6 ng/FDE, the quantity of pheromone collected increased to ca. 17 ng/FDE as methods were refined.

Once the structure was identified, a smaller-scale collection was carried out to quantitate pheromone production from 6000 females feeding on Japanese pumpkin; an average of 34 ng of pheromone/FD over a 10-day period was obtained. In a similar experiment with 1000 nonfeeding females, an average of 15 ng/FD was produced over a 15-day period.

The laboratory bioassays were conducted in a rearing room using a rotating wheel olfactometer (Tashiro et al., 1969). The 1.8-m-diameter wheel rotated at a rate of 0.17 rpm and accommodated 24 cages made from 1-pint Mason jars. Each cage was baited with 10 virgin females or with a chromatographic fraction on a 1.3-cm analytical filter paper disk placed on a 3.5-cm stainless-steel planchet. The males flying within the room responded by flying to the cages; the numbers of males landing on the screened enclosures of each jar were recorded every 5 min for a 30-min period.

The field bioassays were conducted in a 3.6-hectare pomegranate grove in Lindsay, California. A randomized complete block design was used for each test. Each ice cream carton trap was baited either with 40 virgin female mealybugs or with a 5 × 9-mm rubber septum (A.H. Thomas Scientific, Philadelphia, Pennsylvania) impregnated with a CH_2Cl_2 solution of the test sample. Cards (7.6 × 12.7 cm) coated with Tanglefoot® (Tanglefoot Co., Grand Rapids, Michigan) were mounted on the traps to snag the male insects as they approached the lure sources; the numbers of insects trapped were recorded every 3–7 days.

Isolation. The pentane washes of the Porapak Q columns were chromatographed on a 20-g column of silica gel (J.T. Baker, Phillipsburg, New Jersey) activated at 110°C for 2 hr. Increasing concentrations of ether in hexane were used for elution; laboratory bioassay of the fractions showed the active material to be present in the 15% ether fractions. Pheromone-containing fractions were rechromatographed on 20-g of Florisil coated with 20% $AgNO_3$ (Applied Science, State College, Pennsylvania) and activated

overnight at 110° C. The pheromone eluted with 50% ether in hexane as a single compound, as shown by capillary gs chromatography (GC) on a 60-m GOTC column coated with nonpolar SE-52. A few impurities remained, but bioassay of combined fractions indicated that no additional pheromone components were present.

Analytical Instrumentation. The following instruments were used to obtain the data required for the identification of the pheromone. Hewlett-Packard (Avondale, Pennsylvania) gas chromatograph (GC) model 5830; Perkin-Elmer (Norwalk, Connecticut) infrared spectrophotometer (IR) model 621 (samples run as CCl_4 solutions in a 0.1-mm path-length cavity cell), and Finnigan (Sunnyvale, California) mass spectrometer (MS) and data system, models 4000 and 6000, respectively.

RESULTS AND DISCUSSION

Identification. Gas chromatographic retention data were obtained during initial studies when only a few micrograms of the pheromone had been collected and isolated. The compound had GC retention indices (Kovats, 1966) of I = 1573 on polar SP 2340 and I = 1147 on nonpolar SP 2100 columns (each 1.8 m × 3 mm ID; 10% liquid phase on Gas Chrom Q, 100/120 mesh). Reaction of the pheromone with alcoholic KOH eliminated biological activity and produced an alcohol with GC retention indices (I) of 1655 and 1045 on the same two columns. From an examination of the retention data of reference terpene-type alcohols, we concluded that this difference in indices (ΔI) for the polar and nonpolar columns of the hydrolyzed pheromone was consistent with a secondary alcohol function. Acetylation regenerated the original biologically active compound. The acetate function was confirmed by absorptions at 1730 and 1220 cm^{-1} in the IR spectrum of the pheromone.

The electron impact mass spectrum of the pheromone (Figure 1) showed the molecular ion (M^+) at *m/e* 182 (0.6%) and M^+-60 at *m/e* 122 (42%). Chemical ionization (CI) MS confirmed the molecular ion as *m/e* 182, which corresponds to a molecular formula $C_9H_{15}OCOCH_3$. The spectrum of the alcohol (Figure 2) derived from the pheromone gave M^+ at *m/e* 140 (0.6% and M^+-18 at *m/e* 122 (2.5%). The presence of unsaturation in the structure was shown by IR absorptions at 1650 and 905 cm^{-1} which indicated terminal unsaturation, $R_1R_2C{=}CH_2$. Ozonolysis (Beroza and Bierl, 1969) (see identification scheme, Figure 3) of a few micrograms of the pheromone eliminated its biological activity, and MS analysis showed that acetone was produced when the ozonide was cleaved with triphenylphosphine in hexadecane solvent. Thus a second double bond was probably present in a $(CH_3)_2C{=}$ group. Formaldehyde derived by oxidation of the $R_1R_2C{=}CH_2$ group (as identified by IR) could not be detected under the GC-MS conditions used nor were other

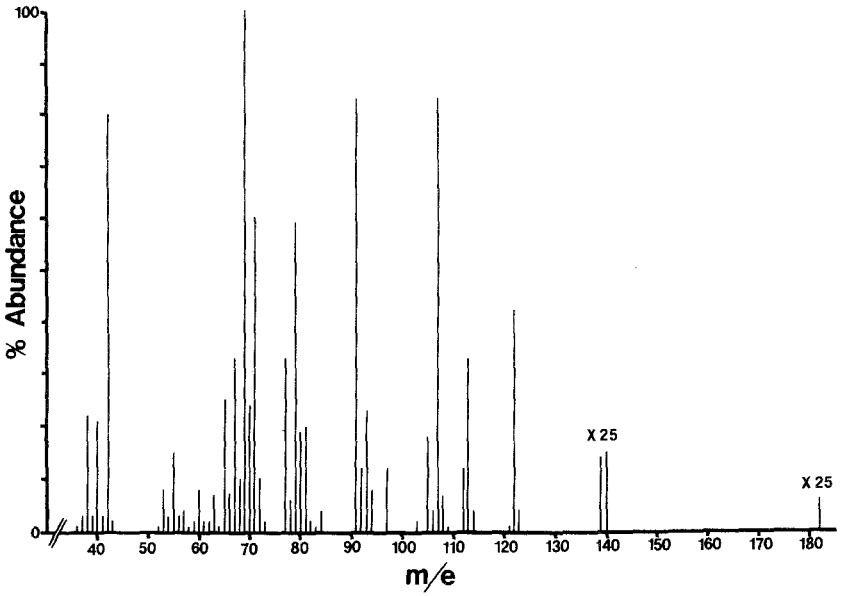

FIG. 1. Mass spectrum of the pheromone.

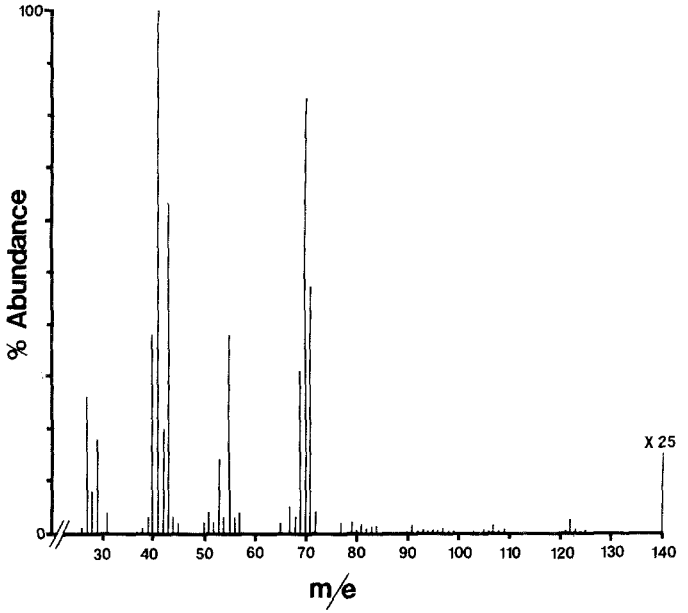

FIG. 2. Mass spectrum of the alcohol derived from the pheromone.

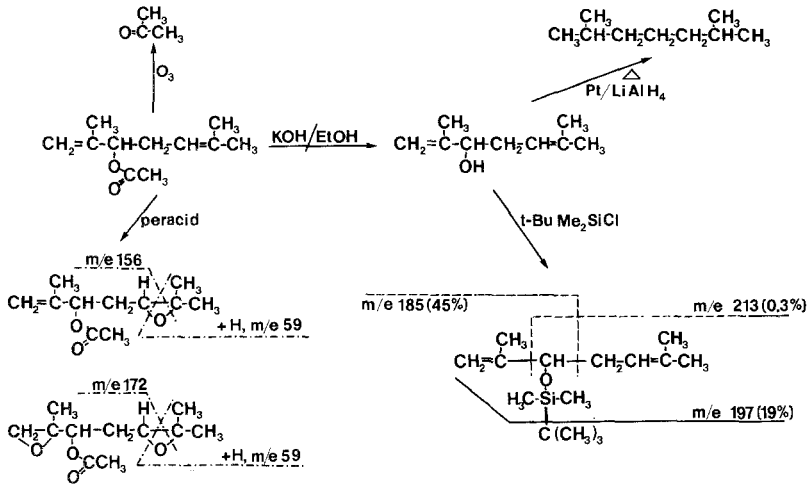

FIG. 3. Identification scheme.

aldehyde or dialdehyde fragments detected. When the pheromone was epoxidized with *m*-chloroperbenzoic acid, a mixture of mono- and diepoxides was obtained. The mass spectra of both compounds showed fragments corresponding to $[(CH_3)_2COH]^+$ (*m/e* 59) and to loss of $(CH_3)_2C-$ (M^+-42), ascribed to transannular cleavage of the 5,6-epoxy group; the base peak was *m/e* 43 for each compound.

With both double bonds characterized and no cyclic structure permitted by the molecular formula, the structure of the hydrocarbon chain and the position of the acetoxy group remained to be identified. The carbon skeleton was characterized by reaction of a few micrograms of the alcohol derived from the pheromone with 1 mg of a mixture (1:1) of 5% Pt on neutral alumina (Nigam, 1966) and $LiAlH_4$ at 250°C (30 min) in a sealed melting-point capillary. Double bonds are saturated and functional groups are removed during this procedure (Bierl-Leonhardt and DeVilbiss, 1981). Nitrogen- or oxygen-containing functional groups in which the hetero atom is attached to a primary carbon atom are eliminated, with the result that both the hetero atom and the adjacent methylene group are lost (Bierl et al., 1969). With this procedure, the alcohol of the pheromone gave a hydrocarbon that was identified as 2,6-dimentylheptane by GC-MS analysis of the reaction products dissolved in hexadecane.

Since the position of double bonds and the presence of a secondary acetoxy group had been established, the structure appeared to be related to 2,6-dimethyl-1,5-heptadiene, with an acetylated alcohol function at the 3 or 4 position. The position of the acetoxy group was established by GC-MS analysis of the *t*-butyldimethylsilyl derivative formed by reaction of a few

micrograms of the pheromone alcohol with the corresponding silyl chloride in pyridine. Characteristic cleavage alpha to the silyl ether group and loss of the *t*-butyl group produced fragments at *m/e* 213 (0.3%) for M^+-C_3H_5, *m/e* 197 (19%) for M^+-*t*-butyl, and *m/e* 185 (45%) for M^+-C_5H_9. This fragmentation pattern was consistent only with the silyl ether group in the 3 position; the similar compound with substitution in the 4 position would yield an abundant fragment at *m/e* 199 for loss of C_4H_7.

The structure of the pheromone was therefore postulated as 2,6-dimethyl-1,5-heptadien-3-ol acetate; the chiral center at C-3 permits the existence of two enantiomers. Reaction of the alcohol derived from the natural pheromone with (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (Mosher's reagent; Dale et al., 1969) produced a single peak at 34.73 min on a 60-m, SP 1000 (polar) capillary column. Similar treatment of synthetic, racemic material (synthesis described below) yielded peaks at 34.73 and 35.25 min corresponding to the two enantiomers; less than 2% of this second enantiomer was present in the natural material. The isolated pheromone, thus shown to be optically active, was found to have a specific rotation of (+) 6.2°; the absolute configuration is yet to be established.

The extracts from the air collections were shown to contain a small amount of the corresponding alcohol in addition to the pheromone acetate. Pheromone was produced by acetylation of inactive chromatographic (silica gel) fractions that contained polar compounds. The ratio of the pheromone acetate to alcohol was found to be 200–250 to 1.

Synthesis. The pheromone was synthesized by the scheme shown in Figure 4. The 2,6-dimethyl-2,5-heptadiene (II) produced by the Wittig reaction was converted to the mono epoxide (III) with 1 equivalent of *m*-chloroperbenzoic acid. Following high-pressure liquid chromatography cleanup on $AgNO_3$-impregnated silica gel, the epoxide was rearranged to 2,6-dimethyl-1,5-heptadien-3-ol with aluminum isopropoxide in refluxing toluene (Terao et al., 1979). The HPLC-purified alcohol was acetylated with acetic anhydride and pyridine to give racemic 2,6-dimethyl-1,5-heptadien-3-ol

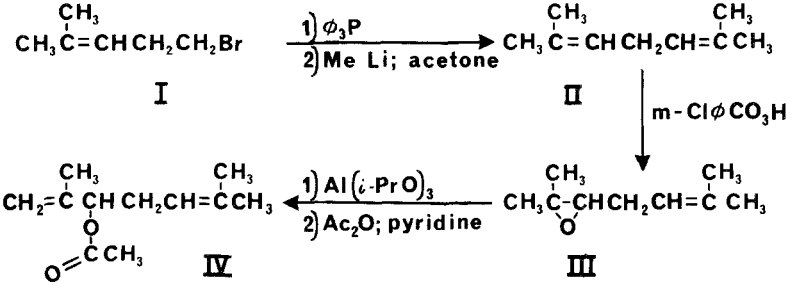

FIG. 4. Synthesis scheme.

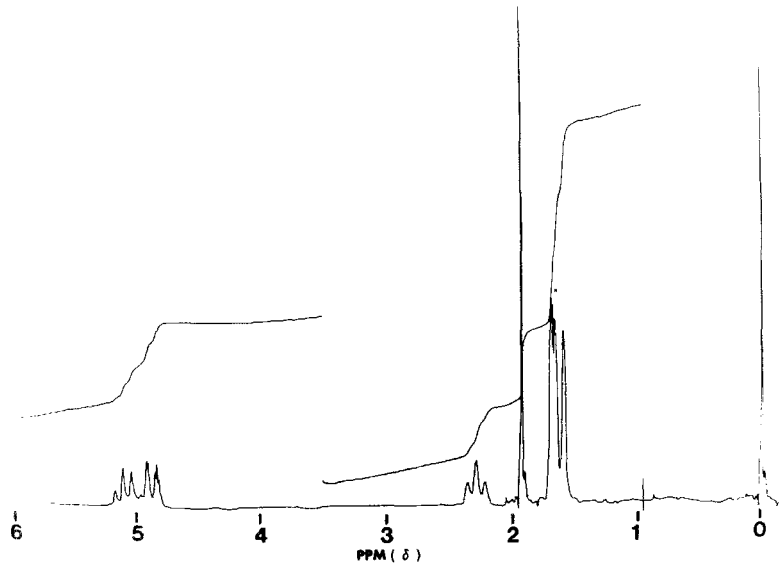

FIG. 5. NMR spectrum of synthetic pheromone, 2,6-dimethyl-1,5-heptadien-3-ol acetate (IV).

acetate (IV). The NMR spectrum (Figure 5) shows the olefinic protons and CHO at 4.8–5.2, CH_2 at 2.3, O_2CCH_3 at 1.94 and methyl branches ca. 1.7 ppm. The NMR spectrum, GC retention data, and mass infrared spectra were identical with those of the isolated pheromone.

The related compounds, 2,6-dimethyl-1,5-heptadien-4-ol acetate, 2,6-dimethyl-1-hepten-3-ol acetate, and 2,5-dimethyl-1,5-hexadien-3-ol acetate, were prepared by similar procedures for bioassay.

Bioassay. The natural and synthetic pheromones were field tested at several concentrations to compare their relative attraction for males. The data

TABLE 1. FIELD BIOASSAY OF NATURAL VS. SYNTHETIC PHEROMONE

Pheromone source	Purity (%)	No. Males Trapped with[a]		
		0.2 μg	2 μg	20 μg
Natural	98	17 z	43 yz	79 xy
Natural	3	10 z	30 z	95 x
Synthetic	97	18 z	27 z	49 xyz
40 Females		10 z	10 z	10 z

[a]Average of 3 replicate traps. Means followed by the same letter(s) are not significantly different at the 5% level of probability based on Duncan's multiple-range test.

TABLE 2. FIELD BIOASSAY OF PHEROMONE AND ANALOGS

Material	Average number males trapped[a]
2,6-Dimethyl-1,5-heptadien-3-ol acetate (natural)	262 y
2,6-Dimethyl-1,5-heptadien-3-ol acetate (synthetic)	220 y
2,6-Dimethyl-1,5-heptadien-4-ol acetate	68 z
2,6-Dimethyl-1-hepten-3-ol acetate	34 z
2,5-Dimethyl-1,5-hexadien-3-ol acetate	50 z
40 Females	177 y

[a]Average of 5 traps; 50 μg/trap. Means followed by the same letter are not significantly different at the 5% level of probability based on Duncan's multiple-range test.

of Table 1 show that the optically active natural pheromone produced male captures at 0.2, 2, and 20 μg/trap similar to those with the racemic synthetic pheromones. In addition to this comparison, the total extract from the air collections (only 3% pheromone) was bioassayed against the isolated material having the same pheromone content; if additional components that alter the attractancy of the pheromone had been present in the trapped volatiles, the trap catch would have been affected. The data in Table 1 suggest that no components trapped from feeding females other than the identified pheromone had a significant effect on attraction.

Table 2 gives a comparison of trap captures with the pheromone and three synthetic analogs which have the acetoxy group in an adjacent position, or only one double bond, or one less carbon atom. Both the natural and synthetic pheromone gave a similarly high trap catch, but the three related compounds showed only weak attraction at best, with much lower catches. Uchida et al. (1981) also obtained a low trap capture (10 males/trap/day) with 2,6-dimethyl-1,5-heptadien-4-ol acetate as compared to the synthetic pheromone (72 males/trap/day).

As reported earlier in this paper, the content of the corresponding alcohol in the emission from the feeding females was about 0.5% of that of the pheromone acetate. Mixtures of the synthetic alcohol, 2,6-dimethyl-1,5-heptadien-3-ol, and its acetate were field tested to measure attraction to males. The data of Table 3 show that 2–20% alcohol added to 40- and 4-μg amounts of the acetate caused an apparent increase in trap catch particularly at the 4-μg level. The statistical evaluation of the data did not, however, show significant differences among the treatments. Further tests will be carried out in the fall of 1981. The alcohol itself was ineffective as an attractant.

Conclusion. The pheromone of the Comstock mealybug, *Pseudococcus comstocki* (Kuwana), has been identified as 2,6-dimethyl-1,5-heptadien-3-ol acetate. Although the natural material is optically active, (+), with an

TABLE 3. EFFECT OF THE ALCOHOL ON THE ATTRACTION OF THE PHEROMONE

Acetate (μg)	Alcohol (μg)	Males trapped[a]	Acetate (μg)	Alcohol (μg)	Males trapped[a]
40	0	784 x	4.0	0	328 xyz
40	0.8	717 xy	4.0	0.08	572 xy
40	4	827 x	4.0	0.4	685 xy
40	8	769 x	4.0	0.8	570 xy
40	20	558 xy	4.0	2.0	241 yz
40	40	415 xyz	4.0	4.0	324 xyz
0	40	51 z	0	4.0	53 z
40 Females		403 xyz	40 Females		403 xyz

[a]Average of 5 replicate traps; total trap captures for October 6 to November 12, 1980. Means followed by the same letter(s) are not significantly different at the 5% level of probability based on Duncan's multiple-range test.

asymmetric carbon at C-3, the synthetic racemic compound has been shown to attract similar numbers of male insects in field bioassay and can be used in traps to monitor populations. The attractiveness of the pheromone may be enhanced by the addition of 5–10% of the corresponding alcohol.

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FIELD BIOASSAY OF *exo*- AND *endo*-BREVICOMIN WITH *Dendroctonus ponderosae* IN LODGEPOLE PINE[1]

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Abstract—Both *exo*- and *endo*-brevicomin reduced the response of flying *Dendroctonus ponderosae* (Coleoptera: Scolytidae) to an attractant composed of *trans*-verbenol and terpenes or of *trans*- and *cis*-verbenol and terpenes in a stand of *Pinus contorta* var. *murrayana*. These data suggest that racemic *exo*- and *endo*-brevicomin may interrupt aggregation in populations of mountain pine beetle colonizing lodgepole pine; functions of the natural chiral compounds are unknown.

Key Words—Coleoptera, Scolytidae, *Dendroctonus ponderosae*, pheromone, *exo*-brevicomin, *endo*-brevicomin, *Pinus contorta*.

INTRODUCTION

The mountain pine beetle (MPB), *Dendroctonus ponderosae* Hopkins, is a destructive bark beetle attacking many species of pine in western North America. Like other *Dendroctonus*, the MPB overcomes and kills its host tree by a mass attack in response to an aggregation pheromone released from the feces of the first females to bore into the bark. *trans*-Verbenol released by MPB in the presence of host-tree terpenes is partially responsible for the aggregative response (Pitman et al., 1968; Pitman, 1971).

Successful colonization of the host tree depends upon infestation by a critical density of mating pairs, which varies from tree to tree and through time (Shrimpton, 1978), sufficient to overcome resin exudation by the tree. If the larval density becomes too great, competition for food can severely limit larval survival (Cole, 1962; Reid, 1963). After a tree is mass attacked,

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aggregation to that tree ceases and the attack switches to adjacent trees (McCambridge, 1967; Geiszler and Gara, 1978). The switching behavior has been hypothesized to be a response either to a decrease in resin exudation and pheromone production (Renwick and Vité, 1970) or to production of an antiaggregation pheromone (Rudinsky et al., 1974). On the basis of laboratory bioassays of walking male beetles emerged from ponderosa pine (*P. ponderosa* Laws.) and a preliminary field test, Rudinsky et al. (1974) suggested that *exo*-brevicomin may be an "anti-aggregative-rivalry pheromone" and that *endo*-brevicomin may also have antiaggregative properties. Both compounds are released by emergent males. Pitman et al. (1978) stated that mass attack was prevented in nine white pine (*P. monticola* Dougl.) baited with pondelure and protected with *exo*-brevicomin, whereas trees similarly baited but without *exo*-brevicomin were mass attacked and killed. However, in lodgepole stands (*P. contorta* Dougl.), *exo*-brevicomin seemed to make baited trees more likely to be mass attacked. In this study we bioassayed the reponse of flying MPB to both *exo*- and *endo*-brevicomin in combination with *trans*- and *cis*-verbenol and terpenes to provide field data on possible antiaggregative properties of brevicomin.

METHODS AND MATERIALS

Brevicomin isomers were bioassayed in a stand composed primarily of lodgepole pine, *P. contorta* var. *murrayana*, with a scattering of ponderosa pine. Infested lodgepole with brood about to emerge were present in the stand, which is about 3 miles west of Paulina Peak in Deschutes County, Oregon, at NE, NE, S6, T21S, R12E, W.M. at 1700 m elevation.

Test compounds were evaporated from open 1/2-dram glass vials placed inside perforated aluminum film cans (Furniss and Schmitz, 1971). Evaporation rate of compounds was estimated by daily monitoring the loss of measured quantities of each compound in the field (Table 1, footnote). The dispensers were wired to the NW edge of the north facing of two 0.25 × 1-m sticky traps made from 6.4-mm (1/4-in.) mesh hardware cloth coated with Stikem Special®. Traps were fastened at breast height to lodgepole pines 22–32 cm DBH that were limbed and wrapped in black plastic (McKnight, 1979) from the base of the tree up to 7 m. Wrapping to this height was considered sufficient protection from initial attacks of MPB, which usually occur on the basal 2 m of the bole, and rarely above 3 m (McCambridge, 1967; Rasmussen, 1974). Beetles collected from the north- and south-facing traps on each tree were combined to represent a single trap per tree. Trees selected to bear traps were 10–30 m apart in three irregular rows.

We obtained *trans*-verbenol of 80% purity that included 10% *cis*-

verbenol from Borregaard of Oslo, Norway. We considered the *cis*-verbenol component acceptable (Pitman, 1971) because it has been identified from the frass of MPB females (Libbey, Ryker, and Rudinsky, unpublished data) and preliminary tests showed the mixture to be attractive. A second test utilized *trans*-verbenol of 99.7% purity from Albany International, Columbus, Ohio. A 1:1 mixture of D-α-pinene and myrcene was included in all treatments. These two terpenes were selected on the basis of known attractancy (Pitman and Vité, 1969; Billings et al., 1976). As MPB have been shown to land on uninfested trees randomly, irrespective of species or terpene composition (Hynum and Berryman, 1980), control traps included terpenes, and blank traps were not included. Synthetic *exo*- and *endo*-brevicomin were from Albany International at 99% purity. A dispenser containing one vial of *exo*-brevicomin evaporated at 4 mg/day as determined by evaporating known amounts in the field. A rate of 12 mg/day was obtained by placing three vials of *exo*-brevicomin in a dispenser. Evaporation rates for *endo*-brevicomin are the same. Myrcene was 78% purity and included 12% limonene, and D-α-pinene was 95% purity, both from K & K Laboratories. *exo*-Brevicomin was tested at 12 mg/day for antiaggregative effect in combination with the *trans*- and *cis*-verbenol mixture (0.5 mg/day) and terpenes in one test, and then tested at 4 mg/day in combination with *trans*-verbenol and terpenes. *endo*-Brevicomin was tested at 4 mg/day with the *trans*- and *cis*-verbenol mixture plus terpenes.

All trees were inspected for attacks each day and again when the tests were completed and the plastic removed. If the protective plastic was breached by a boring female MPB, the catches of that trap for that day and the previous day were excluded, and the paired comparisons in those blocks were eliminated from the data set.

Treatments were assigned to each trap within a row daily by a random numbers table. Trap catches each day in a single row were considered a block of treatments. Variation in density of flying MPB from day to day resulted in variances between blocks of the same treatment that were greater than variances between treatments, and distribution of catches violated the assumption of normality necessary for parametric analysis of variance. Therefore, treatments were compared using Wilcoxon's matched-pairs, signed-ranks test, one-sided (Sokal and Rohlf, 1969, p. 400). This nonparametric test combines comparisons of each pair of treatments matched only within the same blocks. We tested the hypothesis that the catch at the attractant control or the terpene control was equal to the catch at the treatment against the alternative that catch at the treatment was less than catch at the control. Three treatments were tested pairwise against the controls for a total of three possible comparisons per test. By keeping the individual error rate at $\alpha = 0.01$, the simultaneous error rate was $\alpha = 0.03$ for

each test. We chose to accept a moderately high type-I error rate for the study in order to have a reasonable chance of rejecting false hypotheses (cf. Bedard et al., 1980).

RESULTS AND DISCUSSION

Inspection of the trees bearing traps at the completion of the test revealed no previously unnoticed infestations. Unwanted attacks on trap trees were easily detected during the tests due to sudden large increases in trap catches. Invading females were also detected even when no entrance hole was noticeable because boring females gave loud clicking sounds (Rudinsky and Michael, 1973) audible several meters from the tree. However, after two weeks of trapping, over 70% of the original trees bearing traps had been invalidated by one or more attacks, which suggests that an inanimate tree silhouette would have provided a better base for the sticky screens. Beetles responding to the attractants frequently were trapped within 50 cm of the bait; beetles were seldom found higher on the plastic than the top of the sticky trap.

The attractants with and without the *cis*-verbenol isomer were both effective in trapping MPB in the stand of *P. contorta* var. *murrayana*, compared to the terpene control (Table 1). *exo*-Brevicomin evaporated at 12 mg/day reduced catches on traps, and both *exo*- and *endo*-brevicomin evaporated at 4 mg/day also significantly reduced catches (Table 1). Although these data support a possible antiaggregation function for *exo*- and *endo*-brevicomin, the natural, chiral pheromones may function differently.

Data from several sources have suggested that host-specific populations of MPB may have diverged as adaptations to biochemical differences in the hosts (Stock and Amman, 1980; Sturgeon, 1980). During prior attempts to test synthetic *exo*- and *endo*-brevicomin as possible inhibitors of aggregation by flying MPB to sticky traps, a mixture of *trans*-verbenol + α-pinene caught very few beetles (Rudinsky et al., 1974) or was completely unsuccessful in lodgepole pine stands (Rudinsky and Ryker, unpublished data 1978, 1979; Pitman et al., 1978). In contrast, large numbers of MPB were trapped on sticky traps in stands of white pine in Idaho (Pitman et al., 1978). However, these apparent differences could also be due to differences in experimental methodology among these diverse tests. Stock et al. (1978) found electrophoretic evidence for genetic divergence between MPB in lodgepole var. *murrayana* (central Oregon) and populations on several other hosts, including lodgepole var. *latifolia* and white pine. Although *exo*-brevicomin did not protect baited lodgepole pine in the study by Pitman et al. (1978), in our test both *exo*- and *endo*-brevicomin inhibited the response of MPB to the synthetic attractant on sticky traps. However, we cannot designate the compounds produced by the MPB as either aggregation or antiaggregation

TABLE 1. CATCH OF *Dendroctonus ponderosae* IN A LODGEPOLE STAND USING STICKY TRAPS ON PLASTIC-WRAPPED TREES, PAULINA PEAK, DESCHUTES COUNTY, OREGON, 1980

Treatment	Catch/trap/day $\bar{X}$ ± SE	Total catch	No. of matched pairs	
July 17–22				
Terpene control[a]	0.8 ± 0.32	10		a[b]
Terpenes + *t*V + *cis*[c]	7.0 ± 1.73	84	12	b
Terpenes + *t*V + *cis* + *endo*[d]	1.0 ± 0.40	12		a
July 17–23				
Terpene control	1.0 ± 2.78	14		a
Terpenes + *t*V + *cis*	6.3 ± 1.57	87	14	b
Terpenes + *t*V + *cis* + *exo*[e]	1.2 ± 0.55	16		a
July 24–31				
Terpene control	1.8 ± 0.41	18		a
Terpenes + *t*V[f]	16.7 ± 3.61	167	10	b
Terpenes + *t*V + *exo*[g]	2.3 ± 0.55	23		a

[a]Terpenes were a mixture of D-α-pinene, myrcene, and limonene, evaporating at 10 mg/day.
[b]Different letters indicate a significant difference with $\alpha = 0.01$.
[c]*t*V + *cis* = 80% *trans*- and 10% *cis*-verbenol at 0.5 mg/day as a mixture.
[d]*endo* = *endo*-brevicomin at 4 mg/day.
[e]*exo* = brevicomin at 12 mg/day.
[f]*t*V at 0.5 mg/day.
[g]*exo* at 4 mg/day.

pheromones until the natural optical isomers are identified and tested. Also, the large increase in trap catch associated with even a single female MPB having entered the tree through the plastic suggests that our attractant mixture is a weak substitute for the natural attractant. Whether this is due to additional unknown components, optical isomers, or simply greater concentrations of pheromone released is not known. How racemic *exo*-brevicomin would fare against the complete attractant of live female MPB boring in trees in not known for all evaporation rates, nor has *endo*-brevicomin been tested on baited trees.

Attempts to understand how the MPB regulates its own population during host colonization are an important part of our efforts to develop tools useful in pest management strategies for pine forests. Also, insight into possible differences in pheromone production and release, or differences in response to the same compounds by MPB selectively colonizing different species of pine, is essential to an understanding of this important and complex forest insect.

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NEW SYNTHESIS OF ROYAL JELLY ACID

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Abstract—A new synthesis of (*E*)-10-hydroxy-2-decenoic acid (royal jelly acid) is described, starting from 8-bromo-1-octanol via condensation of malonic acid with 8-hydroxyoctanal or 8-acetoxyoctanal.

Key Words—Royal jelly acid, (*E*)-10-hydroxy-2-decenoic acid, honeybee, *Apis mellifera*, Hymenoptera, Apidae

INTRODUCTION

Ten-carbon aliphatic compounds are prominent among those isolated from the honeybee *Apis mellifera* (L.). In particular, the royal jelly, secreted by the mandibular glands of worker bees, contains an important amount of (*E*)-10-hydroxy-2-decenoic acid (**A**) (Royal Jelly Acid).

$$HO{-}(CH_2)_7{-}CH{=}CH{-}COOH \qquad A$$

This acid is interesting because of its relationship to queen pheromone: 9-oxo and (*E*)-9-hydroxy-2-decenoic acid.

Pain et al. (1974) have shown that parallels exist between the yearly cycle of the acid and the secretions of the queen pheromones, which are responsible for the attraction of young honeybees, and the inhibition of their ovaries and ability to produce queen cells.

Since 1960, many syntheses for this acid alcohol have been proposed, condensation of various aldehydes with malonic acid being frequently used (Fray, 1960; Fusii, 1960; Sisido, 1962; Smissman, 1964; Fournet, 1965; Bestmann, 1966).

We have found two new methods which give a faster synthesis and higher yields; these start with 8-bromo-1-octanol and use as intermediates three aldehydes obtained by the oxidation of a $-CH_2Br$ group by pyridine oxide.

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These three aldehydes, 8-hydroxyoctanal, its tetrahydropyranyl (THP) derivative, and 8-acetoxyoctanal then undergo malonic synthesis, and by decarboxylation, the acid is obtained.

METHODS AND MATERIALS

Infrared data were obtained with a Beckman IR-18 A instrument; nuclear magnetic resonance data were obtained with a Varian A-60 instrument. The reaction schemes are shown in Figures 1 and 2.

8-Bromo-1-octanol 1

A solution of 1,8-octanediol in petroleum ether (bp 50–70°) was refluxed with 35% HBr aqueous solution, under continuous extraction during 15 hr. Fractional distillation yielded 75% of the bromo derivative; bp 92° / 0.5 mm.

8-Hydroxyoctanal THP Ether 3

The THP ether of 8-bromo-1-octanol (29.3 g, 0.1 mol) was added to a suspension of pyridine-*N*-oxide (19 g, 0.2 mol) and $NaHCO_3$ (22 g, 0.25 mol) in 180 ml of anhydrous toluene. The mixture was stirred overnight under reflux, and water was added (500 ml). After extraction with benzene and drying ($MgSO_4$), the solvents were removed, and distillation yielded 12 g, bp 128° / 0.6 mm (70%) of the aldehyde 3; IR, 1730 (CO) cm^{-1}.

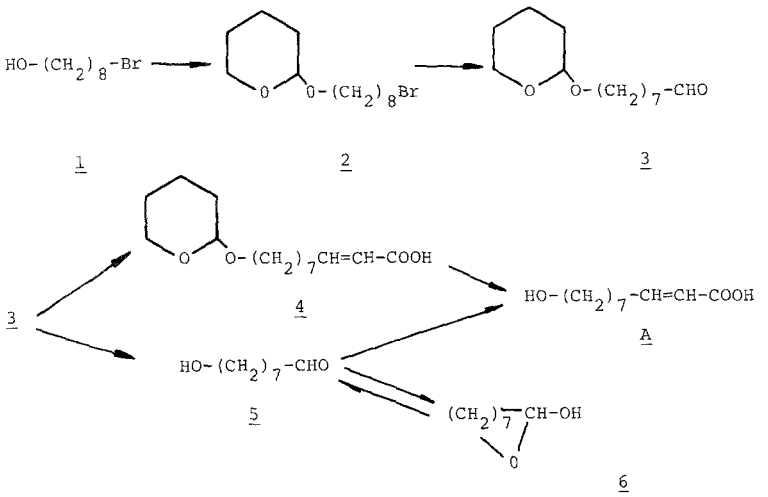

FIG. 1. Synthesis of acid A by method 1.

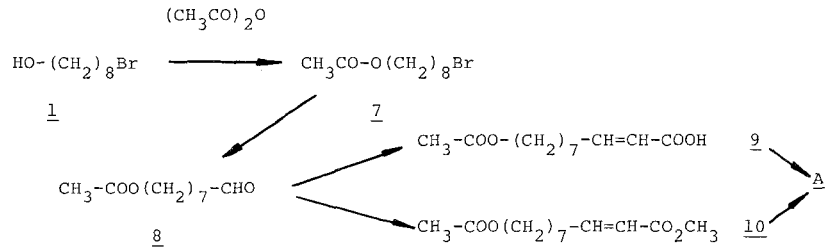

FIG. 2. Synthesis of acid A by method 2.

(E)-10-Hydroxy-2-decenoic Acid, A

Route a. The aldehyde 3 (22.8 g, 0.1 mol) was added to a solution of malonic acid (10.4 g, 0.1 mol) in 50 ml of pyridine. The mixture was stirred overnight at ambient temperature, warmed 2 hr at 100°, and cooled. Water (50 ml) was added, and the mixture was extracted with ether. The ether solution was washed with dilute HCl, and extracted with 5% NaOH (3 × 50 ml). Acidification gave the acid 4 as an oil.

The oil 4 was dissolved in methanol (50 ml), HCl 5% (100 ml) was added, and the mixture was refluxed for 4 hr, cooled, and extracted with ether. The acid A was recrystallized from ether–petroleum ether (50:50) to yield 9 g (48%); mp, 64–65°.

Route b. Compound 3 was carried through the inverse sequence (1) acid hydrolysis (80% yield) gave the 8-hydroxyoctanal 5 (bp 90–95° / 0.5 mm) and (2) malonic synthesis as described for the previous preparation gave the acid alcohol A (66% yield).

8-Acetoxyoctanal 8. Compound 1 was converted to the aldehyde 8 by the sequence: (1) acetylation with acetic anhydride (96% yield) to the ester 7 (bp 140° / 12 mm) and (2) treatment with pyridine-*N*-oxide as described for the preparation of aldehyde 3. Overall yield of 8 from 1 was 55–60%; bp 138° / 15 mm.

RESULTS AND DISCUSSION

Method 1: Via 8-Hydroxyoctanal 5

On treatment with dihydropyran by acid catalysis, 8-bromo-1-octanol, 1, gives the THP derivative 2 (96%); bp 113–114° / 0.3 mm. This product, either distilled or in crude form, is then oxidized under reflux by the pyridine-*N*-oxide in toluene method oxidation of —CH_2Br group to —CHO (Stowell, 1970), to give the corresponding ether aldehyde 3 (70%). Two routes are then possible.

Route a. The aldehyde 3 undergoes malonic condensation. The action of malonic acid in the presence of piperidine, followed by decarboxylation by heating, gives the ethylenic acid 4 which, by acid hydrolysis, gives the required acid A (yield: 48% starting from aldehyde 3). The acid 4 is used in crude form.

Route b. Acid hydrolysis of compound 3 gives the 8-hydroxyoctanal 5 (yield: 80%, bp 95–97° / 0.5 mm) which, by the action of malonic acid followed by decarboxylation gives the acid alcohol A with a yield of 66% (Sisido, 1962). This preparation of 8-hydroxyoctanal gives much better yields than the methods published previously: Action of KCN on chlorohydrin followed by reduction of the nitrile (Colonge, 1969), or using 2-hydroxycyclooctanone as starting material (Hurd, 1952).

It must be noted that the linear form of the 8-hydroxyoctanal is in equilibrium with the cyclic tautomer 6 (2-oxacyclononanol), shown by the NMR to be present as 20% in solution of $CDCl_3$.

The overall yield of the four steps starting from 8-bromo-1-octanol is around 35%.

Method 2: Via 8-Acetoxyoctanal

The esterification of 8-bromo-1-octanol 1 by acetic anhydride gives 8-acetoxy-1-bromooctane 7 with a yield of 96%. The acetate 7 is then transformed into 8-acetoxyoctanal 8 (yield, 60%), by pyridine-*N*-oxide in the same way as for THP ether 2 in method 1. There are then two possible routes.

Route a. The ester aldehyde 8 is treated with malonic acid in pyridine to give, after decarboxylation, an acid ester 9 (yield, 75%). This product is then saponified by KOH in an aqueous alcoholic medium. After acidification, ether extraction gives the required acid A (yield, 82%). It is not necessary to purify the ester 9, as it can be used in its crude form. Overall yield of the four steps is 35%.

Route b. The ester aldehyde 8 is converted to the olefin 10 by a Wittig reaction (Bestmann, 1966). After the elimination of triphenyl phosphine oxide by zinc chloride, the diester formed (10) is saponified with KOH. This gives acid A, with a yield of 55% (it is not necessary to isolate the diester 10). Overall yield is 32%. Bestmann (1966) did not isolate the aldehyde 8 which was prepared in situ via six steps starting from suberic acid.

In conclusion, it is possible to prepare (*E*)-10-hydroxy-2-decenoic acid (A) in four steps, starting from 8-bromooctoctanol 1, and to obtain an overall yield of 35%.

The pure product, from which it is hard to eliminate water, is a white solid which melts at 64–65° after recrystallization from a mixture of ether–petroleum ether (50:50) (lit. mp 64–65°, Fray, 1960). The *E* structure is confirmed by IR spectra (absorption at 990 cm^{-1}) and NMR spectra (coupling constant = 16 Hz) for the ethylenic protons. This compound is used in biological tests on social insects.

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TASTE PREFERENCES OF THE COMMON VAMPIRE BAT (*Desmodus rotundus*)[1]

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Abstract—Taste preference tests, with simultaneous presentation of treated and untreated food, were administered to 24 common vampire bats (*Desmodus rotundus*). The bats received brief exposures to four different stimuli representing sweet, salty, sour, and bitter tastes, each at four different concentrations. Despite a strong location bias, the bats significantly ($P < 0.01$) avoided the highest concentrations of the salty, sour, and bitter tastes. Consumption of the sweet stimulus at all concentrations was similar to that of the untreated standard. Vampires evidently can discriminate based on taste, although their ability is apparently poorly developed when compared with some euryphagous species such as the rat. Hence, taste is probably not a factor in host selection by the vampire.

Key Words—Taste, taste preference, *Desmodus rotundus*, citric acid, sucrose, sodium chloride, quinine, vampire bat.

INTRODUCTION

The common vampire bat (*Desmodus rotundus*) depends solely on blood from living hosts for sustenance. Yet within the confines of their extreme stenophagia, vampires do prefer certain types of hosts (Goodwin and Greenhall, 1961; Turner, 1975). Factors influencing these preferences are unknown and may range from simply the availability or abundance of hosts to their accessibility, vulnerability, degree of domestication, or to some unique

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aspect of their detectability such as size, odor, or color. Although little is known about the sensory basis of food selection by noninsectivorous bats (Suthers, 1970), it seems likely that vampires utilize various sensory systems for locating and selecting hosts.

Several investigators have studied aspects of the olfactory, visual, and auditory senses of vampires (Mann, 1960; Schmidt and Greenhall, 1971; Pru and Briceno, 1972; Suthers, 1966; Shumake et al., 1977), but few have studied the vampire's sense of taste. Park and Hall (1951) described the gross anatomy of the vampire tongue, showing that taste buds are present. Suthers (1970) summarized the results of investigations by Fishman and others, in which electrophysiological responses to gustatory stimuli were compared in several species, including the vampire. They applied test solutions to fungiform papillae on the tip of the tongue of test animals and recorded the steady-state height of the integrated action potentials of the chorda tympani nerve. These studies demonstrated that vampires have highly functional taste receptors.

The study reported here was designed to further evaluate the sense of taste by examining the vampire's preference for sweet, salty, sour, and bitter.

METHODS AND MATERIALS

We used 24 adult vampire bats. They were captured in Mexico, shipped to our laboratory in Denver, individually housed in wire cages ($34 \times 17.5 \times 17.5$ cm), and adapted to feeding on fresh defibrinated cattle blood from glass feeding tubes.

We measured the preferences of each animal using an automated electronic testing apparatus which has been previously described in detail by Thompson and Grant (1971). The device (Figure 1) is based on the principle of the brief-exposure, foods-together technique described by Young and Kappauf (1962). By this technique, the test animal is given a traditional two-choice situation; however, the apparatus is programed such that the animal briefly samples each food alone before the two foods are presented together for choice behavior. Temporal and spatial positional habits are minimized by alternating the sequence and positions in which the choices are presented. According to Young (1967), the technique yields a cross-section of relative levels of acceptability before postingestional factors influence results. The foods-together technique was used by Young (1945, 1966, 1967), Young and Madsen (1963), Young and Greene (1953), and Christensen (1962) to rate the palatability of liquid foods and thus appeared particularly applicable to this study.

Fresh defibrinated whole bovine blood was treated with sucrose, citric acid, sodium chloride, or quinine hydrochloride to represent the basic taste categories of sweet, sour, salty, and bitter, respectively. Molar concentrations

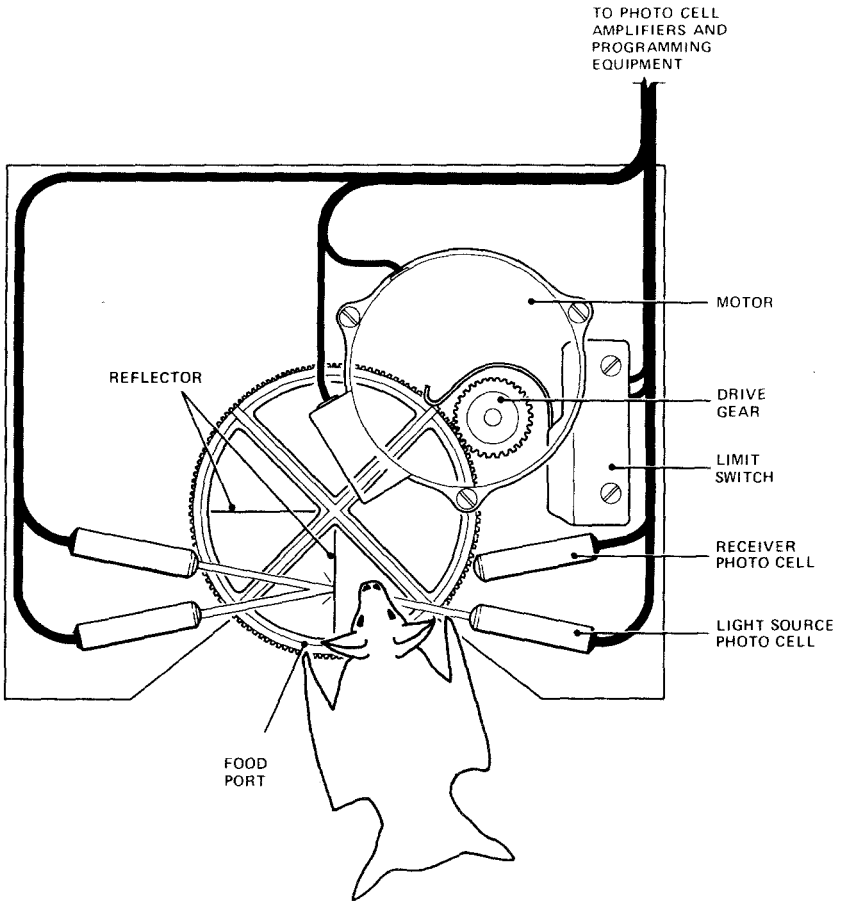

FIG. 1. Automated electronic testing apparatus for measuring taste preference behavior (modified from Thompson and Grant, 1971).

from 1×10^{-4} to $1 \times 10^{-1.5}$ were used for citric acid and quinine, and molar concentrations from 6.6×10^{-4} to 3.3×10^{-1} were used for sucrose and sodium chloride. Four concentrations of each taste (Table 1) were used for the preference evaluations. Fresh untreated defibrinated blood was used as the alternate choice. The preference test was replicated (treated vs. untreated positions reversed) and mean preference values were calculated for each bat. Tests were conducted betwen 0800 and 1100 each day before the bats received their regular daily blood ration. Fresh test samples (both treated and untreated) were prepared before each test and were at room temperature (about 21° C) before measurements were made.

Equal amounts (4.0 cc) of treated or untreated blood were weighed and

TABLE 1. TASTE STIMULUS TEST CONCENTRATIONS

Stimulus	Concentration (g/100 ml of blood)			
	C^1	C^2	C^3	C^4
Sucrose	0.034	5.682	11.364	17.114
Sodium chloride	0.006	0.971	1.942	2.925
Citric acid	0.002	0.021	0.210	1.150
Quinine hydrochloride	0.004	0.040	0.400	2.188

pipetted into each of the small compartments and 8.0 cc were placed in the large compartments (see Figure 1). The two large quadrants are used for the nonchoice food-sampling positions and the remaining two quadrants are bisected for the choice positions. Placement of treated or untreated blood was reversed for alternate bats. As the bats fed from the preference testers, the compartmentalized food trays rotated in sequence to various positions. In a typical test cycle, the bat samples the untreated food, samples the treated food, chooses between the two presented simultaneously, samples the treated food, samples the untreated food, and again chooses between the two together but with positions reversed from the first exposure. The time each animal spent feeding at each compartment was automatically recorded on digital counters. Immediately after each bat had made sixteen 6-sec choices, the test apparatus was removed from the cage and the blood remaining in each compartment was weighed. Preference was calculated according to the following formula.

$$\text{Consumption preference } (\%) = \frac{\text{amount treated blood consumed}}{\text{total blood consumed}} \times 100$$

Preferences were analyzed by two-way analysis of variance.

RESULTS AND DISCUSSION

The vampires exhibited a stereotyped behavior when confronted with the two-choice situation in the study. That is, some bats consistently drank from the left (or right) side of the preference tester whereas others drank from the first compartment that rotated toward them, regardless of treatment. There were no significant differences ($P > 0.05$) in preference for untreated blood versus blood treated with sucrose. However, bats avoided the salty, sour, and bitter tastes. Preferences at the highest concentrations (C_4) of these tastants were significantly lower ($P < 0.01$) than for untreated blood. A graphic comparison of the mean preference responses to each taste stimulus is given in Figure 2.

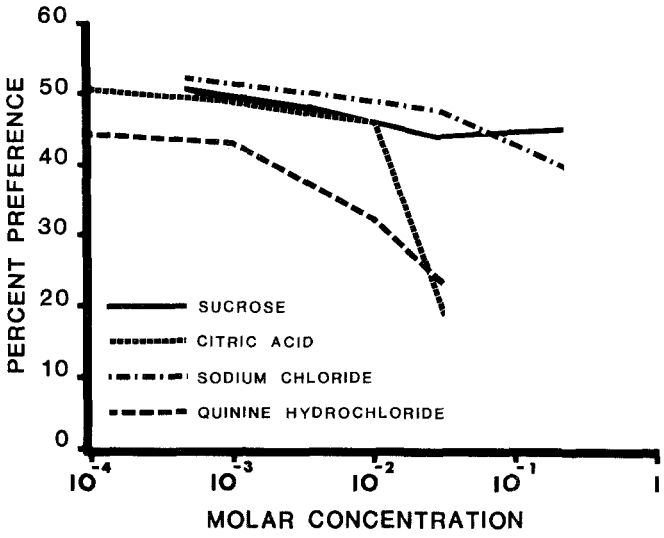

FIG. 2. Mean consumption preference responses of vampire bats to basic taste stimuli of sweet, sour, salty, and bitter.

Our results generally agree with those summarized by Suthers (1970) in that vampires responded to the sweet and salty tastes less than to the bitter and sour ones. The strong avoidance of citric acid at the highest concentration level is also in agreement with Suthers' (1970) report that the response of *Desmodus* to a sour stimulus exceeded that of all other species tested.

Our results do not correspond to the findings of Fishman, who reported (personal communication) no difficulty in recording integrated nerve responses to these stimuli in the same range of concentration. Perhaps the bats detected the sweet or salty tastes but were indifferent to them. Alternately, receptor responses may not have been processed or integrated to the extent necessary to produce a change in preference response. Of course it is frequently difficult to infer or predict behavioral responses from electrophysiological data. For example, the rat shows a relatively low chorda tympani nerve response to quinine or sucrose but both produce very pronounced hedonic preference changes (Fishman, 1971; Shumake et al., 1971).

Our study, and those summarized by Suthers, indicate that vampires can discriminate tastes. The other chemical sense, olfaction, generally serves a variety of functions (e.g., mate selection, avoidance of enemies, guidance), but the function of taste is limited to regulation of ingestion of nutrients and possibly the avoidance of toxic substances (Kare and Ficken, 1963). Because of the extremely specialized diet of the vampire, it is reasonable to assume that the sense of taste in vampires, although present, is not very functional. The

results of our study would tend to support such a hypothesis. If this is true, it is probably a contributive factor to the high efficacy of the systemic method of vampire control described by Thompson et al. (1972) and Bullard and Thompson (1977). The toxicant present in the bovine bloodstream apparently elicits no aversive response from the vampires which would result in cessation of feeding. Efficacy of control with this technique is generally 95% or greater. And, if this hypothesis regarding the vampires' sense of taste is valid, then it follows that prey selection is due to other factors such as those discussed at the beginning of this report.

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MISIDENTIFICATION BY WILD RABBITS, *Oryctolagus cuniculus,* OF GROUP MEMBERS CARRYING THE ODOR OF FOREIGN INGUINAL GLAND SECRETION
II. Experiments with All-Female Groups

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Abstract—The results of experiments designed to clarify the behavioral function of the odor of the secretion from the inguinal glands of rabbits, *Oryctolagus cuniculus*, are presented. Members of groups of female rabbits were smeared with odoriferous materials, and the behavior of their pen-mates towards them was recorded. Eight sources of natural rabbit odors and a commercial perfume were used in 180 tests involving 120 adult female wild-type rabbits. A high proportion of the animals smeared with the inguinal gland secretions from unfamiliar females were attacked by their pen-mates. In contrast, treatment with inguinal gland secretions from unfamiliar male rabbits; macerated inguinal gland materials from unfamiliar males and females, respectively; urine from unfamiliar females; and macerated anal gland material from unfamiliar females resulted in only minor disruption to the normal social relationships between the group members which was not significantly different to that caused by treatment with a commercial perfume.

Key Words—Wild rabbit, *Oryctolagus cuniculus*, female groups, inguinal glands, strange odors, misidentification.

INTRODUCTION

General observations of the behavioral role of the inguinal gland secretion of rabbits, *Oryctolagus cuniculus*, have indicated that its odor is involved in the identification of individuals, groups, and possibly sex. The aim of the current studies is to gather experimental evidence to substantiate these views.

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An earlier paper of this series dealt with the reaction of all-male groups of rabbits towards members experimentally treated with the inguinal gland secretions from unfamiliar animals (Hesterman and Mykytowycz, 1982). In this paper the results of similar experiments using all-female groups are reported. As in the previous series, sources of odor other than inguinal gland secretions were used for comparison.

METHODS AND MATERIALS

The ethological basis and methodology were the same as those for experiments on all-male groups described in an earlier paper (Hesterman and Mykytowycz, 1982).

Animals. The majority of the 120 adult, female, wild-type rabbits used in the experiments originated from field populations, but some were born in captivity. They were kept in groups of three individuals in 3 × 3 m outdoor pens. A shelter box of standard design was attached to the side of each pen. Water and food, in the form of lucerne hay, were provided ad libitum.

To facilitate visual identification of rabbits by observers the fur of each animal was marked in a distinctive pattern with a black dye.

The social order within each group was assessed by frequent observations of the rabbits' behavior from a watchtower and physical examination of animals for injuries and general condition. Groups were used for experimentation only after the dominance hierarchy had been completely stable for at least two months.

Sources of Odor. Nine sources of odor were tested. These were: the secretions from the inguinal glands of the lowest-ranking animals in each group; the inguinal gland secretions from unfamiliar males and females, respectively; urine from unfamiliar female rabbits; a commercially available perfume; and macerated anal gland materials and macerated inguinal gland materials from unfamiliar males and females.

Macerated anal gland materials were used in an attempt to obtain information on the behavioral effect of this source of odor since it is impractical to obtain pure anal gland secretions in the quantities necessary for the experiments. Macerated inguinal gland materials were also included as a check on the efficacy of this means of obtaining gland secretions.

Preparation of the odor sources other than the macerated gland materials was identical to that described for the all-male group experiments.

Macerated Inguinal Gland Materials. Inguinal glands were removed from freshly shot adult rabbits and frozen in the field. In the laboratory one composite gland (sebaceous and sudoriferous parts) from each of 20 animals was cleaned of nonglandular tissues and the total collection weighed. During the removal and cleaning of the glands, care was taken to ensure that they were

not contaminated with any sebum from the inguinal sacs. The glands were macerated and mixed with an appropriate volume of water to give a suspension containing 50 mg gland material per milliliter. Aliquots of 0.5 ml of the suspension were sealed into glass ampules and stored at −18° C until required.

Macerated Anal Gland Materials. The anal glands were collected in the same way as the inguinal glands. Only the anterior lobes of the anal glands were used, since it is extremely difficult to separate the posterior parts from the muscle and connective tissues. The anterior lobes from 20 animals were macerated to produce an aqueous suspension containing 50 mg gland material per milliliter. The suspension was divided into 0.5-ml aliquots and stored at −18° C until required.

Selection of Animals and Application of Odors. Preliminary observations showed that, as in the all-male groups, only higher ranking individuals would react uninhibitedly towards odor-smeared companions. The odors under test were therefore always applied to the lowest-ranking individuals of the groups since this option promised to be the most sensitive in revealing any effects of the treatments.

All odor preparations were applied by means of a cottonwool swab and were rubbed thoroughly into the fur of the hindquarters, flanks, and head of the selected rabbits.

Experimental Procedures. Testing procedures were identical to those described for the all-male series. The odor under test was applied to the third-ranking individual without removing it from the shelterbox and with minimum possible disturbance to the other two animals. The behavior of the group was then observed for 30 min, starting from the time when any member of the group emerged from the shelter box.

The incidences and durations of the following forms of behavior for each rabbit were recorded: agonistic behavior (chasing, biting), following other rabbits, sniffing at other rabbits, mounting, grooming, and exploration. The data were recorded by one observer using a multichannel event recorder.

To assess the long-term effects of the odor applications, all rabbits were examined on each of the three days following the test and their body weights, general conditions, and fresh injuries were recorded.

The nine odor sources were each tested on 20 groups during three separate periods. Tests with the smeared animals' own inguinal gland secretions, perfume, male inguinal gland secretion, and female inguinal gland secretion were carried out over a period of 30 days. After a 14-day interval the male macerated anal gland, female macerated anal gland, male macerated inguinal gland, and female macerated inguinal gland materials were tested over a further period of 45 days. The same 20 groups of females were employed in the tests of the above-listed odor sources. The female urine was tested separately on another occasion using other groups of females.

Treatment of Data. Only the incidences of attacks and injuries are considered in this report. The more detailed data collected during these experiments together with those from the all-male group series and the results of further experiments on mixed-sex groups will be presented elsewhere.

Statistical comparisons between the incidences of aggression or injuries in the different treatments were carried out by means of the test statistic:

$$T = \frac{p_1 - p_2}{\sqrt{\frac{npq}{n-1}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where p_1 = proportion of tests in which aggression or injuries occurred in treatment 1.

p_2 = proportion of tests in which aggression or injuries occurred in treatment 2.

p = proportion of tests in which aggression or injuries occurred in treatments 1 and 2 combined.

q = proportion of tests in which aggression or injuries did not occur in treatments 1 and 2 combined.

n_1 = number of tests in treatment 1.

n_2 = number of tests in treatment 2.

n = number of tests in treatments 1 and 2 combined.

RESULTS AND DISCUSSION

The number of tests in which rabbits were attacked or injured are shown in Figure 1 and Table 1.

From Figure 1 it can be seen that the lowest frequency of aggression towards the smeared animals occurred when their own inguinal gland secretions were used as the source of odor (1 test). There were slightly, but not significantly, higher frequencies of aggression in the tests using perfume (5 tests), urine, macerated anal glands, and macerated inguinal glands from unfamiliar females, and macerated inguinal glands and inguinal gland secretions from unfamiliar males (2–4 tests). Treatment with macerated anal glands from unfamiliar males produced a moderately high frequency of aggression towards the smeared rabbits (10 tests), but this is not significantly different ($P > 0.05$) from the results for perfume. However, the difference between the number of tests in which aggression was displayed towards the smeared and first- or second-ranking (unsmeared) animals is significant ($P < 0.05$).

Only treatment with strange female inguinal gland secretion resulted in an unequivocal increase in the frequency of aggression displayed by pen-

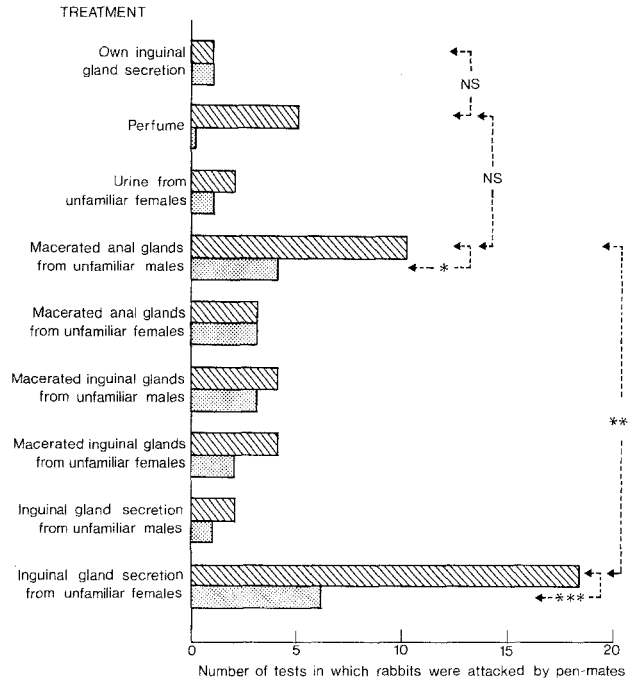

FIG. 1. The number of tests in which rabbits, *Oryctolagus cuniculus*, smeared with various odors were attacked by pen-mates in all-female groups. Comparable data for unsmeared members of the groups are also shown. $N = 20$ for each odor treatment. Hatched columns = smeared rabbit. Stippled columns = unsmeared rabbit. NS = not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

mates towards the smeared females (18 tests). This frequency is significantly greater than that observed in the tests with male macerated anal gland ($P < 0.01$) and is highly significantly different from the frequency of aggression directed towards the unsmeared animals in the same tests ($P < 0.001$).

Table 1 shows the numbers of dominant and second-ranking females seen to attack the smeared rabbits as well as the separate data on injuries. The dominant females were more frequently aggressive towards the treated animals than the second-ranking does. This difference is most obvious and statistically significant ($P < 0.05$) in the tests with inguinal gland secretion from unfamiliar females.

A high proportion of the does smeared with inguinal gland secretion from unfamiliar females were subsequently found to have sustained injuries (14 out of 20). Treatment with the macerated anal gland material from males also resulted in three of the does receiving injuries, but this was a significantly

TABLE 1. NUMBER OF TESTS IN WHICH SUBORDINATE RABBITS, *Oryctolagus cuniculus*, SMEARED WITH VARIOUS ODORS WERE ATTACKED BY DOMINANT AND SECOND-RANKING PEN-MATES OR SUSTAINED INJURIES WITHIN THREE DAYS OF TREATMENT[a]

	Number of tests			
	Observed attacks			
Odor	By dominant	By second ranker	Injured	
Subordinate females' own inguinal gland secretion	1	0	0	
Perfume	5	0	0	
Strange female urine	2	1	0	
Strange male macerated anal gland	6	3	3	
Strange female macerated anal gland	2	2	0	
Strange male macerated inguinal gland	4	1	0	
Strange female macerated inguinal gland	4	0	0	**
Strange male inguinal gland secretion	2	0	0	
	*[b]			
Strange female inguinal gland secretion	15	6	14	

[a]Twenty tests were performed in each odor treatment.
[b]$*P < 0.05$; $**P < 0.01$.

lower incidence ($P < 0.01$) than in the tests with inguinal gland secretion from females.

Thus, both the frequency of attacks and the resulting injuries indicate that the females reacted very aggressively towards subordinate group members carrying the odor of inguinal gland secretions from unfamiliar does.

Surprisingly, the inguinal gland secretion from unfamiliar males did not provoke an aggressive reaction. This is in contrast to our earlier observations on the territorial behavior of mixed-sex pairs of rabbits where the sex of an intruder did not affect aggressive responses by the resident females (Mykytowycz and Hesterman, 1975).

In this respect the results also differ from those obtained with all-male groups in which the pen-mates attacked subordinates carrying the odor derived from the inguinal gland secretion of strange females as well as strange males. It seems that the appearance of a male in all-female groups is at least tolerated, if not welcomed. These observations are consistent with the suggestion that the presence of males is necessary to stimulate sexual activity in female rabbits (Myers and Poole, 1962), and the observation that in mice, the odor of males induces estrus in females (Bronson, 1979).

The difference in the reactions of females to pen-mates smeared with

inguinal gland secretions from male and female rabbits shows clearly that the odor from this source contains information which can be used to identify the sex of the donor animal.

The failure in this study of the macerated inguinal glands to release aggression seems to support the view that bacterial action on the sebum in the inguinal pouches may be essential for the formation of odor signals (Albone and Perry, 1976). The results of the tests with male macerated anal gland materials are not clear-cut. Although treatment with this source of odor did not induce a significantly greater frequency of aggression towards the smeared females than occurred in the perfume tests, the difference between smeared and unsmeared animals within the anal gland series is just significant ($P < 0.05$). However, the evaluation of the results from the tests with macerated gland materials—both inguinal and anal—must be tentative in view of the fact that the concentrations of active components contained in the preparations compared to the pure gland secretions is not known.

Since the results of a more detailed statistical treatment of the other behavioral parameters measured during the course of these tests, together with those from experiments with all-male and mixed-sex groups, will be presented in another paper, lengthy discussion of the results presented so far is premature. It is important to point out, however, that the data presented here for all-female groups support the hypothesis that the odor from the inguinal glands of rabbits functions for the purposes of individual or group recognition. There is strong evidence that rabbits can also identify the sex of other individuals from their inguinal gland odor.

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STRUCTURE–ACTIVITY RELATIONSHIPS IN SEX ATTRACTANTS FOR NORTH AMERICAN NOCTUID MOTHS[1]

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Abstract—Sex attractants known for 145 species of noctuid moths have many common features both as to chemical constituents and to their relationships in blends. The great majority of constituents are straight-chain (*Z*)-alkenols, -alkenals, or -alkenyl acetates of even carbon number (10 through 16). The unsaturation is nonterminal in odd-numbered positions (5 through 11). In effective lures, these components are blended in specific ratios and the components in a sex pheromone or sex attractant blend are structurally related by "one-change" steps. This means that any blend component differs from one or more other components by a single structural alteration, such as a change in double bond position, or a change in carbon chain length, or a change in the oxygen function. For the few multicomponent systems known in detail, the central place in the "one-change" framework is occupied by the predominant blend component. Different patterns of occurrence of lure components occur in the subfamilies Acronictinae, Noctuinae, Hadeninae, Cuculliinae, Amphipyrinae, Heliothidinae, Plusiinae, Acontiinae, and Pantheinae, and some subfamilies are as yet without known lures. Some guiding principles for elucidation of blend compositions for unstudied species are presented; these guidelines can also be used in improvement of some synthetic blends of unsatisfactory quality.

Key Words—Alkenyl compounds, pheromones, chemotaxonomy, decenyl, dodecenyl, tetradecenyl, hexadecenyl, trapping, Lepidoptera, Noctuidae.

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INTRODUCTION

Since 1960 the discovery of chemical systems which can attract male moths over long distances has gone on at an ever-increasing rate. Many sex pheromone blends have been isolated and identified from insect species, and many synthetic sex attractant blends have been demonstrated to behave like sex pheromones. In some cases an artificial blend has subsequently been shown to be essentially the natural pheromone. In this paper the term sex pheromone is used to mean an attractive (or coattractive) compound or compounds shown to be produced by the responding species, and sex attractant to mean any similarly biologically active chemical system whether naturally occurring or not. Moreover it can be assumed that in every case presented here, the virgin female is the pheromone producer and the males the entities attracted by sex pheromones or sex attractants. Since most available information on "attraction" still derives from field trapping data, lure power has here been taken as the prime criterion of sex attractant activity, and field trapping with all its shortcomings has been accepted here as a method for assessing the potency of chemical systems. The topic of this article is not, therefore, the behavioral biology of moths in attractant systems, but rather some aspects of the structural chemistry of successful sex attractants. We believe that an appreciation of these aspects in noctuids can assist future identifications of sex pheromone systems in the Lepidoptera.

The Noctuidae family (Lepidoptera) comprises about 1500 species in temperate North America and includes cutworms, armyworms, and some loopers. Most of these species are rare or uncommon, and only a few percent are recognized as significant pests. Adults of most species are nocturnal fliers, although some crepuscular and a few diurnal species (e.g., *Heliothis ononis* D.&S.) are known. Taxonomy of the family has exercised many workers, with highly divergent results (Dyar, 1902; McDunnough, 1938; Forbes, 1954). The system used in this paper is that of McDunnough as modified in the Canadian National Collection and exemplified in Rockburne and Lafontaine (1976). Some common synonyms have, however, been retained, for example: *Euxoa altera* for *E. lutulenta*; *Heliothis zea* for *Helicoverpa zea*; and *Feltia ducens* for *F. jaculifera*.

NATURE AND SOURCES OF DATA

Knowledge of sex attractant systems for lepidoptera has developed mainly by two parallel routes: (1) identification of sex pheromones, and (2) field screening of chemicals for attractancy. In the former route the species is targeted and the chemical identities are unknowns at the outset; in the latter route the chemicals are known but the species to be attracted are not specified.

TABLE 1. STRUCTURAL GRID OF COMPOUNDS BIOLOGICALLY ACTIVE IN NOCTUID SEX ATTRACTANT BLENDS[a]

Carbon chain length	Double bond position					
	3	5	7	9	11	13
Acetates						
10	0	10	0	0	–	–
12	0	10	44	5	0	–
14	0	6	15	59	7	0
16	0	1	4	4	40	0
18	0	0	0	0	0	0
Aldehydes						
10	0	0	0	0	–	–
12	0	0	2	0	0	–
14	0	0	3	8	2	0
16	0	0	2	4	22	0
18	0	0	0	0	0	0
Alcohols						
10	0	0	0	0	–	–
12	0	0	7	0	0	–
14	0	0	0	2	1	0
16	0	0	0	1	20	0
18	0	0	0	0	0	0

[a]Numbers are frequency of occurrence of the compound in Table 2.

Compounds shown by either of these routes to be attractive or coattractive to noctuid male moths are summarized in Table 1. The general distribution of compounds indicated in this table has already been noted by others (e.g., Priesner et al., 1975; Roelofs, 1979; Steck et al., 1979d).

Caution must be used in interpreting field screening results because very slight chemical impurities can give rise to false positive or false negative results (Steck et al., 1980b). Note (Table 2) that many noctuid blends have optimal minor component contents of 1% or less; contents of 0.1% or even 0.01% are quite sufficient for biological activity. Since most chemical lure components have, historically, been less than 99.9% pure even before possible degradation in the field, we suspect that some data in the literature result from chemical contaminants. In the case of positive trapping results with impure (or at least undefined) lures, the attraction may be entirely due to the major lure component, may be caused by some one impurity, or may arise from a fortuitous multicomponent effect. Possible field degradation of highly pure or well-defined lures introduces yet another complication which presumably will vary with the chemical nature of the lure (e.g., monoene or diene), the releaser system used, and the physical environment in which the observations are

made (e.g., humid or arid regions, the former favoring hydrolytic degradation).

To be recognized as attractive in trapping tests, a chemical or blend must consistently lure male moths into traps in numbers significantly greater than are obtained in unbaited control traps. Where blends are involved, a constituent may be regarded as coattractive if its presence in the lure blend results in significantly increased trapping relative to that with lures lacking the component. Some preconditions must be met in the testing process; for example, that the chemicals used are sufficiently pure for the purpose; that purely mechanical factors, such as "carrier" or "ballast" effects on blend release, are not introduced during tests; or that the traps used are compatible with the purposes of the test. The term "significantly greater" is ultimately arbitrary and depends in part upon the trap design used. With most species, "significance" may be attached to very small numbers of captures; lure efficiency is quite a different matter which requires some independent assessment of the available population. Most of our laboratory's trapping work has employed horizontal cone-orifice live traps (Steck and Bailey, 1978) which, because moths are required to land and enter in very close proximity (2-3 cm) to the dispenser, can furnish some measure of the postflight stages of male response. Of course, for success they must be so baited as to elicit a fairly full spectrum of male response steps. In addition, such traps almost completely exclude chance entry of moths.

We have reexamined and confirmed many previously reported noctuid lures, using high-purity materials in field trapping tests. In many cases we have been able to improve earlier systems or to provide better-defined synthetic blends. For the purposes of this manuscript, however, we have accepted at face value all literature reports for which no evaluation has been possible.

Besides field trapping and pheromone isolation, the techniques of electrophysiology (receptor site identification and electroantennal scans) and behavioral analysis have provided additional means of assessing the potential and actual responses of male noctuids to pheromone-like chemicals.

GENERAL CHARACTERISTICS OF NOCTUID SEX ATTRACTANTS

Table 2 lists sex attractants for about 145 noctuid species found in North America. Underlying many of these data are electroantennogram studies (e.g., Chisholm et al., 1975) and chemical isolations as well as field trapping results. Representing only ca. 10% of the available species, the table is incomplete indeed, but from it some generalizations can be made about the chemical compounds in noctuid sex attractants and the sex attractant blends as systems.

TABLE 2. SEX ATTRACTANTS FOR SOME NORTH AMERICAN NOCTUID MOTHS, LISTED BY SUBFAMILIES[a]

Species	Best known attractant	References
Subfamily Acronictinae		
Acronicta grisea (Walker)	*Z*7-12 : Ac + *Z*9-12 : Ac (M)	Steck et al., 1977a
Simyra henrici (Grote)	*Z*7-14 : Ald (L)	
Subfamily Noctuinae		
Actebia fennica (Tauscher)	1 : 1 *Z*11-14 : Ac + *Z*7-12 : Ac (L)	Steck et al., 1979d (A)
Agrotis ipsilon (Hufnagel)	5 : 1 *Z*7-12 : Ac + *Z*9-14 : Ac (M)	Hill et al., 1979 (P)
A. orthogonia (Morrison)	5 : 1 *Z*7-12 : Ac + *Z*5-12 : Ac (H)	Struble and Swailes, 1978 (A)
A. obliqua (Smith)	*Z*5-14 : Ac (L)	
A. venerabilis (Walker)	100 : 10 : 1 *Z*5-10 : Ac + *Z*7-12 : Ac + *Z*7-12 : Ald (H)	Steck et al., 1979c (A)
A. vetusta (Walker)	50 : 1 *Z*11-16 : Ac + *Z*9-14 : Ac (M)	
A. volubilis (Harvey)	100 : 1 *Z*5-14 : Ac + *Z*7-14 : Ac (L)	
Amathes c-nigrum (L.)	*Z*7-14 : Ac (L)	Roelofs and Comeau, 1970 (A) Bestmann et al., 1979 (P)
	20 : 1 *Z*7-14 : Ac + *Z*5-14 : Ac (H)	Steck et al., 1980b (A)
A. collaris (G & R)	1 : 1 *Z*7-14 : Ac + *Z*9-14 : Ac (M)	Steck et al., 1979d (A)
Chersotis juncta (Grote)	*Z*11-16 : Ac + *Z*11-16 : Ald (L)	
Cryptocala acadiensis (Bethune)	*Z*11-16 : Ac + *Z*9-14 : Ac + *Z*9-14 : Ald (L)	
Euagrotis tepperi (Smith)	10 : 1 : 1 *Z*11-16 : Ac + *Z*9-14 : Ac + *Z*7-12 : Ac (H)	
Eurois astricta (Morrison)	1 : 5 : 5 *Z*11-16 : Ac + *Z*9-14 : Ac + *Z*11-16 : Ald (M)	Underhill et al., 1977b (A)
E. occulta (L.)	30 : 100 : 1 *Z*11-16 : Ac + *Z*9-14 : Ac + *Z*11-16 : Ald (H)	Steck et al., 1976 (A)
Euxoa acornis (Smith)	4 : 1 : 5 *Z*9-14 : Ac + *Z*9-14 : Ald + *Z*11-16 : Ac (H)	Underhill et al., 1977b (A)
E. albipennis (Grote)	10 : 1 : 1 *Z*5-10 : Ac + *Z*7-12 : Ac + *Z*7-12 : OH (H)	Steck et al., 1979c (A)

TABLE 2. (Continued)

Species	Best known attractant	References
E. altera (Smith)	50:1 Z11-16:Ac + Z9-14:Ac (M)	
E. auxiliaris (Grote)	100:1:10 Z5-14:Ac + Z7-14:Ac + Z9-14:Ac (H)	Struble and Swailes, 1977a (A) Struble, 1981b (A)
E. basalis (Grote)	5:5:1 Z7-12:Ac + Z7-12:OH + Z5-12:Ac, *or* 5:5:1 Z7-12:Ac + Z7-12:OH + Z5-10:Ac (H)	
E. campestris (Grote)	Z5-10:Ac (L)	Underhill et al., 1981 (A)
E. cicatricosa (G. & R.)	Z7-12:Ac (L)	
E. dargo (Strecker)	9:1 Z9-14:Ac + Z11-16:Ac (L)	
E. declarata (Walker)	Z5-10:Ac (M)	Underhill et al., 1981 (A)
E. divergens (Walker)	200:1 Z9-12:Ac + Z7-12:Ac (L)	
E. drewseni (Staudinger)	Z7-14:Ac + Z5-12:Ac	Struble and Swailes, 1978 (A)
E. flavicollis (Smith)	1000:1 Z9-14:Ac + Z7-12:Ac (H)	
E. manitobana (McDunnough)	4:1 Z9-14:Ac + Z7-14:Ac (M)	
E. messoria (Harris)	20:1 Z11-16:Ac + Z7-16:Ac (H)	Struble et al., 1977a (A)
E. mimallonis (Grote)	Z5-10:Ac (L)	
E. obeliscoides (Guenée)	500:1 Z9-14:Ac + Z7-12:Ac (M)	
E. ochrogaster (Guenée)	500:5:2:1 Z5-12:Ac + Z7-12:Ac + Z9-12 Ac + Z5-10:Ac (H)	Steck et al., 1980a (A); Steck et al., 1982 (A)
	200:2:1:1–200:6:2:1 (same compounds) (H)	Struble et al., 1980 (P); Struble, 1981a (A)
E. olivia (Morrison)	Z5-10:Ac (L)	Underhill et al., 1981 (A)
E. perolivalis (Smith)	4:5:1 Z11-16:Ac + Z9-14:Ac + Z11-16:OH	Struble et al., 1977b (A)
E. pestula (Grote)	100:1:1 Z5-10:Ac + Z7-12:Ac + Z7-12:Ald (M)	
E. plagigera (Morrison)	5:1 Z9-14:Ac + Z9-14:OH (M)	

E. pleuritica (Grote)	5 : 1 : 1 *Z*7–12 : Ac + *Z*7–12 : OH + *Z*5–12 : Ac, *or* 5 : 1 : 1 *Z*7–12 : Ac + *Z*7–12 : OH + *Z*5–10 : Ac (H)	Steck et al., 1978 (A)
E. ridingsiana (Grote)	500 : 20 : 1 *Z*9–14 : Ac + *Z*11–16 : Ac + *Z*7–12 : Ac(H)	
E. rockburnei (Hardwick)	*Z*5–10 : Ac (L)	Underhill et al., 1981 (A)
E. scandens (Riley)	*Z*7–12 : OH (L)	
E. servita (Smith)	*Z*7–14 : Ac + *Z*7–14 : Ald (L)	
E. tessellata (Harris)	1 : 1 *Z*7–16 : Ac + *Z*5–16 : Ac, *or* 200 : 1 *Z*7–16 : Ac + *Z*5–14 : Ac (H)	Struble et al., 1981 (A)
E. tristicula (Morrison)	200 : 1 : 500 *Z*9–12 : Ac + *Z*9–14 : Ac + *Z*7–12 : Ac (M)	Steck et al., 1979a (A)
Feltia ducens (Walker)	2000 : 1 : 1 *Z*11–16 : Ac + *Z*9–14 : Ac + *Z*7–12 : Ac (M)	
Paradiarsia littoralis (Packard)	100 : 1 *Z*7–12 : Ac + *Z*5–12 : Ac (L)	
Peridroma saucia (Hübner)	1 : 1 *Z*11–16 : Ac + *Z*9–14 : Ac (M)	Struble et al., 1976 (A)
Spaelotis clandestina (Harris)	*Z*7–14 : Ald (M)	Steck et al., 1979d (A)
Subfamily Hadeninae		
Aletia oxygala (Grote)	20 : 1 *Z*11–16 : Ald + *Z*11–16 : Ac (M)	
Anhimella contrahens (Walker)	10 : 1 *Z*11–16 : Ald + *Z*11–16 : OH (M)	
Ceramica picta (Harris)	200 : 2 : 1 *Z*11–14 : Ac + *Z*11–16 : Ac + *Z*9–14 : Ac (H)	
Faronta diffusa (Walker)	9 : 1 *Z*11–16 : Ac + *Z*11–16 : Ald (M)	Underhill et al., 1977b (A)
Hypocoena rufostrigata (Packard)	100 : 1 : 1 *Z*11–16 : Ald + *Z*11–16 : OH + *Z*9–14 : Ald (M)	
Lacinipolia lorea (Guenée)	*Z*7–14 : Ac (L)	Roelofs and Comeau, 1971 (A)
L. renigera (Stephens)	*Z*9–14 : Ac (L)	
L. vicina (Grote)	5 : 5 : 1 *Z*11–16 : Ac + *Z*9–14 : Ac + *Z*7–14 : Ac (H)	
Leucania commoides (Guenée)	4 : 4 : 1 *Z*11–16 : Ac + *Z*9–14 : Ac + *Z*11–16 : OH (H)	Struble et al., 1977b (A)
L. linda (Franclemont)	19 : 1 *Z*9–14 : Ac + *Z*11–14 : Ac	Roelofs et al., 1976 (A)

TABLE 2. (Continued)

Species	Best known attractant	References
L. multilinea (Walker)	4:4:1 *Z*11-16:Ac + *Z*9-14:Ac + *Z*11-16:OH (H)	Struble et al., 1977b (A)
L. phragmatidicola (Guenée)	*Z*9-14:Ac	Roelofs and Comeau, 1971 (A)
Mamestra configurata (Walker)	19:1 *Z*11-16:Ac + *Z*9-14:Ac (H)	Underhill et al., 1977a (P)
Morrisonia confusa (Hübner)	*Z*11-16:Ac	Roelofs and Comeau, 1970 (A)
Nephelodes minians (Guenée)	*Z*11-16:Ald (L)	Underhill et al., 1977b (A)
Orthodes crenulata (Butler)	*Z*11-16:Ac	Roelofs and Comeau, 1970 (A)
Orthosia hibisci (Guenée)	*Z*9,*E*12-14:Ald; *or* *Z*9-14:Ald (H); 100:1 *Z*9-14:Ald + *Z*11-14:Ald (H)	Hill and Roelofs, 1978 (A)
Polia assimilis (Morrison)	100:1 *Z*11-14:Ac + *Z*9-14:Ac (H)	Steck et al., 1982 (A)
P. atlantica (Grote)	1000:5:1 *Z*11-16:Ac + *Z*11-16:Ald + *Z*11-14:Ald (H)	Steck et al., 1980b (A)
P. discalis (Grote)	10:1 *Z*9-14:Ac + *Z*11-14:Ac (M)	
P. grandis (Boisduval)	*E*9-14:Ac	Roelofs and Comeau, 1971 (A)
P. ingravis (Smith)	100:1 *Z*9-14:Ac + *Z*11-16:Ac (M)	Steck et al., 1982 (A)
P. lilacina (Harvey)	10:1 *Z*7-14:Ac + *Z*9-14:Ac (M)	
P. nevadae (Grote)	*Z*11-16:Ac + *Z*11-16:Ald (L)	
P. purpurissata (Grote)	100:1 *Z*9-14:Ac + *Z*7-14:Ac (L)	
P. segregata (Smith)	10:1 *Z*7-14:Ac + *Z*11-14:Ac (M)	
P. tacoma (Strecker)	100:1 *Z*9-14:Ac + *Z*9-14:Ald (H)	
Pseudaletia unipuncta (Haworth)	*Z*11-16:Ac + *Z*11-16:OH (M)	Hill and Roelofs, 1980 (P:*Z*11-16:Ac);
	5000:10:2:1 *Z*11-16:Ac + *Z*11-16:OH + *Z*11-16:Ald + *Z*9-14:Ac (H)	McDonough et al., 1980 (P:*Z*11-16:Ac + *Z*11-16:OH); Steck et al., 1980b (A)
Pseudorthodes vecors (Guenée)	*Z*11-16:Ac	Roelofs and Comeau, 1970 (A)
Scotogramma farnhami (Grote)	100:1:1 *Z*11-16:Ald + *Z*9-14:Ald + *Z*9-16:Ald (M)	Steck et al., 1982 (A)
Scotogramma trifolii (Rottemburg)	9:1 *Z*11-16:Ac + *Z*11-16:OH (H)	Struble and Swailes, 1975 (A); Underhill et al., 1976 (P)

Sideridis rosea (Harvey)	10:1 *Z*9–14:Ac + *Z*11–14:Ac; *or* 2:1 *Z*9–14:Ac + *Z*11–16:Ac (M)	Struble et al., 1976 (A)
Xylomyges curialis (Walker)	*Z*11–16:OH	McDonough et al., 1982 (P)
X. dolosa (Grote)	*Z*7–12:Ac (L)	Steck et al., 1979d (A)
Subfamily Cuculliinae		
Cucullia florea (Guenée)	*Z*9–14:Ac (L)	
C. intermedia (Speyer)	*Z*9–14:Ac (L)	Roelofs and Comeau, 1970 (A)
C. omissa (Dod)	*Z*9–14:Ac (L)	
C. postera (Guenée)	100:1 *Z*9–14:Ac + *Z*9–14:Ald (M)	
C. speyeri (Lintner)	*Z*9–14:Ac (L)	
Fishia hanhami (Grote)	8:1 *Z*5–14:Ac + *Z*9–14:Ac (H)	Steck et al., 1979d (A)
F. derelicta (Hampson)	3:1 *Z*7–12:Ac + *Z*9–14:Ac (M)	Underhill et al., 1977b (A)
Homohadena infixa (Walker)	200:1 *Z*7–14:Ac + *Z*9–14:Ac (H)	Steck et al., 1982 (A)
Lithomoia solidaginis (Hübner)	*Z*11–16:Ald (L)	Underhill et al., 1977b (A)
Lithophane unimoda (Lintner)	*Z*9–14:Ac (L)	
L. thaxteri (Grote)	*Z*9–14:Ac (L)	Steck et al., 1979d (A)
Oncocnemis lepipuloides (McDunnough)	*Z*7–12:Ac + *Z*9–14:Ac (M)	
O. piffardi (Walker)	500:1 *Z*9–14:Ac + *Z*7–12:Ac (M)	Steck et al., 1982 (A)
Parastichtis discivaria (Walker)	*Z*11–16:Ac + *Z*11–16:Ald (L)	
Pyreferra citrombra (Franclemont)	*Z*7–12:Ac + *Z*9–14:Ac	Roelofs and Comeau, 1971 (A)
Rancora albida (Guenée)	*Z*9–14:Ac (L)	
Sunira bicolorago (Guenée)	*Z*11–16:Ac + *Z*9–16:Ac (L)	
Sutyna profunda (Smith)	*Z*11–16:Ac + *Z*9–16:Ac (L)	
Xanthia lutea (Ström)	*Z*9,*E*12–14:Ald (L)	
Subfamily Plusiinae		
Anagrapha falcifera (Kirby)	10:1 *Z*7–12:Ac + *Z*7–12:OH (M)	Steck et al., 1979b (A)
Argyrogramma verruca (Fabricius)	94:6 *Z*7–12:Ac + *E*7–12:Ac	McLaughlin et al., 1975 (A)
Autographa ampla (Walker)	*Z*7–12:Ac	Roelofs and Comeau, 1970 (A)
A. biloba (Stephens)	*Z*7–12:Ac	Roelofs and Comeau, 1970 (A)
A. egena (Guenée)	*Z*5–12:Ac	Kaae et al., 1973 (A)
A. californica (Speyer)	*Z*7–12:Ac + *Z*7–12:Formate	Butler et al., 1977 (A)
	10:1 *Z*7–12:Ac + *Z*7–12:OH (H)	Steck et al., 1979b (A)

TABLE 2. (Continued)

Species	Best known attractant	References
A. flagellum (Walker)	*Z*7-12 : Ac + *Z*7-14 : Ac (M)	Steck et al., 1979d (A)
A. precationis (Guenée)	*Z*7-12 : Ac (L)	Roelofs and Comeau, 1971 (A)
Chrysaspidia contexta (Grote)	*Z*7-12 : Ac (L)	Roelofs and Comeau, 1970 (A)
C. putnami (Grote)	100 : 1 *Z*5-12 : Ac + *Z*7-12 : Ac (L)	Steck et al., 1982 (A)
C. venusta (Walker)	1 : 1 *Z*7-12 : Ac + *Z*5-12 : Ac (M)	Steck et al., 1977a (A)
Plusia aereoides (Grote)	*Z*7-12 : Ac (L)	Roelofs and Comeau, 1970 (A)
Pseudoplusia includens (Walker)	*Z*7-12 : Ac	Tumlinson et al., 1972 (P)
Rachiplusia ou (Guenée)	*Z*7-12 : Ac	Roelofs and Comeau, 1970 (A)
Syngrapha epigaea (Grote)	20 : 1 *Z*7-12 : Ac + 14 : Ac (L)	
S. rectangula (Kirby)	*Z*7-12 : Ac (L)	
Trichoplusia ni (Hübner)	93 : 7 *Z*7-12 : Ac + 12 : Ac (M);	Berger, 1966 (P); Bjostad et al., 1980 (P);
	200 : 1 *Z*7-12 : Ac + *Z*7-14 : Ac (M)	Steck et al., 1980b (A)
T. oxygramma (Geyer)	96 : 4 *Z*7-12 : Ac + *E*7-12 : Ac	McLaughlin et al., 1975 (A)
Subfamily Amphipyrinae		
Agroperina cogitata (Smith)	20 : 1 *Z*11-16 : Ac + *Z*9-14 : Ac (M)	
A. dubitans (Walker)	20 : 1 *Z*11-16 : Ac + *Z*9-14 : Ac (M)	Underhill et al., 1977a (A)
Andropolia contacta (Walker)	*Z*7-12 : Ac + *Z*5-12 : Ac (M)	Steck et al., 1977a (A)
Apamea indela (Smith)	200 : 50 : 1 *Z*11-16 : Ac + *Z*11-16 : OH + *Z*11-16 : Ald (M)	Steck et al., 1982 (A)
A. interoceanica (Smith)	*Z*9-14 : Ac (L)	Roelofs and Comeau, 1970 (A)
A. velata (Walker)	*Z*11-16 : Ac	Roelofs and Comeau, 1971 (A)
Crymodes devastator (Brace)	100 : 50 : 2 *Z*11-16 : Ald + *Z*11-16 : Ac + *Z*11-16 : OH (H)	Steck et al., 1980c (A)
Enargia infumata (Grote)	500 : 1 *Z*11-16 : Ac + *Z*9-14 : Ac (M)	
Ipimorpha pleonectusa (Grote)	100 : 1 *Z*11-16 : Ald + *Z*11-16 : OH (M)	
Helotropha reniformis (Grote)	20 : 1 *Z*11-16 : Ac + *Z*7-16 : Ac (L)	Struble et al., 1977a (A)
Nedra ramulosa (Guenée)	*Z*11-14 : OH	Roelofs and Comeau, 1970 (A)
Oligia bridghami (G & R)	500 : 1 *Z*11-16 : Ac + *Z*11-16 : OH (L)	

O. mactata (Guenée)	500:1 Z11-16:Ac + Z11-16:OH (L)	
Protagrotis niveivenosa (Grote)	Z11-16:Ald + Z9-14:Ac (L)	
Spodoptera dolichos (Fabricius)	Z9,E12-14:Ac	Mitchell and Tumlinson, 1973 (A)
S. eridania (Cramer)	4:1 Z9-14:Ac + Z9,E12-14:Ac	Jacobson et al., 1970 (P): Mitchell and Doolittle, 1976 (not A)
S. exigua (Hübner)	Z9,E12-14:Ac	Brady and Ganyard, 1972 (P)
S. frugiperda (J.E. Smith)	10:1 Z9-12:Ac + Z9-14:Ac	Sekul and Sparks, 1967, 1976 (P); Jones and Sparks, 1979 (P)
S. praefica (Grote)	Z7-12:Ac	Birch, 1977 (P)
Subfamily Heliothidinae		
Heliothis ononis (D & S)	Z11-16:Ald (L)	
H. phloxiphaga (G & R)	Z11-16:Ald (L)	Kaae et al., 1973 (A)
H. subflexa (Guenée)	Z9-16:Ald + Z11-16:Ald + Z9-16:Ac + Z11-16:Ac + Z7-16:Ac[+ Z9-16:OH + Z11-16:OH]	Teal et al., 1981 (P)
H. virescens (Fabricius)	ca. 30:1 Z11-16:Ald + Z9-14:Ald	Roelofs et al., 1974 (P); Tumlinson et al., 1975 (P);
	Z11-16:Ald + Z9-14:Ald[+ Z9-16:Ald, Z7-16:Ald + Z11-16:OH + 14:Ald + 16:Ald]	Klun et al., 1979, 1980b (P)
H. zea (Boddie)	ca. 60:1 Z11-16:Ald + Z9-16:Ald [+ Z7-16:Ald + 16:Ald]	Klun et al., 1979, 1980a (P)
Schinia bina (Guenée)	7:3 Z11-16:Ald + Z11-16:Ac (L)	Underhill et al., 1977b (A)
S. meadi (Grote)	Z11-16:Ald (L)	
Subfamily Acontiinae		
Lithacodia albidula (Guenée)	Z5-12:Ac + Z7-14:Ac (L)	
Subfamily Pantheinae		
Raphia frater (Grote)	Z7-12:OH (L)	Weatherston et al., 1974

[a]In references, (A) indicates a synthetic sex attractant, (P) a sex pheromone confirmed synthetically as the attractant demonstrated. Not included are subfamilies such as Agaristinae, Catocalinae, Euteliinae, and Sarrothripinae, which have no known attractants. References are the latest or best sources for more detailed descriptions of species attractants but may in some cases refer to blends less complex than the ones listed in column 2. Unreferenced systems are unpublished work from the authors' laboratory. Systems examined by the authors have been assigned letters L (low), M (medium), or H (high) roughly—and perhaps subjectively—indicating the lure potency as assessed against light traps or other population indicators.

Chemical Components. All the known attractant chemicals are straight-chain alcohols, acetates, or aldehydes, the functional group being always on a terminal carbon atom. No branched noctuid pheromones are reported in the literature, although some compounds have electrophysiological activity (Priesner et al., 1977).

The carbon chains known are C_{10}, C_{12}, C_{14} and C_{16}; odd-numbered chain lengths often can substitute but yield inferior systems. All isolated sex pheromones have even-numbered chains.

In the vast majority of cases a single double bond is present in the molecule. A few dienes, notably in *Spodoptera* species, are sex pheromone components, but it should be noted that almost all dienes reported attractive for noctuids are of a type recently shown capable of substituting in some respects for monoolefinic constituents (Chisholm et al., 1980). Some diolefinic sex pheromones are well known in Old World noctuids: (9*Z*,11*E*)-9,11-tetradecadienyl acetate and (9*Z*,12*E*)-9,12-tetradecadienyl acetate from *Spodoptera littoralis* (Boisduval) and *S. litura* (Fabricius) (Tamaki and Yushima, 1974); (*E*)-9,11-dodecadienyl acetate from *Diparopsis castanea* (Hampson) (Nesbitt et al., 1975); and (10*E*,12*E*)-10,12-hexadecadienal from *Earias insulana* (Kehat et al., 1979; Hall et al., 1980). Also, there are indications that some North American species including *Apamea cinefacta* (Grote) and *Oncocnemis chandleri* (Grote) respond only to diolefinic attractants (Chisholm et al., unpublished results).

The double bond configuration is *Z*—that is, *cis*—in almost every noctuid sex attractant, and in all noctuid sex pheromones the attractive components are *Z* isomers.

The unsaturation is located in an odd-numbered position on the chain, but a position not lower than 5 nor higher than 11 as counted from the functional group. Terminal unsaturation is not known in North American species.

The main compounds so far known to be involved in noctuid sex attraction thus form a tightly limited structural group. Table 1 shows this in a checkerboard of chain length vs. *Z* unsaturation point, with the number in each square the frequency of occurrence of that radical in Table 2. The majority (75%) of all the attractant chemicals are accounted for by just three radicals: (*Z*)-7-dodecenyl, (*Z*)-9-tetradecenyl, and (*Z*)-11-hexadecenyl. Frequency distribution is so graded as to suggest that few if any new attractant molecules of this type will be discovered among noctuids of the subfamilies shown in the table. Some peripheral radicals such as (*Z*)-3-decenyl, (*Z*)-3-dodecenyl, (*Z*)-11-octadecenyl, and (*Z*)-13-octadecenyl are inviting possibilities. We have screened some blends based on (*Z*)-3- and (*Z*)-13-analogs, on terminal olefins, and on C_8 and C_{18} molecules, but with consistently negative results for noctuids.

This subfamily qualification is important, for throughout many subfamilies (e.g., Agaristinae, Catocalinae, Euteliinae, Sarrothripinae) not a single species has been linked to a defined attractant. This may reflect a lack of investigative attention or even the absence of sex pheromones, but it suggests that these taxa may require sex attractant molecules fundamentally different from those effective in Noctuinae, Hadeninae, Cucullinae, Amphipyrinae, Plusiinae, and Heliothidinae.

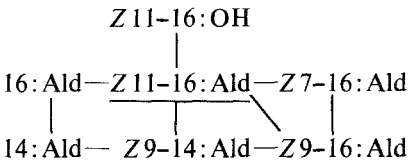
Sex Attractant Blends. Because such a limited number of molecular structures are potential attractants, it is not surprising that blends, exploiting "permutation-combination" possibilities, always seem to be needed for efficient and specific attraction of noctuid males. Single pure compounds are seldom, if ever, satisfactory lures, and occasional reports of high attractancy—especially in the earlier literature—probably indicate coattractive trace contaminants in the chemical samples used. Considering the results in Table 2, these generalizations about blends can be offered:

1. Blends are always required for maximal attractancy.

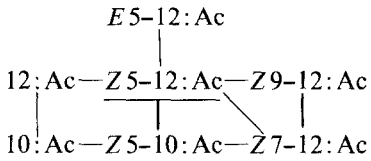
2. A very wide range of optimal component ratios is possible, from 1:1 up to at least 5000:1 (*Pseudaletia unipuncta*).

3. Blends of three or more coattractants are required with a considerable number of species, and complex sex pheromones may prove general in the noctuids.

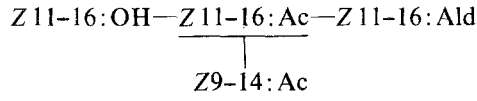
4. Olefinic components in an effective blend are nearly always structurally related by "one-change" steps. That is, components may, from one to another, have different carbon chain lengths, or different double bond positions (counted from either end of the chain), or different functional groups, but only one of these differences. Thus, for example, *Heliothis virescens*' main coattractive sex pheromone constituents are *Z* 11–16: Ald and *Z*9–14: Ald (double bond in this case at position 5 from the terminal methyl end) differing only in carbon chain length. The compounds 14: Ald, 16: Ald, *Z*7–16: Ald, *Z*9–16: Ald, and *Z*11–16: OH, also reported from *H. virescens* females (Klun et al., 1980b), are only "one change" removed from *Z*11–16: Ald or *Z*9–14: Ald. In *Heliothis zea* pheromone the coattractive constituents are *Z* 11–16: Ald and *Z*9–16: Ald, differing only in double bond position. The compounds 16: Ald and *Z* 7–16: Ald were also found in this sex pheromone (Klun et al., 1980a). The structural "one-change" relationships in these pheromone blends can be shown diagrammatically:



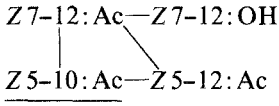
In the multicomponent sex pheromone of *Euxoa ochrogaster* (Struble et al., 1980) the relationships are:



The sex pheromone of the armyworm (*Pseudaletia unipuncta*) is reported as Z 11-16: Ac + Z 11-16: OH (Hill and Roelofs, 1980; McDonough et al., 1980), but high attractancy in lures requires in addition 0.02–0.05% Z 11-16: Ald and 0.01–0.02% Z 9-14: Ac (Steck et al., unpublished results). The blend relationships are thus:



A final example concerns a synthetic blend for *Euxoa albipennis* in which each component contributes to overall potency:



Of all the entries of Table 2, only two (*Actebia fennica* and *Protagrotis niveivenosa*) violate the "one-change" rule. Neither of these blends is a strong lure and both are probably parapheromone mixtures.

We can offer no biochemical explanation for the "one-change" phenomenon, although its universal occurrence in lepidopterous lures suggests that a fundamental biogenetic or sensory principle is involved. In most cases, too, the lines of relationship are most numerous around the principal component of the blend. Where the difference between two coattractants in a blend is one of bond position, the two positions are usually nearest odd-numbered carbons such as 5 and 7, 7 and 9, or 9 and 11. This is not necessarily so, however. For *Fishia hanhami* (blend Z 5-14: Ac + Z 9-14: Ac) the compound Z 7-14: Ac is a powerful trapping inhibitor even at 0.1% content, and for *Polia segregata* (blend Z 7-14: Ac + Z 11-14: Ac) the analog Z 9-14: Ac is similarly inhibitory (Steck et al., 1979d). On the other hand trace quantities of Z 9-16: Ac in the lure (Z 7-16: Ac + Z 11-16: Ac) for *Euxoa messoria* may enhance the attraction of males (Steck et al., unpublished results).

FACTORS AFFECTING POTENCY AND SPECIFICITY OF SEX ATTRACTANTS

Some of these have already been suggested. Specifically there exist five physical-chemical factors of prime importance in structure-activity relationships:

Right Constituents. This is not so obvious as it seems, because parapheromones, compounds which are not natural to a species but nonetheless have pheromone-like action upon the males, are commonly encountered in noctuid lures. Table 2 lists alternative lures of roughly comparable potency for *Euxoa basalis*, *E. pleuritica*, *E. tessellata*, *Orthosia hibisci*, and *Sideridis rosea*. In each case a parapheromone seems to be operative in (at least) one lure system. Our experiences indicate that there is usually a better and a poorer system in such instances, and we might regard the more attractive system as having the "right" constituents. However, this is not always so, and a judgment is not always feasible as to what is "right." For the purposes of this manuscript we therefore accept both members of dual systems which have comparable lure power, realizing that one, if not both, of the systems may be parapheromonal in nature.

On the subject of parapheromones, we have found in general that they retain the same functional group and formal *Z* double bond position as the component or components for which they substitute. Different carbon chain lengths are a common feature, so that *Z* 11–16:Ac or *Z* 11–15:Ac substitute to some extent for *Z* 11–14:Ac in lures for *Sideridis rosea*; *Z* 5–11:Ac for *Z* 5–10:Ac or *Z* 5–12:Ac in *Euxoa ochrogaster* (Struble 1981a); *Z* 5–16:Ac or *Z* 5–15:Ac for *Z* 5–14:Ac in *Amathes c-nigrum* (Steck et al., unpublished results); and so forth. Chisholm et al. (1980) have shown the parapheromonal action of some dienes among noctuids, while Priesner et al. (1977) have indicated possible parapheromonal properties of alkyl-branched pheromone analogs.

Completeness of Blends. As in other insect families, complete blends are critical to lure power and specificity in noctuid lures. Males of the red-backed cutworm *Euxoa ochrogaster*, unresponsive to pure *Z* 5–12:Ac, can be attracted to 100:1 blends of *Z* 5–12:Ac + *Z* 7–12:Ac (Steck et al., 1980a), but addition of 0.2–0.3% of either of *Z* 5–10:Ac or *Z* 9–12:Ac increases trap captures 2- to 3-fold. Addition of traces of both these components gives a 6- to 8-fold overall increase in captures (Steck et al., 1982; Struble 1981a), so that the trapping effects (although not necessarily the behavioral effects) of these two components in this system are the same. If *Z* 5–12:Ac is omitted from lures, the attractancy of the remaining blend not surprisingly falls to zero. Moreover, if *Z* 7–12:Ac is lacking in an otherwise complete lure, trapping similarly drops to near zero. Thus there appears to be a hierarchical order for blend components with the primary component (here *Z* 5–12:Ac) necessary

before the secondary component (*Z*7–12:Ac) can have a behavioral effect, and both components necessary before tertiary and quaternary components such as *Z*9–12:Ac and *Z*5–10:Ac can come into play. In *P. unipuncta* the hierarchical order is (1) *Z*11–16:Ac, (2) *Z*11–16:OH, (3 and 4) *Z*11–16:Ald and *Z*9–14:Ac; in *E. albipennis* the order is (1) *Z*5–10:Ac, (2) *Z*7–12:Ac, (3 and 4) *Z*7–12:OH and *Z*5–12:Ac. In all examples known to us, the primary component is also the principal mass component of the blend. It is not yet known whether component hierarchies reflect behavioral responses to individual chemicals or whether concerted actions are involved.

Attractant blends are usually improved by incorporating progressively smaller quantities of minor components, some or all of which may be trace constituents. Without these, the lure will perform below its potential.

The principle of "one-change" structural relationships and the structure–activity grid give rise to guidelines for candidate components which can be useful for completing a sex pheromone or sex attractant whose composition is incompletely known. For example, the only *Z* compounds related by "one-change" steps to main component *Z*11–16:Ac and within the biologically active limits of Table 1 are *Z*7–16:Ac,*Z*9–16:Ac, *Z*9–14:Ac, *Z*11–14:Ac, *Z*11–16:OH, and *Z*11–16:Ald. If one accepts (1) the need for blends, (2) the noctuid requirement for *Z* isomer, (3) the structure-activity grid of Table 1, and (4) the "one-change" rule, then it follows not merely that one or more of the six related compounds above may be present, but rather that one or more must be present—perhaps as trace coattractants—in a noctuid sex attractant blend based on *Z*11–16:Ac. The noctuid sex attractants and pheromones now known almost all accord with this conclusion, which holds equally well for principal components other than *Z*11–16:Ac. We have found this a very useful hypothesis in development of multicomponent synthetic attractants for noctuids. Where other coattractant compounds are present in a sex attractant, they can be expected to relate in a "one-change" way to the principal coattractant, or to one another, or in both these ways. Thus for the example of *Z*11–16:Ac and *Z*9–14:Ac as principal and secondary coattractants, respectively, these further coattractants are known: *Z*7–12:Ac (*Feltia ducens* and *Euagrotis tepperi*); *Z*7–14:Ac (*Lacinipolia vicina*); *Z*11–16:OH (*Leucania commoides*, *L. multilinea*, and *Euxoa perolivalis*); *Z*11–16:Ald (*Eurois astricta and E. occulta*); and *Z*9–14:Ald (*Euxoa acornis* and *Cryptocala acadiensis*).

Optimal Ratios in Blends. Multicomponent attractants function best at a unique composition. Deviations from this composition cause loss of trapping power and possibly loss of trapping specificity also. The components *Z*11–16:Ac and *Z*9–14:Ac together give sex attractant lures for many noctuids depending on their ratio in the blend; 500:1 lures *Enargia infumata* whereas

50:1 attracts *Mamestra configurata*, 1:1 brings *Peridroma saucia* to traps, 1:4 brings *Eurois occulta*, and 1:100, *Polia Ingravis*. Ratios affects specificity in three-component lures too: Z11–16:Ac + Z11–16:OH + Z11–16:Ald lures any of *Crymodes devastator* (25:1:50), *Apamea indela* (200:50:1), *Polia atlantica* (2000:1:5), or *Pseudaletia unipuncta* (5000:10:1).

An interesting and but recently appreciated subject is the use of lures containing "trace" coattractant compounds which function best at levels below 1% of the total blend. "Trace" blends have been reported in *Heliothis* spp. (Klun et al., 1979, 1980a,b; Teal et al., 1981); for *Polia atlantica* and *Pseudaletia unipuncta* (Steck et al., 1980b); and in *Euxoa ochrogaster* (Struble et al., 1980; Struble, 1981a). More than two dozen additional examples appear in Table 2. Trace components are probably key factors in performance of most noctuid sex attractants; use of very-high-ratio lure blends is then critical for optimal attraction.

Use of insufficiently pure synthetic compounds has from time to time led some workers (including the authors) to believe that a lure is simpler than is really the case. Two examples are instructive. *Pseudaletia unipuncta* (Hill et al., 1980) and *Feltia ducens* (Underhill et al., unpublished results) both yield Z11–16:Ac as the only readily detectable sex pheromone component. *F. ducens* females attract *P. unipuncta* males in the field and both species are lured by pure synthetic Z11–16:Ac. Yet the full blends for these species also contain, respectively, Z11–16:OH + Z11–16:Ald + Z9–14:Ac and Z7–12:Ac + Z9–14:Ac + other possible components.

Blend Release Rate. Sex attractants perform best near a unique release rate, with much higher or lower release rates giving inferior performance in trapping. The actual parameter involved may be the number of attractant molecules reaching the insect sensory apparatus per unit time, but release rate from the lure source is the most easily measured control of this. In field situations release rates suffer night-to-night variations because of changes in factors such as temperture and wind velocity. Male response potential may also fluctuate with these and other factors, so that field captures cannot always be neatly expressed in release rate terms. In general it is safe to say that, experimentally, trapping increases from zero linearly with the logarithm of release rate up to a maximum, following which an analogous decrease to zero occurs. While differences in the maximum point certainly exist among species, the majority of noctuids seem to respond best to release rates of 10–100 ng/h of total blend, under field trapping conditions.

In blends where components have different volatilities (i.e., different vapor pressures), the applied composition will not be the composition released into the air. Initially the most volatile component makes up a somewhat greater fraction of the airborne blend that expected from the

applied composition, but as this component is lost from the dispenser it later makes up less of the blend than expected. The compositions given in Table 2 are applied ratios.

Chemical Discriminators. Lure specificity as well as potency usually increases in blended lures, and a positive correlation exists between a compound's ability to enhance a given lure's potency and its ability to inhibit the approach of nontarget species which would otherwise be attracted to the same lure. Some instances of this have been noted already (Ganyard and Brady, 1971; Steck et al., 1977b; Klun et al., 1980b). This effect opens the possibility of interspecies communication in nature by chemicals; the available evidence suggests this happens wherever components are emitted with a sex pheromone, which (whether they elicit any conspecific response or not) selectively inhibit the approach of alien species. Such compounds have been termed chemical discriminators (Steck et al., 1979b); they are useful in achieving sex attractant specificity in synthetic blends. For species where calling periods serve to distinguish chemically similar pheromones, specificity may not be attainable with synthetic lures (Teal et al., 1978; Underhill et al., 1981).

CHEMOTAXONOMIC VALUE OF SEX ATTRACTANT CHEMICALS

At the species or genus levels, patterns of sex attractant components appear to have little chemotaxonomic significance. For example, the genus *Euxoa* uses a bewildering array of olefinic blends. In a few instances where extremely close taxonomic relationships exist, the sex attractants involved are also very similar. Examples include the *Euxoa* groups *flavicollis*, *obeliscoides*, *ridingsiana* (Z9–14:Ac + Z7–12:Ac) and *campestris*, *declarata*, *rockburnei* (Z5–10:Ac); species of the genus *Cucullia* (Z9–14:Ac); and *Heliothis* spp. (Z11–16:Ald). The trio *Eurois* (formerly *Peridroma*) *occulta*, *E. astricta*, and *Peridroma saucia* all share Z11–16:Ac + Z9–14:Ac as sex attractant components. Some other obvious close relations can be seen in Table 2, but against them must be set the chemical diversity evident in the large genera *Agrotis*, *Euxoa*, and *Polia*. The overall lack of chemotaxonomic relationships may reflect in part the unsettled (or at least artificial) nature of lepidopteran taxonomy at these levels, genera being frequently distinguished by characteristics which in other phyla or classes would not serve to differentiate species [see Dyar (1902) and Forbes (1954) for alternative taxonomic treatments of the Noctuidae].

It is at the subfamily level that sex attractants take on clearer chemotaxonomic relationships. The distribution of olefinic components in sex attractants of various noctuid subfamilies is far from homogeneous. Table 3 shows the patterns based on available data. Insufficient data make generalizations on many groups impossible, but in the main subfamilies we note:

TABLE 3. RADICAL FREQUENCY AND (PERCENT DISTRIBUTION) OF SEX ATTRACTANT COMPONENTS IN 7 NOCTUID SUBFAMILIES[a]

Radical	Acronictinae (2 spp.)	Noctuinae (46 spp.)	Hadeninae (34 spp.)	Cucullinae (19 spp.)	Plusiinae (18 spp.)	Amphipyrinae (19 spp.)	Heliothidinae (7 spp.)
Z5-10:	0	10 (10)	0	0	0	0	0
Z5-12:	0	5 (5)	0	0	3 (11)	1 (3)	0
Z7-12:	1 (33)	26(26)	1 (1)	4 (14)	21 (85)	2 (6)	0
Z9-12:	1 (33)	3 (3)	0	0	0	1 (3)	0
Z5-14:	0	5 (5)	0	1 (4)	0	0	0
Z7-14:	1 (33)	9(9)	5 (7)	1 (4)	1 (4)	0	0
Z9-14:	0	24 (23)	21 (32)	16 (54)	0	7 (24)	1 (5)
Z11-14:	0	1 (1)	8 (11)	0	0	1 (3)	0
Z5-16:	0	1 (1)	0	0	0	0	0
Z7-16:	0	3 (3)	0	0	0	1 (3)	2 (11)
Z9-16:	0	0	2 (3)	2 (8)	0	0	5 (26)
Z11-16:	0	15 (15)	31 (46)	5 (16)	0	17 (58)	11 (58)

[a]Based on data of Table 2.

1. All Z5–10: radicals, as Z5–10:Ac, occur in Noctuinae.

2. Plusiinae require only the three tightly connected radicals Z5–12:, Z7–12:, and Z7–14: (100% of entries).

3. Heliothidinae require mostly Z11–16: (58% of entries) as Z11–16:Ald (in all species) and seem not to utilize C_{12} components.

4. Cucullinae and Hadeninae are broadly similar in structural responses but the latter are more frequently associated with Z11–14: and less frequently with Z7–12:.

5. Amphipyrinae very frequently require Z9–14: and Z11–16: (82% of entries).

6. Noctuinae have the widest structural scope, weighted towards molecules with double-bond 5 carbons removed from the methyl end (76% of entries).

The extent to which diolefinic compounds can elicit male responses in noctuids is not yet known. In some species dienes have been demonstrated as sex pheromone components, and certain types of dienes have been shown to substitute generally for related monoenes (Chisholm et al., 1980). However, diolefinic attractants seem to be the exception rather than the rule among the Noctuidae, and too little is reported to allow a meaningful dicussion of their distribution patterns.

Hill noted (1979) that some *Catocala* moths could be attracted to traps baited with lures "similar" to live female *Hyphantria cunea* (Drury) (family Arctiidae). No details were given, however, nor were the species named.

CONCLUSION

The noctuids use, for attractant purposes, a rather restricted repertory of chemicals, potency and specificity being largely matters of compound combinations and ratio permutations. The systems are not random, however, but follow consistent patterns which give rise to generalizations of a predictive nature concerning the chemical arrays that will prove attractive to male moths—especially in cases where a principal pheromone or attractant is already known. If the generalizations above are correct, future discovery of sex pheromones and development of synthetic sex attractants for noctuids will be governed by these factors:

1. In Noctuinae, Hadeninae, Cucullinae, Amphipyrinae, Heliothidinae, Plusiinae, and probably other subfamilies, the chemical structures involved will be restricted to those indicated in Table 1.

2. Multicomponent blends will be required whose components are related by the "one-change" rule.

3. Most new or improved attractants will contain one or more trace components.

4. In subfamilies as yet unstudied new structural types of attractants may be found.

Note Added in Proof—The sex pheromone of the Catocalinae species *Caenurgina erechtea* (Cramer) has been shown to consist of (3*Z*, 6*Z*, 9*Z*)-3, 6, 9-eicosatriene and (3*Z*, 6*Z*, 9*Z*)-3, 6, 9-heneicosa-triene (Underhill et al., in preparation).

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EFFECTS OF PHEROMONE COMPONENTS AND THEIR DEGRADATION PRODUCTS ON THE RESPONSE OF *Heliothis* SPP. TO TRAPS[1,2]

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Abstract—None of the isolated degradation products of (*Z*)-11-hexadecenal [(*Z*)-11-HDAL] affected the catches of either tobacco budworm [*Heliothis virescens* (F.)] or bollworm [*H. zea* (Boddie)] moths when dispensed with pheromone from cotton dental rolls in cone traps. Also, none of the degradation products of (*Z*)-9-tetradecenal [(*Z*)-9-TDAL] had an effect on trap catches of tobacco budworm moths. Two of the three chemicals that have previously been identified in ovipositor washes of tobacco budworms but that are absent in those of bollworms caused a reduction in capture of bollworms: (*Z*)-9-TDAL (1.0 μg/trap) caused a 96% reduction in trap catch and (*Z*)-11-hexadecen-1-ol (20.0 μg/trap) caused a similar reduction. Tetradencenal (40 μg/trap) had no effect on trap catch.

Key Words—Sex pheromone, *Heliothis virescens*, tobacco budworm, *Heliothis zea*, bollworm, virelure, Lepidoptera, Noctuidae.

INTRODUCTION

Sex pheromone components have been isolated and identified for the tobacco budworm, *Heliothis virescens* (F.) (Roelofs et al., 1974; Tumlinson et al., 1975; Klun et al., 1980a) and for the bollworm, *Heliothis zea* (Boddie) (Klun

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

et al., 1980b). (*Z*)-11-Hexadecenal [(*Z*)-11-HDAL], (*Z*)-9-hexadecenal [(*Z*)-9-HDAL], (*Z*)-7-hexadecenal [(*Z*)-7-HDAL], and hexadecanal are found in rinses of the ovipositors of both species; however, rinses of the ovipositors of the tobacco budworm also contain (*Z*)-9-tetradecenal [(*Z*)-9-TDAL], tetradecanal, and (*Z*)-11-hexadecen-1-ol. Klun et al. (1980a) observed that traps baited with (*Z*)-11-HDAL (115.5 μg), (*Z*)-9-HDAL (4.5 μg), (*Z*)-7-HDAL (2.6 μg), and (*Z*)-9-TDAL (6.9 μg) captured tobacco budworm males but no bollworm males, even though adults of that species were concurrently abundant with tobacco budworms in the field. They suggested that (*Z*)-9-TDAL was a deterrant to bollworm attraction and concluded that one or more of the three chemicals found in the rinses of tobacco budworm ovipositors but not in similar rinses from bollworms influenced specificity between the two species. Recently, Shaver and Ivie (1981) isolated and identified several products formed during the degradation of virelure [(*Z*)-11-HDAL and (*Z*)-9-TDAL], the synthetic sex pheromone of the tobacco budworm. These products from (*Z*)-11-HDAL were: (*Z*)-11-hexadecenoic acid, *cis*- and *trans*-11,12-epoxyhexadecanoic acid, *cis*- and *trans*-11,12-epoxyhexadecanal, *cis*- and *trans*-10,11-epoxypentadecanal, (*Z*)-10-pentadecenal, (*Z*)-5-pentadecene, *cis*- and *trans*-5,6-epoxypentadecane, and tetradecene. The products degradation products obtained from (*Z*)-9-TDAL were: (*Z*)-9-tetradecenoic acid, *cis*- and *trans*-9,10-epoxytetradecanoic acid; *cis*- and *trans*-9,10-epoxytetradecanal; *cis*- and *trans*-8,9-epoxytridecanal, (*Z*)-8-tridecenal, (*Z*)-5-tridecene, *cis*- and *trans*-5,6-epoxytridecane, and dodecene.

Tests described herein were conducted to determine the effects of (1) the degradation products of virelure on the entrapment of adult tobacco budworms and bollworms in traps baited with the appropriate pheromone and (2) certain components of the tobacco budworm pheromone on the entrapment of bollworms.

METHODS AND MATERIALS

Degradation products of (*Z*)-11-HDAL and (*Z*)-9-TDAL were formed by allowing hexane solutions of these compounds to stand in the laboratory under fluorescent lights for at least 2 weeks. Acidic degradation products were extracted as their salts from the hexane solutions with 0.1 N NaOH. The alkaline solutions were then neutralized with HCl, and the acids recovered with hexane (Shaver and Ivie, 1980). The acidic degradation products of (*Z*)-11-HDAL included (*Z*)-11-hexadecenoic acid (65%), *cis*-11,12-epoxytetradecanoic acid (31%), and *trans*-11,12-epoxyhexadecanoic acid (4%), while those of (*Z*)-9-TDAL included (*Z*)-9-tetradecenoic acid (68%), *cis*-9,10-epoxytetradecanoic acid (29%), and *trans*-9,10-epoxytetradecanoic acid (3%).

The relative amounts of the acids were determined by first converting them to the respective methyl esters by reaction with diazomethane and then analyzing the reaction mixtures with gas chromatography on 1.7-m × 0.65-cm glass columns packed with 1.95% SP2401 + 1.6% SP2250 on 80/100 mesh Supelcoport.

The hydrocarbon products formed during the degradation of (*Z*)-11-HDAL and (*Z*)-9-TDAL were separated by elution from silica gel columns with hexane. Hydrocarbons produced during the degradation of (*Z*)-11-HDAL included (*Z*)-5-pentadecene (85%) and tetradecene (position of unsaturation not determined, 15%), and those formed from (*Z*)-9-TDAL included (*Z*)-5-tridecene (90%) and dodecene (position of unsaturation not determined, 10%).

Other degradation products were isolated by preparative gas chromatography on 1.7-m × 0.65-cm glass columns packed with 1.95% SP2401 + 1.6% SP2250 on 80/100 mesh Supelcoport. These degradation products were *cis*- and *trans*-9,10-epoxytetradecanal (95% *cis* isomer) and (*Z*)-8-tridecenal from (*Z*)-9-TDAL and *cis*- and *trans*-9,10-epoxyhexadecanal (95% *cis* isomer) and (*Z*)-10-pentadecenal from (*Z*)-11-HDAL.

Cotton dental roll dispensers containing virelure [(*Z*)-11-HDAL, 245 μg and (*Z*)-9-TDAL, 15 μg] were used as standards in tests to assess the effects of each degradation product on trap catch of tobacco budworms. Similar dispensers containing all four components of the bollworm pheromone [(*Z*)-11-HDAL, 231 μg; (*Z*)-9-HDAL, 9.0 μg; (*Z*)-7-HDAL, 5.2 μg; and hexadecanal, 22 μg] were used as standards for bollworm tests.

Three pheromone components that are found in tobacco budworms but not in bollworms [(*Z*)-9-TDAL, tetradecanal, and (*Z*)-11-hexadecen-1-ol] were tested individually with the bollworm pheromone for their effects on trap catches of bollworm moths. In the first test, (*Z*)-9-TDAL was added in concentrations of 0.05, 0.2, and 1 μg/dispenser; this corresponds to 0.02%, 0.035%, and 0.37% of the total mixture, respectively. We originally had planned to add up to twice the relative concentration found in rinses of tobacco budworm ovipositors (5.5% of total pheromone), but preliminary tests showed that trap catches of bollworm were reduced to zero with as much as half the relative amount found in tobacco budworm ovipositor rinses. This test was conducted for six nights, with six replicates per night.

In another test, (*Z*)-9-TDAL was formulated in rubber septa (formulation 1171 red, The West Co., Phoenixville, Pennsylvania) with 2.5 mg of the four-component bollworm pheromone to give 0.01%, 0.1%, 1%, and 5% (*Z*)-9-TDAL. The test was run for four nights with six replicates per night, and fresh baits were prepared after the second night.

All tests of tobacco budworm adults were conducted in a cotton field. Bollworm tests were conducted in corn fields (May–June) and cotton fields (July–September). All fields were located in Burleson County, Texas. Cone

traps (Hartstack et al., 1979) were set up in straight lines at the perimeters of the fields and treatments were randomized nightly. Dispensers were prepared each night and placed in the traps between 9:30 and 11:00 PM so that the most volatile compound would be available during the period of peak moth activity.

RESULTS AND DISCUSSION

None of the degradation products of (*Z*)-11-HDAL caused a significant change in the response of bollworm moths to traps baited only with the four-component bollworm pheromone (Table 1). Although the traps baited with pheromone + the acidic decomposition products of (*Z*)-11-HDAL caught more insects than traps containing the pheromone alone (35.1 moths/night compared to 30.2 moths/night), the difference is not significant.

TABLE 1. EFFECT OF DEGRADATION PRODUCTS OF (*Z*)-11-HDAL ON TRAP CATCHES OF *Heliothis zea* MALES IN CONE TRAPS BAITED WITH COTTON DENTAL ROLL DISPENSERS CONTAINING 267.2 μg OF A FOUR-COMPONENT PHEROMONE[a]

Degradation products	$\bar{X}$ ± SE/trap/night
Test 1	
Pheromone only	39.6 ± 2.04 a[c]
(*Z*) + (*E*)-11,12-Epoxyhexadecanal, 190 μg[b]	58.6 ± 4.29 a
(*Z*) + (*E*)-11,12-Epoxyhexadecanal, 900 μg	44.7 ± 3.79 a
Test 2	
Pheromone only	30.2 ± 1.51 a
(*Z*) + (*E*)-HDAL acids, 200 μg[d]	35.1 ± 1.66 a
Test 3	
Pheromone only	117.1 ± 5.4 a
(*Z*)-10-Pentadecenal, 5 μg	120.2 ± 7.1 a
(*Z*)-10-Pentadecenal, 25 μg	95.0 ± 5.5 a
Test 4	
Pheromone	48.6 ± 1.8 a
(*Z*)-11-HDAL hydrocarbons (25 μg)[e]	46.5 ± 1.4 a

[a]The amounts (%) of each component in each dispenser were: (*Z*)-11-hexadecenal, 231.0 μg (87%); (*Z*)-9-hexadecenal, 9.0 μg (3%); (*Z*)-7-hexadecenal, 5.2 μg (2%); and hexadecenal, 22 μg (8%).
[b]Ca. 95% *Z* isomer.
[c]Numbers followed by the same letter are not significantly different, $P = 0.05$, Duncan's new multiple-range test.
[d]Mixture of (*Z*)-11-hexadecenoic acid (65%) and *cis* + *trans*-11,12-epoxyhexadecanoic acid [31% *cis*, 4% *trans*].
[e]Mixtures of (*Z*)-5-pentadecene (85%) and tetradecene (15%).

TABLE 2. EFFECT OF DEGRADATION PRODUCTS OF (*Z*)-11-HDAL AND (*Z*)-9-TDAL ON TRAP CATCHES OF *Heliothis virescens* IN CONE TRAPS BAITED WITH COTTON DENTAL ROLL DISPENSERS CONTAINING PHEROMONES[a]

Degradation products	$\bar{X}$ ± SE/trap/night
Test 1	
Virelure standard	5.9 ± 0.3 a[c]
(*Z*) + (*E*)-9,10-Epoxytetradecanal, 15 μg[b]	5.1 ± 0.2 a
(*Z*) + (*E*)-11,12-Epoxyhexadecanal, 60 μg[b]	4.9 ± 0.2 a
Test 2	
Virelure standard	9.4 ± 0.2 a
(*Z*)-9-TDAL acids (250 μg)[d]	8.8 ± 0.2 a
(*Z*)-11-HDAL acids (250 μg)[e]	10.3 ± 0.3 a
Test 3	
Virelure standard	13.6 ± 0.3 a
(*Z*)-8-Tridecenal (2 μg)	12.1 ± 0.3 a
(*Z*)-10-Pentadecenal (15 μg)	13.0 ± 0.5 a
Test 4	
Virelure standard	14.5 ± 0.4 a
(*Z*)-9-TDAL hydrocarbons (10 μg)[f]	14.2 ± 0.4 a
(*Z*)-11-HDAL hydrocarbons (25 μg)[g]	17.3 ± 1.3 a

[a]Each trap was baited with a cotton dental roll containing 245 μg (*Z*)-11-hexadecenal + 15 μg (*Z*)-9-tetradecenal.
[b]Ca. 95% *Z* isomer.
[c]Numbers followed by the same letter are not significantly different, $P = 0.05$, Duncan's new multiple-range test.
[d]Mixture of (*Z*)-9-tetradecenoic acid (68%) and *cis* + *trans*-9,10-epoxytetradecanoic acid, 29% *cis* and 3% *trans*.
[e]Mixture of (*Z*)-11-hexadecenoic acid (65%) and *cis* + *trans*-11,12-epoxyhexadecanoic acid, 31% *cis* and 4% *trans*.
[f]Mixture of (*Z*)-5-tridecene (90%) and dodecene (10%).
[g]Mixture of (*Z*)-5-pentadecene (85%) and tetradecene (15%).

Likewise, none of the degradation products of (*Z*)-11-HDAL and (*Z*)-9-TDAL significantly affected the potency of virelure in attracting tobacco budworms to the trap (Table 2). Traps baited with 25 μg of (*Z*)-11-HDAL hydrocarbons plus virelure caught about 19% more moths than traps baited with virelure alone, but this difference was not significant.

The addition of (*Z*)-9-TDAL to the four-component bollworm pheromone at a rate as low as 0.2 μg/trap significantly reduced the capture of bollworm males (Table 3). Traps containing 1.0 μg of (*Z*)-9-TDAL combined with the four-component bollworm pheromone caught an average of only 0.6 bollworm males/trap/night, but also caught a few tobacco budworm moths. In this case, the concentration of (*Z*)-9-TDAL represented 0.37% of the total

TABLE 3. EFFECT OF 3 *H. virescens* PHEROMONE COMPONENTS NOT FOUND IN *H. zea* ON TRAP CATCH OF *H. zea* IN CONE TRAPS BAITED WITH COTTON DENTAL ROLL DISPENSERS CONTAINING 4 COMPONENTS

Component	Amount (μg/trap)[a]	$\bar{X}$ ± SE/trap/night
(*Z*)-9-Tetradecenal	0.00	17.3 ± 0.56 a[b]
	0.05 (0.02%)	12.8 ± 0.43 a
	0.20 (0.07%)	4.5 ± 0.02 b
	1.00 (0.37%)	0.6 ± 0.01 b
(*Z*)-11-Hexadecen-1-ol	0.00	14.7 ± 0.09 a
	5.00 (1.85)	5.2 ± 0.40 b
	10.00 (3.70)	3.0 ± 0.20 bc
	20.00 (7.50)	0.6 ± 0.01 c
Tetradecanal	0.00	86.2 ± 4.10 a
	10.00 (3.7)	88.9 ± 5.40 a
	20.00 (7.5)	75.8 ± 4.10 a
	40.00 (15.0)	64.6 ± 3.40 a

[a]Each dispenser contained (*Z*)-11-hexadecenal (231.0 μg), (*Z*)-9-hexadecenal (9.0 μg), (*Z*)-7-hexadecenal (5.2 μg), and hexadecenal (22 μg) plus indicated quantity (μg) of test compound.

[b]Numbers followed by the same letter are not significantly different at $P = 0.05$, Duncan's new multiple-range test.

bollworm pheromone mixture. (Its relative concentration in heptane washes of tobacco budworm ovipositors is ca. 5%.)

(*Z*)-11-Hexadecen-1-ol at 5 μg/trap (the lowest concentration tested), reduced the capture of male bollworm moths when used with the four-component pheromone (Table 3). Traps baited with dispensers containing 20 μg of (*Z*)-11-hexadecen-1-ol plus the bollworm pheromone mixture caught an average of only 0.6 moths/night/trap. This concentration represents ca. 7.5% of the total bollworm pheromone in the dispensers, and is approximately equal to the concentration (6%) of (*Z*)-11-hexadecen-1-ol in heptane washes of tobacco budworm ovipositors.

Tetradecanal had no significant influence on captures of bollworm moths, even at the highest concentration (40 μg/trap or 15% of bollworm pheromone) tested. This compound constituted about 2% of the total components of heptane washes of tobacco budworm ovipositors (Klun et al., 1979).

When (*Z*)-9-TDAL was formulated with the bollworm pheromone mixture in rubber septa, significant decreases in the capture of bollworm moths were obtained with concentrations as low as 2.5 μg (*Z*)-9-TDAL/septum (Table 4). Traps baited with rubber septa containing 2.5 mg bollworm

TABLE 4. EFFECT OF (*Z*)-9-TETRADECENAL ON TRAP CATCHES OF *Heliothis zea* IN CONE TRAPS WITH PHEROMONE FORMULATED IN RUBBER SEPTA[a]

(*Z*)-9-Tetradecenal/trap (μg)	$\bar{X}$ moth/trap/night ± SE
0	25.4 ± 1.03 a[b]
0.25	21.0 ± 0.88 ab
2.50	16.5 ± 0.69 b
25.00	1.4 ± 0.18 c[c]
250.00	0.1 ± 0.01 c[d]

[a]Each rubber septum contained 2.5 mg of the four-component *H. zea* pheromone plus the indicated quantity of (*Z*)-9-tetradecenal.
[b]Numbers followed by the same letter are not significantly different (Duncan's new multiple-range test, $P = 0.05$).
[c]Traps averaged 6.6 *H. virescens*/trap/night.
[d]Traps averaged 34.6 *H. virescens*/trap/night.

pheromone plus 25 μg (*Z*)-9-TDAL caught an average of 1.4 bollworm moths/trap/night, but caught an average of 6.6 tobacco budworm moths/trap/night. Traps baited with 2.5 mg bollworm pheromone plus 250 μg (*Z*)-9-TDAL caught an average of 0.1 male bollworm moths/trap/night and 34.6 male tobacco budworm moths/trap/night.

The results indicate that the degradation products of (*Z*)-11-HDAL tested had no effect on catches of either tobacco budworm or bollworm moths when the traps were baited with their respective pheromone. Also, the degradation products of (*Z*)-9-TDAL that were tested had no effect on trap catches of tobacco budworm.

Since four of the seven compounds that have been found in the tobacco budworm are also found in the bollworm, it is likely that the presence or absence of one or more of the three chemicals found only in the tobacco budworm has an important role in determining the specificity of the two species in their response to pheromone-baited traps and, possibly, specificity in mating, as suggested by Klun et al. (1980a). Indeed, in our test, two of the three chemicals did influence trap catch of bollworms. This information could be useful in programs utilizing mating disruption techniques to suppress populations of *Heliothis* spp.

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OVIPOSITION DETERRING PHEROMONE IN *Anastrepha fraterculus* FLIES

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Abstract—*Anastrepha fraterculus* (Wiedemann) females were found to deposit a water-soluble, durable, oviposition-deterring pheromone during ovipositor dragging on fruit after egg-laying. We present evidence that the occurrence of pheromone deposition after egg-laying, the amount deposited, and departure from the fruit without additional egg-laying after pheromone deposition are flexible traits in *A. fraterculus*, varying in expression according to fruit size and other factors. Unlike *Rhagoletis, A. fraterculus* males were not arrested by the pheromone.

Key Words—Pheromone, *Anastrepha fraterculus*, Diptera, Tephritidae oviposition deterrent.

INTRODUCTION

More than 35 species of phytophagous insects are known to release epideictic pheromones which regulate intraspecific spacing patterns on exhaustible food resources (Prokopy, 1981a). Among these are 13 species of frugivorous Tephritidae which release oviposition-deterring pheromone (ODP), including 11 species of *Rhagoletis* flies, the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), and the Caribbean fruit fly, *Anastrepha suspensa* (Loew).

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann), is a major pest of native and introduced tree fruits in Brazil (Malavasi et al., 1980) and other countries of South and Central America. Among its hosts are Surinam cherry, *Eugenia uniflora* L., and guava, *Psidium guajava* L. Like *A.*

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suspensa (Prokopy et al., 1977), ovipositing *A. fraterculus* females bore with the ovipositor through the fruit skin into the flesh, usually lay only one egg per boring puncture (we found 40 eggs in 39 egg-containing punctures in Surinam cherries in nature), and usually drag the ovipositor on the fruit surface after egg-laying.

Here, we demonstrate that *A. fraterculus* females deposit ODP during ovipositor dragging. We present data on the longevity and aqueous solubility of the ODP, the response of the males to ODP, and the influence of fruit characteristics on amount of ODP released and other aspects of postoviposition behavior.

METHODS AND MATERIALS

All experiments were conducted in a laboratory under ambient temperature and humidity conditions of 25 ± 3° C and 60 ± 10% relative humidity. All flies used in experiments originated from pupae from infested guavas collected in nature. They were maintained in 30 × 30 × 30-cm cages containing water and a mixture of sucrose and protein hydrolysate, with about 40 females and 15 males per cage. They were assayed when mature, 2–3 weeks old. Males assayed for response to ODP were confined without females for 5 days prior to assay. In addition (Table 2), we conducted observations of wild fly behavior on abundantly fruiting guava trees in nature and on a field-caged, 2-m, nonfruiting guava tree on which 24 clean guava fruits were hung and 20 lab-maintained females were released on the day of observation.

Test fruits consisted of fresh-picked, uninfested Surinam cherries (average 13 mm diam, range 12.5–13.5 mm) and guavas (average 25 mm diam, range 24.0–26.0 mm). Both were readily accepted as oviposition sites, but both were available in limited supply. Hence, we also used artificial fruits (13 or 25 mm diam) comprised of red-dyed agar spheres enveloped in parafilm (Boller, 1968). These were moderately accepted for oviposition.

The preassay and experimental assay procedures used for tests in Table 1 were essentially identical to those outlined by Prokopy (1981b). Except for the second treatment in experiment 3, each ODP-marked fruit received a standardized number of 15 ovipositor dragging circles. This number was slightly less than the largest number of dragging circles (17) made on a 13-mm fruit by any fly observed and was obtained, using fly transfer procedures (Prokopy, 1981b), from draggings by 2–4 females. To determine aqueous solubility of ODP, artificial fruits marked with ODP were rinsed for 3 min in 20 ml of distilled water (25° C) prior to testing. Artificial fruits tested 5 days after being marked with ODP, as well as comparable control fruits, were kept dry at 25° C, 100% relative humidity until testing.

In experiment 1 of Table 3, each of 27 females was offered a succession of

four clean 13-mm artificial fruits, four clean 25-mm artificial fruits, and four clean 25-mm guavas, with the sequence of fruit types varied in systematic fashion. In experiment 2 of Table 3, 10 females per cage were allowed to choose among a representative of each of these three fruit types during a 10-min period. In the Table 4 experiment, a female was allowed to oviposit in a 13- or 25-mm artificial fruit and immediately thereafter was gently transferred (Prokopy, 1981b) to a different 13- or 25-mm artificial fruit for dragging. In each replicate of this experiment, the same female was used for all treatments, with treatment order varied in systematic fashion. In experiment 1 of Table 3 and in the Table 4 experiment, a single test fruit was the only fruit present in the cage.

RESULTS

Assay females were significantly deterred from boring into Surinam cherries which had received previous borings and ovipositor draggings (Table 1, experiment 1). They were not deterred from boring into artificial fruit where other females had bored but were prevented from dragging the ovipositor, whereas they were significantly deterred from boring into artificial fruit

TABLE 1. BORING FREQUENCY OF *A. fraterculus* FEMALES INTO UNMARKED FRUITS (CONTROLS) AND FRUITS THAT HAD EARLIER RECEIVED BORING ATTEMPTS (= + BORE), OVIPOSITOR DRAGGING IN AMOUNT OF 15 CIRCLES PER FRUIT (= + DRAG), OR OTHER TREATMENTS AS INDICATED[a]

Experiment	Days, treatment to testing	Fruit	Treatment	No. females arriving on fruit	% attempting to bore
1	0	S. cherry	+ bore, + drag	22	18a
		S. cherry	Control	22	86b
2	0	Artificial	+ bore, no drag	33	82b
		Artificial	No bore, + drag	33	15a
		Artificial	Control	33	79b
3	0	Artificial	No bore, + drag	30	23a
		Artificial	No bore, + drag (5 circles)	30	60b
		Artificial	Control	30	97c
4	0	Artificial	No bore, + drag	29	28a
		Artificial	No bore, + drag (rinsed in water)	29	72b
5	5	Artificial	No bore, + drag	30	23a
		Artificial	Control	30	80b

[a]All fruits = 13 mm diam. All tests in laboratory cages. Values in each experiment not followed by the same letter are significantly different at the 0.05 level according to chi-square test criterion.

where, through fly transfer procedures, other females had not bored but did drag the ovipositor (Table 1, experiment 2). Five ovipositor dragging circles on a 13-mm artificial fruit conferred moderate (significant) oviposition deterring activity, but at a significantly reduced level compared to 15 circles (Table 1, experiment 3). Rinsing ovipositor-dragged artificial fruit in water sharply reduced or eliminated oviposition deterrence (Table 1, experiment 4), but ovipositor-dragged artificial fruit maintained under dry conditions for 5 days remained highly deterrent (Table 1, experiment 5). Assay males spent less time per visit, although not significantly so, on 13-mm ovipositor-dragged artificial fruit (43 ± 82 sec, $N = 63$) than on 13-mm clean artificial fruit (76 ± 99 sec, $N = 63$).

Observations of postoviposition behavior on Surinam cherries and guavas in lab cages, in the field-caged tree, or in nature revealed the following: In lab cages, 94% ($N = 71$) of females which had laid an egg in Surinam cherries subsequently dragged the ovipositor. Significantly fewer females (56%, $N = 48, p < 0.01$) dragged the ovipositor after laying an egg in guavas. In lab cages, 84% ($N = 85$) of females which ovipositor-dragged on Surinam cherries had laid an egg, compared with 90% ($N = 31$, no significant difference) in which ovipositor dragging on guavas was preceded by egg-laying (we have no explanation for the manifestation of ovipositor dragging by a small percentage of females in the absence of egg-laying, nor do we know if any ODP was deposited in the process). Ovipositor dragging on both hosts appeared random in direction. It was not continuous in all cases, being occasionally interrupted for several seconds for cleaning. The average duration of dragging was approximately the same (37–44 sec, no significant differences) on Surinam cherries in lab cages and on guavas in lab cages, in the field-caged tree, and in trees in nature (Table 2). More females (although not

TABLE 2. POSTOVIPOSITION BEHAVIOR OF *A. fraterculus* FEMALES ON HOST FRUITS IN DIFFERENT LOCATIONS[a]

Fruit[b]	Location of observations	No. females observed dragging ovipositor	Mean duration of dragging (sec)	% dragging females which departed fruit afterward
Guava (25 mm)	Lab cage	29	38 ± 32a	21a
S. cherry (13 mm)	Lab cage	85	39 ± 23a	38a
Guava (25 mm)	Field cage	30	44 ± 19a	70b
Guava (25 mm)	Nature	35	37 ± 22a	91c

[a]Values in each column not followed by the same letter are significantly different at the 0.05 level according to t tests (column without %) or the chi-square test criterion (column with %).

[b]Guava fruits in nature and the field cage may have been marked with pheromone prior to arrival of observed females. All fruits in lab cages were clean.

TABLE 3. INFLUENCE OF FRUIT TYPE (ALL CLEAN) ON OVIPOSITION AND POSTOVIPOSITION BEHAVIOR OF *A. fraterculus* FEMALES IN NO-CHOICE (EXPERIMENT 1) OR CHOICE (EXPERIMENT 2) TESTS IN LABORATORY CAGES[a]

Experiment	Fruit	No. females arriving	Arriving females attempting to bore		Boring females which oviposited		Ovipositing females which dragged afterward		Mean duration of dragging (sec)	Dragging females which departed fruit afterward	
			N	%	*N*	%	*N*	%		*N*	%
1	Guava (25 mm)	108	67	62a	44	66a	26	59b	35 ± 28ab	5	19b
	Artificial (25 mm)	108	55	51ab	41	75a	27	66b	27 ± 13b	11	41b
	Artificial (13 mm)	108	44	41b	34	77a	30	88a	42 ± 26a	20	67a
2	Guava (25 mm)	69a	30	43a							
	Artificial (25 mm)	53b	13	25b							
	Artificial (13 mm)	46b	7	15b							

[a]See footnote *a* of Table 2.

significantly so) departed the fruit without additional boring after ovipositor dragging on Surinam cherries in lab cages than on guavas in lab cages, and significantly more departed after dragging on guavas in nature than on guavas in the field-caged tree or in lab cages (Table 2).

Certain of these observations suggested that fruit characteristics might influence postoviposition behavior. Indeed, subsequent tests in lab cages showed that among females which had just oviposited (1) significantly more dragged the ovipositor afterward when on 13-mm artificial fruit than when on 25-mm artificial fruit or guavas; (2) the duration of ovipositor dragging was significantly longer on 13-mm than on 25-mm artificial fruit (duration on guavas was intermediate); and (3) significantly more ovipositing females departed 13-mm artificial fruit after ovipositor dragging than departed guavas or 25-mm artificial fruit (Table 3, experiment 1). These relationships were so despite the fact that under no-choice (Table 3, experiment 1) as well as choice (Table 3, experiment 2) test conditions, arriving females attempted boring significantly less often into 13-mm artificial fruit than into guavas (boring frequency into 25-mm artificial fruit was intermediate). During experiment 1, we counted the number of ovipositor dragging circles and found significant positive correlation ($P < 0.001$) between duration of ovipositor dragging and number of dragging circles on each fruit type: 13-mm artificial fruit, $r = 0.828$; 25-mm artificial fruit, $r = 0.962$; guavas, $r = 0.883$.

Furthermore (Table 4), when females were allowed to oviposit in 25-mm artificial fruit and were immediately transferred to other 25-mm or 13-mm artificial fruit for ovipositor dragging, they dragged significantly longer on the 13-mm fruit. Similarly, ovipositing females transferred from 13-mm artificial fruit dragged significantly longer on other 13-mm fruit than on 25-mm artificial fruit. Moreover, significantly more females departed the 13-mm fruit after dragging than departed the 25-mm fruit.

TABLE 4. POSTOVIPOSITION BEHAVIOR OF *A. fraterculus* FEMALES AFTER TRANSFER FROM ONE CLEAN ARTIFICIAL FRUIT TO ANOTHER IN LABORATORY CAGES[a]

No. females assayed	Size of artificial fruit in which female oviposited (mm)	Size of artificial fruit to which female transferred for ovipositor dragging (mm)	Mean duration of dragging (sec)	% dragging females which departed fruit afterward
19	25	13	65 ± 63a	42a
19	13	13	59 ± 41a	53a
19	25	25	36 ± 21b	11b
19	13	25	26 ± 13b	11b

[a]See footnote *a* of Table 2.

Some of the resulting values for treatments of this Table 4 experiment were different from those for comparable treatments of experiment 1, Table 3. We have no explanation for these differences, other than each experiment was conducted using a different protocol and during a different week.

DISCUSSION

Our findings convincingly demonstrate that *A. fraterculus* females deposit a water-soluble, durable, oviposition-deterring pheromone during ovipositor dragging on the fruit surface after egg-laying. These characteristics parallel those of *A. suspensa* (Prokopy et al., 1977). The ecological significance of ODPs is postulated to be mediation of uniformity in egg distribution among available hosts, and hence effective utilization of available natural resources for larval development (Prokopy, 1981a,b). This does, in fact, appear to be the case in *A. fraterculus*, as sampling of the distribution of *A. fraterculus* eggs among Surinam cherries in nature revealed a variance (0.87) considerably less than the mean (1.36 eggs per cherry) (Malavasi et al., unpublished data). Surinam cherries are capable of supporting to maturity an average of 1.64 *A. fraterculus* larvae per fruit (Malavasi et al., unpublished data).

The fact that *A. fraterculus* males were not arrested by the ODP does not parallel the situation in *Rhagoletis* species, where ODP acts to arrest males on the fruit surface, alerting them to the recent presence of females on the host tree (Prokopy, 1980). This enhances the probability of courtship encounters between males and females. Unlike *Rhagoletis* flies (Prokopy, 1980), mating initiation in *A. fraterculus* is not confined to host trees (Malavasi et al., unpublished data), thereby precluding strong selection pressure for male response to ODP.

Our finding that fruit size influences the occurrence and duration of ovipositor dragging after egg-laying was unexpected. It is the first suggestion among tephritids that ODP deposition may be a variable behavior whose expression is host-dependent.

Evidence suggests that this sort of phenomenon may occur also in a species of *Hylemya* flies (Zimmerman, 1980).

Specifically, our data show that compared with postoviposition behavior on 25-mm artificial fruits, *A. fraterculus* females on 13-mm artificial fruits of identical quality (i.e., identical physical and chemical composition) dragged the ovipositor in a significantly greater proportion of cases for a significantly longer time. They also dragged in a significantly greater proportion of cases after ovipositing in 13-mm Surinam cherries compared with 25-mm guavas. The fact that duration of ovipositor dragging was highly correlated with

number of dragging circles, coupled with the fact that a greater number of dragging circles confers greater deterrence (Table 1, experiment 3), suggests a strong positive relation between duration of dragging and amount of ODP deposited. Together, these data suggest that *A. fraterculus* females ovipositing in artificial fruit are apparently able to regulate the amount of pheromone deposited according to fruit size. Small artificial fruits receive more ODP than larger fruits of equal quality. The data in Table 4 clearly demonstrate that it is during postoviposition behavior, and not during preoviposition behavior or during oviposition itself, wherein the female acquires the necessary information for determining how much ODP to release on artificial fruits. Whether the nature of the information is fruit size per se (as might be measured during the course of ovipositor dragging) or frequency of tarsal receptor (Crnjar and Prokopy, 1982) contact with ODP is uncertain. It is also uncertain whether the greater duration of ovipositor dragging on small compared with large artificial fruits of equal quality characterizes the dragging duration pattern on small compared with large real fruits of equal quality. The duration of dragging on 25-mm guavas and 13-mm Surinam cherries in lab tests was nearly identical (Table 2), but differences in fruit quality may have played a confounding role.

Finally, our data demonstrate that, as with ODP deposition, departure of *A. fraterculus* females from a fruit without additional boring after ovipositor dragging is not a fixed trait. It may vary according to the surroundings, availability of other fruits in the vicinity, prior experience of the females, or fruit size or quality. Thus, where fruits were small (13 mm), of possibly inferior quality (artificial), or ample other fruits were available (guavas in nature), females departed after ovipositor dragging more often than where fruits were larger (25 mm), of possibly better quality (guavas), or no other fruits were available (guavas in lab cages) (text and Tables 2, 3, and 4).

Recent evidence (Averill and Prokopy, unpublished data) from studies of *R. pomonella* suggests that, as with *A. fraterculus*, the occurrence and amount of ODP deposition and other aspects of postoviposition behavior are not fixed traits but may vary according to fruit size and/or quality, ecological conditions, and possibly also the genetic structure of the population. Additional experiments on both these species are needed to determine if there is a common pattern in the expression of these behaviors and to ascertain the ecological and evolutionary implications.

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DEFENSIVE AND PHEROMONAL SECRETION OF THE TERGAL GLAND OF *Aleochara curtula*[1] I. The Chemical Composition

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Abstract—The defensive secretion from the tergal gland of the staphylinid beetle, *Aleochara curtula*, acting as a supplementary mating stimulant, was investigated by gas–liquid chromatography, mass spectrometry, and IR spectroscopy. The reservoir contains a complex mixture of hydrocarbons, aliphatic aldehydes, and substituted 1,4-benzoquinones. *n*-Undecane, 1-undecene, and (*Z*)-4-tridecene were identified as the major hydrocarbon components. The main aldehydes are *n*-dodecanal and (*Z*)-5-tetradecenal, and the chief quinones are toluquinone and 2-methoxy-3-methyl-1,4-benzoquinone, the latter being established structurally by comparison of the mass spectra of the three synthesized isomers. Quantitative GLC analyses revealed no sex specificity of the relative concentrations of the compounds.

Key Words—*Aleochara curtula* (Goeze), Coleoptera, Staphylinidae, tergal gland secretion, hydrocarbons, aliphatic aldehydes, substituted 1,4-benzoquinones, *n*-undecane, 1-undecene, (*Z*)-4-tridecene, n-dodecane, (*Z*)-5-tetradecenal, toluquinone, 2-methoxy-3-methyl-1,4-benzoquinone.

INTRODUCTION

Within the staphylinid beetles, a tergal gland supported with a large reservoir is restricted to the subfamily Aleocharinae (Araujo, 1978). Histological investigations on the epidermal glands were made to interpret the behavioral adaptations of myrmecophilous and termitophilous Aleocharinae in com-

[1]Coleoptera: Staphylinidae (Aleocharinae)

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parison to the free-living species (e.g., Pasteels, 1968; Kistner and Pasteels, 1969). The secretion of the tergal gland of some species has been proved to have defensive properties (Jordan, 1913; Pasteels, 1968; Hölldobler, 1970). In *Aleochara curtula* (Goeze), which is a most abundant species found with carcasses, intraspecific communication by components of the tergal gland secretion was studied and their role as additional mating stimulants established (Peschke, 1982). These effects support the female sex pheromone from epicuticular lipids (Peschke, 1978a).

The chemical composition of the tergal gland secretion of two closely related Aleocharinae has also been investigated in *Lomechusa strumosa* (Blum et al., 1971) and *Drusilla canaliculata* (Brand et al., 1973), but much more comparative data on tergal gland chemistry are needed in order to make safe conclusions concerning the chemical taxonomy, the function of each compound, and the evolution of this versatile group of beetles. In the present paper we report our chemical investigations on the tergal gland secretion of *A. curtula* as a basis for subsequent behavioral investigations.

METHODS AND MATERIALS

Collection of Secretion. A. curtula was reared in the laboratory according to Fuldner (1968) and Peschke (1978a). Sexes were separated immediately after emergence. Beetles were freshly killed by freezing, and the tergal gland secretion was collected by inserting a triangle of filter paper between the sixth and seventh abdominal tergites. The triangles from 100 males or females were collected in 1 ml *n*-hexane. This extract was stored at −17° C and could be used for GLC without further preparation.

Chemical Isolation and Identification. Gas–liquid chromatographic analyses (GLC) were carried out on a Shimadzu GC-6A instrument with hydrogen flame detectors under standard conditions of hydrogen (25 ml/min), air (250 ml/min), and nitrogen flow (30 ml/min), employing the routine temperature program (70–190° C at 8° C/min column temperature, 210° C detector and injector temperature). Comparison of retention times and cochromatographics were run on four liquid phases (3% or 10% Carbowax 20 M, 10% SP 1000, 10% SE 30), using Chromosorb W AW 80/100 mesh as a solid support and made up in 2-m × 3-mm glass columns.

Quantitative determination of the compounds was carried out by GLC with a Shimadzu ITX-4 integrator. The peak areas were calibrated to mass by injection of mixtures of weighed standards.

For preparative GLC a 2-m × 4-mm steel column supported with 10% Carbowax 20 M on Chromosorb W AW 80/100 mesh was used. Individual substances were trapped by liquid nitrogen.

Ozonolysis was carried out according to the procedure of Beroza and

Bierl (1967), and the resulting aldehydes were identified by GLC retention times (3% Carbowax 20 M, 5 min at 70° C, then temperature programmed from 70° to 190° C at 8° C/min).

Gas chromatography–mass spectrometry was carried out on a Varian 2700 gas chromatograph coupled to a Varian CH 7 mass spectrometer through a two-stage Watson Biemann separator using an all-glass system for the connection. Temperatures were 250° C for the separator and the connecting capillary tube. The mass spectra were run at an electron energy of 70 eV.

IR spectra of purified compounds dissolved in C_6D_6 were taken with a Perkin-Elmer 377 Granting infrared spectrophotometer equipped with a 2-μl microcell.

Synthesis of Substituted 1,4-Benzoquinones. 2-Methyl-1,4-benzoquinone was purchased from Fluka. 2-Methoxy-3-methyl-1,4-benzoquinone, 2-methoxy-5-methyl-1,4-benzoquinone, and 2-methoxy-6-methyl-1,4-benzoquinone were synthesized by oxidation with potassium nitrosodisulfonate (Fremy's radical) of 2-methoxy-3-methylaniline, 2-methoxy-5-methylaniline, and 2-methoxy-6-methylaniline, respectively (Zimmer et al., 1971). The amines were obtained from the corresponding nitrocresols by methylation with dimethylsulfate, followed by Raney nickel-catalyzed reduction with hydrazine hydrate.

The purity of the synthesized benzoquinones was controlled by GLC and found to be >95%. In the case of 2-methoxy-6-methyl benzoquinone, the structure of a 1,4-benzoquinone was self-evident from the synthesis. For the 2-methoxy-3-methyl isomer and the 2-methoxy-5-methyl isomer, the possibility of a 1,2-benzoquinone structure was ruled out because both compounds failed to give a phenazine derivative with orthophenylenediamine.

The synthetic isomers were separated by GLC on a 6-ft × 2-mm ID glass column packed with 3% OV 225 on Gas Chrom Q and operated with a helium flow of 30 ml/min and a temperature program of 100° C with 4° C/min.

Synthetic hydrocarbons and *n*-dodecanal were purchased from Fluka, Roth, or Sigma. A sample of (*Z*)-5-tetradecenal was obtained from Dr. O. Vostrowsky, Erlangen.

RESULTS

General Features of Secretion. Like other Aleocharinae (Araujo, 1978; Jordan, 1913; Pasteels, 1968; Kistner and Pasteels, 1969), *A. curtula* possesses a tergal gland with a bilobate reservoir formed by an invagination of the intersegmental membrane between the sixth and seventh abdominal tergites (Figure 1). The volume of the reservoir is approximately 0.2 mm^3, as determined by measuring histological preparations.

The secretion is immediately sucked from the reservoir by inserting a

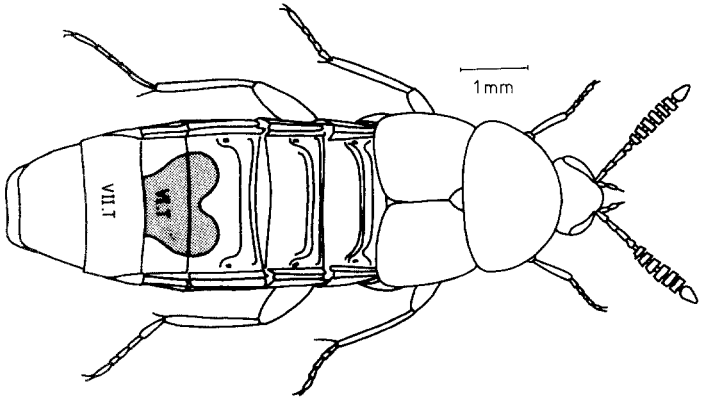

FIG. 1. Dorsal view of *A. curtula* with the reservoir of the tergal gland (dotted area; VI.T, VII.T: sixth and seventh abdominal tergites). No sex specificity was recognized in gross morphology.

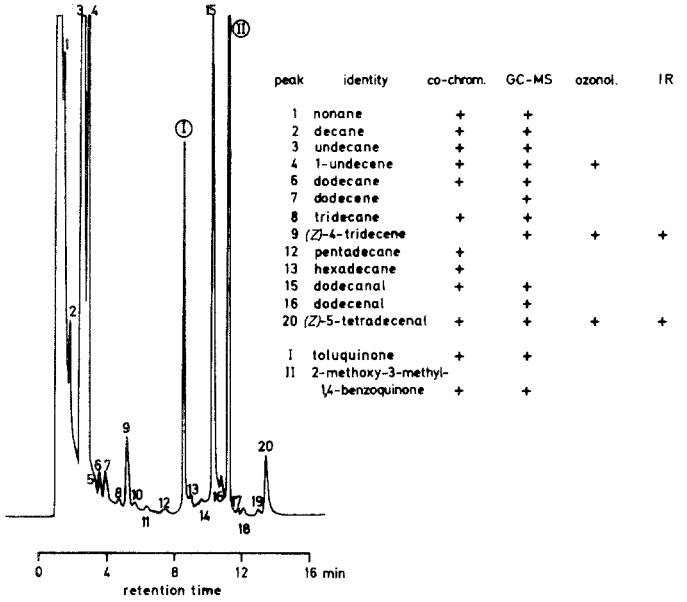

FIG. 2. Gas chromatogram of the tergal gland secretion of 20-day-old *A. curtula* females (3% Carbowax 20 M, 70–190° C at 8° C/min) and identification of compounds by cochromatography, gas chromatography–mass spectrometry, and IR spectroscopy.

triangle of filter paper. The bright yellow color, as well as the pungent odor of the secretion and its ability to cause a red or brown staining of the human skin, implied the presence of quinones in the mixture.

GLC analysis on different columns showed that the secretion contained at least 22 different compounds (Figure 2). Most of the mass spectra obtained by combined GC-MS showed features characteristic for aliphatic hydrocarbons and aldehydes, i.e., low-intensity molecular ions and series of fragments differing by 14 mass units. Two of the major compounds, however, had very prominent molecular ions. They could only be chromatographed on 10% SE 30 and 3% Carbowax 20 M but were not seen in the chromatograms when 10% SP 1000 or 10% Carbowax 20 M was used.

Identification of compounds of the tergal gland secretion of *A. curtula* was carried out for females 20 days after emergence.

Identification of Quinones. One of the two compounds (peak I) with strong molecular ion (*m/e* 122) cochromatographed with authentic 2-methyl-1,4-benzoquinone and had an identical mass spectrum. The molecular ion of the second compound (peak II) at *m/e* 152 implied the structure of a toluquinone with one additional methyl group and an oxygen function. Since no trimethylsilylated product was obtained upon treatment with silylating agents, a hydroxy group was unlikely, and the structure of a methoxytoluquinone was assumed. For unequivocal identification of this compound, the three possible isomers, 2-methoxy-3-methyl-1,4-benzoquinone, 2-methoxy-5-methyl-1,4-benzoquinone, and 2-methoxy-6-methyl-1,4 benzoquinone, were synthesized and their mass spectra (Figure 3) and GLC retention times (4.5,

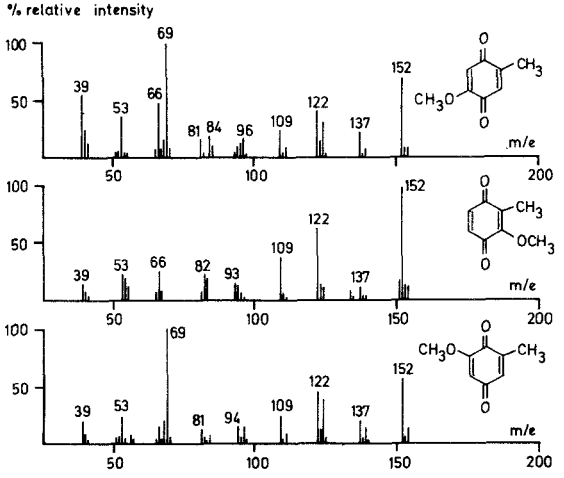

FIG. 3. Electron impact mass spectra of the isomeric methoxy-methyl-1,4-benzoquinones.

11.5, and 12.0 min, respectively, on 3% OV 225) were determined. The secretion constituent had a mass spectrum identical with that of authentic 2-methoxy-3-methyl-1,4-benzoquinone, and it also cochromatographed with the synthetic compound on both GLC columns, 10% SE 30 and 3% Carbowax 20 M. The two other isomers were not present in the solution.

The structure of 2-methoxy-3-methyl-1,4-benzoquinone was substantiated not only by the synthetic route but also by the compound's mass spectrum. Whereas the mass spectra of the isomeric compounds 2-methoxy-5-methyl-1,4-benzoquinone and 2-methoxy-6-methyl-1,4-benzoquinone are very simlar, both having their base peak at m/e 69, the mass spectrum of 2-methoxy-3-methyl-1,4-benzoquinone lacks the m/e 69 ion (Figure 3). High-resolution measurements on the spectrum of 2-methoxy-5-methyl-1,4-benzoquinone (Bowie et al., 1966) have shown that the peak at m/e 69 is composed of the ions $C_3HO_2^+$ (90%) and $C_4H_5O^+$ (10%). The same fragmentation is possible for 2-methoxy-6-methyl-1,4-benzoquinone; this explains the abundant m/e 69 (Figure 3). In contrast, neither of these ions can be formed upon fragmentation of 2-methoxy-3-methyl-1,4-benzoquinone. Instead, analogous ions, although with lower intensity, are observed at m/e 83 and 54 (Figure 3).

Identification of Lipids. Standard GLC analysis of lipid compounds were run on a 10% Carbowax 20 M column, which gave results similar to those obtained on 10% SP 1000 liquid phase. Twenty peaks were detected, and 10 substances could be identified by comparison of the retention times with those of synthetic standards. Cochromatography revealed the same results. The mass spectra of 11 major compounds were compared with those of authentic samples (Figure 2).

n-Alkanes with carbon chain length from C_9 to C_{16} were identified. Undecane is the main component of the secretion; nonane, decane, and dodecane occur in smaller amounts, whereas tridecane, pentadecane, and hexadecane could be detected in trace amounts only.

n-Alkenes Peak 4, which appeared shortly after that of undecane, was identified as an undecene by GC-MS. The native substance cochromatographs with synthetic 1-undecene. Ozonolysis of native undecene obtained by preparative GLC gave decanal as the main reaction product. Thus, 1-undecene was established. However, other aldehydes were also produced in minor amounts by ozonolysis, indicating that numerous undecene isomers and C_{11} molecules with multiple unsaturation should be expected. Similar procedures employing GC-MS, cochromatography, and ozonolysis showed the occurrence of 4-tridecene in quantities even smaller than those determined in the undecene mixture. The IR spectrum of the substance purified by preparative GLC showed no absorption at 960 cm^{-1}, thus indicating the lack of a *trans*-distributed olefin bond. Therefore, the occurrence of (*Z*)-4-tridecene is assumed. Another unsaturated hydrocarbon, dodecene, was

TABLE 1. COMPARISON OF COMPOSITION OF TERGAL GLAND SECRETION OF YOUNG (0 DAYS) AND OLD (20 DAYS) MALES AND FEMALES OF *A. curtula* IN PERCENT PEAK AREA[a]

Peak	Substance	Amount (μg/gland), ♀♀, 20 days	% peak area			
			0 days		20 days	
			♂♂	♀♀	♂♂	♀♀
A						
1	Nonane	–	+	+	+	+
2	Decane	–	0.6	0.9	0.8	0.9
3	Undecane	96.9	74.2	75.5	75.8	76.4
4	1-Undecene	10.9	6.7	7.0	6.3	7.1
5	–	–	0.2	0.4	+	+
6	Dodecane	2.7	0.4	0.7	0.3	0.2
7	Dodecene	–	0.4	0.3	0.3	0.3
8	Tridecane	–	+	0.1	+	+
9	(*Z*)-4-Tridecene	2.9	2.6	2.5	1.3	1.5
10	–	–	0.2	0.2	0.1	0.1
11	–	–	0.1	0.2	+	+
12	Pentadecane	$2 \cdot 10^{-2}$	+	+	+	+
13	Hexadecane	$2 \cdot 10^{-2}$	+	+	0.1	+
14	–	–	+	+	+	+
15	Dodecanal	21.4	9.7	7.9	12.6	10.4
16	Dodecenal	–	0.7	0.7	0.5	0.7
17	–	–	0.4	0.4	0.2	0.5
18	–	–	0.2	0.2	+	+
19	–	–	0.3	0.3	0.2	0.1
20	(*Z*)-5-Tetradecenal	4.7	3.2	2.6	1.4	1.4
B						
1-20	Total lipids	~150	79.9	80.6	86.6	87.2
I	Toluquinone	24	11.1	17.4	2.1	1.5
II	2-Methoxy-3-methyl-1,4-benzoquinone	130	9.0	2.0	11.3	11.3

[a](A). Lipid compounds separated on 10% Carbowax 20 M. (B). Amounts of quinones separated on 10% SE 30, relative to the total lipid content. plus: Substance detected, but <0.1%. Absolute amounts of some compounds were calculated for 20-day-old females; minus: not determined.

identified, but the position of the double bond remains unknown. Peak 10 seems to be another unsaturated C_{13} hydrocarbon; it was not further analyzed.

Among the aldehydes, one of the major compounds in the tergal gland secretion of *A. curtula* (peak 15) was identified as *n*-dodecanal. The next major aldehyde is (Z)-5-tetradecenal (peak 20), as established by GC-MS and by ozonolysis, which yielded nonanal as the main product. The IR spectrum showed no absorption at 960 cm^{-1}, thus indicating the *cis* configuration. Peak

16 is an unsaturated C_{12} aldehyde with an unknown position of the double bond.

Quantitative Determination of Secretion Compounds of Males and Females of Different ages. For quantitative evaluations, peak areas were determined from chromatograms of the secretion of 100 beetles combined in 1 ml *n*-hexane. Absolute amounts of some compounds were calculated by comparison with peak areas of the corresponding synthetic substances at different concentrations (Table 1). Only secretion compounds of 20-day-old females were determined. GLC traces of secretions from males or females, respectively, obtained immediately after emergence or 20 days later, did not show substantial differences in the relative composition of lipid compounds after calculation of peak areas from separations on 10% Carbowax 20 M (Table 1, A). Freshly emerged males and females produced somewhat more toluquinone and less 2-methoxy-3-methyl-1,4-benzoquinone than 20-day-old beetles, as determined by calculation of peak areas from separation on 10% SE 30 and compared to the total content of lipids (Table 1, B). Only comparison among beetle groups was intended by calculation of percent peak areas, in order to also include the minor, partly unidentified substances which could not be calibrated for mass. The response of the FID to various classes of compounds is obviously quite different. The total peak areas of chromatograms of 20-day-old males or females were quite similar, but those of newly emerged beetles amounted to only about 10% of the total peak areas of old *A. curtula*.

DISCUSSION

Both sexes of *Aleochara curtula* possess a tergal gland. No sex specificity of the chemical composition of its secretion could be detected. The chemistry of this secretion is very similar to that of *Drusilla canaliculata* (Brand et al., 1973), which is an Aleocharinae species too, but belongs to the tribe Zyrasini instead of Aleocharini. In both species, undecane is the main saturated hydrocarbon accompanied by smaller amounts of 1-undecene. While tridecane occurs only in trace amounts in the secretion of *A. curtula*, it has been identified as the major hydrocarbon of another Zyrasini, *Lomechusa strumosa* (Blum et al., 1971). In *D. canaliculata* and *A. curtula* (*Z*)-4-tridecene is the major C_{13} hydrocarbon. Further saturated hydrocarbons, pentadecane and hexadecane, are produced in minor amounts by *A. curtula*. The defensive secretions of *Hesperus semirufus* and *Philonthus politus* (Staphylininae) also contain tridecane and pentadecane, but this secretion originates from pygidial glands, which are not homologous with the tergal gland of Aleocharinae. The predominant occurrence of terpenoid compounds in pygidial secretions emphasizes this difference (Abou-Donia et al., 1971; Bellas et al., 1974;

Dettner, 1980; Fish and Pattendan, 1975; Schildknecht et al., 1976; Wheeler et al., 1972). The secretion from the sternal gland of *Eusphaleurum longipenne* (Omaliinae) contains 3-methyl-butyric acid and *trans*-hex-2-enal (Klinger and Maschwitz, 1977).

The aldehyde mixture of the tergal gland secretion of *A. curtula* also resembles that of *D. canaliculata*. Dodecanal and (*Z*)-5-tetradecenal are the main aldehydes. Long-chain *n*-aldehydes are found in the defensive secretion of millipedes (*E*-2-dodecenal; Wheeler et al., 1964) and have been identified as male or female sex pheromones of moths (Dahm et al., 1971; Röller et al., 1968; Leyrer et al., 1973; Roelofs et al., 1971; Underhill et al., 1977; Weatherston et al., 1971).

Brand et al. (1973) discussed the possible relationships of the biosynthesis of hydrocarbons and aldehydes, basing their reasoning on the homologous position of the double bonds. We assume that in *A. curtula* undecane and dodecanal, as well as (*Z*)-4-tridecene and (*Z*)-5-tetradecenal, also have common precursors.

Toluquinone and 2-methoxy-3-methyl-1,4-benzoquinone represent major components of the tergal gland secretion of *A. curtula*. They have also been identified in *D. canaliculata* (Brand et al., 1973), but in *L. strumosa* the latter was lacking (Blum et al., 1971). Toluquinone is a common compound of arthropod defensive secretions, often occurring with hydrocarbons (Weatherston and Percy, 1970; Eisner and Meinwald, 1966; Schildknecht, 1968). 2-Methoxy-3-methyl-1,4-benzoquinone is very common in millipedes (Weatherston and Percy, 1970), but not frequently found in beetles (Tenebrionidae; Tschinkel, 1975; Carabidae: Eisner et al., 1977; Schildknecht et al., 1968).

The chemical investigations on only three Aleocharinae do not permit extensive conclusions on evolution, but they do demonstrate that variations in the composition of the tergal gland secretion are more striking between members of one tribe with different life habits (Zyrasini: *L. strumosa* and *D. canaliculata*) than between *A. curtula* and *D. canaliculata*, which are free-living species from different tribes. Perhaps the different composition of the secretion of *L. strumosa* in an adaption to myrmecophily; however, an extended comparative investigation is necessary. On the other hand, detailed behavioral studies on the function of each compound of defensive secretions of Aleocharinae are lacking. Defensive properties have been demonstrated for some species by Jordan (1913), Pasteels (1968), and Hölldobler (1970). Kemner (1923) assumed that mating behavior of *A. curtula* could be managed by odors, probably from the tergal gland. However, only their role as an additional aphrodisiac could be expected for this secretion, because sex specificity of the composition could not be detected. Nevertheless, the role of the tergal gland secretion of *A. curtula* as an additional mating stimulant has been demonstrated by Peschke (1982), acting synergistically with the essential

female sex pheromone from the epicuticular hydrocarbons (Peschke, 1978a; Peschke, in preparation).

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ABSENCE OF FARNESOL IN STRAWBERRY AND HOP FOLIAGE

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Abstract—The farnesol content of foliage has been proposed as an indicator of susceptibility to the two-spotted spider mite in various crops. The use of this chemical "marker" was investigated for strawberry and hop varieties. No farnesol was detectable in any of the varieties tested. The techniques necessary for analysis and identification of farnesol from foliage are discussed.

Key Words—Farnesol, strawberry, hop, two-spotted spider mite, Acari, Tetranychidae, *Tetranychus urticae*, plant resistance, resistance mechanism.

INTRODUCTION

The two-spotted spider mite, *Tetranychus urticae* Koch, is one of the most persistent and ubiquitous pests of agricultural crops (review: van de Vrie et al., 1972). It is a serious economic problem of strawberry and other berryfruit in many countries, and it is a major problem on strawberries grown for export in New Zealand. Pest management would be greatly assisted if varieties resistant to *T. urticae* could be developed. Much attention has been given to the breeding of such varieties, and to the chemical nature of host-plant resistance to this pest. It was recently proposed by Regev and Cone (1975b), and Regev (1978) that mite population levels could be positively correlated with farnesol content in hop and in strawberry leaves. This hypothesis looked promising as a "marker" technique for the rapid selection and breeding of resistant varieties and was closely investigated for possible use against the two-spotted mite on New Zealand strawberries.

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METHODS AND MATERIALS

Preliminary Trials. Four cultivars of strawberry (*Fragaria ananassa* Duchesne) grown commercially in New Zealand, Redgauntlet, Sequoia, Cambridge Favourite, and Tioga, were established in plots at Mt. Albert Research Centre, DSIR, Auckland, in April 1979.

Six grams of fresh mature leaves (or its dried equivalent, 1 g) were finely ground and extracted in solvent under the conditions of Table 1. Duplicate samples had 60 μg synthetic farnesol added before treatment.

The filtrate was evaporated in vacuo (water aspirator) to dryness. It was further refined by chromatography on a Florisil column (5 g), eluted successively with 25 ml each of petroleum ether, petroleum ether–ether (9:1), and ether. Each of the elutes was concentrated to a final volume of 120 μl under a stream of nitrogen, and analyzed by gas–liquid chromatography (GLC) (Varian Aerograph 1800, with flame ionization detectors), using nitrogen as the carrier gas.

Three columns, 2 m $\times$ 0.2 mm ID stainless steel, were used, packed with (1) 10% Silar 10CP (polar); (2) 5% Carbowax 20 M (polar); or (3) 10% OV-101 (nonpolar), all on Chromosorb W-AW-HMDS 100–120 mesh, and the temperature was maintained at 170°C. A synthetic farnesol standard of 50 ppm (50.64 μg/μl) was used for comparison with the leaf extracts. All subsequent extractions were made from fresh foliage, using absolute methanol.

Seasonal Analysis. This analysis was replicated regularly through the growing season (September 1979–February 1980), using fresh leaf ether extract from the four strawberry cultivars. Fruit is harvested from mid-October through to early February (for Tioga, Sequoia, and Cambridge Favourite; Redgauntlet is harvested 3–4 weeks later); thus foliage was sampled prior to and through the harvest season.

In addition, single analyses were made of oven-dried leaf extracts from Tioga and Sequoia, and fresh leaf ether extract from 9 hop cultivars (*Humulus lupulus* Linn.). The hop leaves were collected from the Riwaka

TABLE 1. LEAF TREATMENTS AND SOLVENTS TESTED FOR EXTRACTION OF FARNESOL FROM STRAWBERRY

Solvent	Fresh leaf	Freeze dried	Oven dried[a]
Methanol	x	x	x
$CHCl_3$–Ethanol	x	x	
Petroleum ether	x		

[a]48 hours at 50°C.

Research Station, DSIR, in February 1980, from cultivars known from field observations to be differently susceptible to *T. urticae* infestation. Counts of mite populations on the leaves were made concurrently with all analyses. The ether extracts were further analyzed by GC-MS and TLC.

Gas chromatography–mass spectrometry (GC-MS) was performed on a Varian 2700 gas chromatograph, coupled via a membrane separator (at 180° C) to an AEI-MS 30 mass spectrometer, operated at 20 eV (low resolution) and 70 eV (high resolution). Helium was used as the carrier gas. Fractions with the same retention time as synthetic farnesol on GLC were collected and concentrated in tubes packed with glass wool coated with 10% Silar 10CP after the procedure of Murray et al. (1972). These fractions were analyzed by mass spectrometry. Similarly, the four major isomers in the standard synthetic farnesol solution were collected and characterized separately by GC-MS.

For thin-layer chromatography (TLC), each of the ether extracts was spotted on to a 20 × 10-cm thin-layer plate coated with silica gel. After development in petroleum ether–ether (1 : 1) to a distance of 20 cm, the plates were air dried at room temperature and exposed to iodine vapor until the spots stained dark brown. Synthetic farnesol was used as a reference on either side of the plate.

RESULTS

Preliminary Trials. Preseason work (July–September 1979) with 2-year-old Tioga plants yielded no detectable farnesol from the leaves. To ensure that a reliable procedure was obtained before the season began, synthetic farnesol (60 μg) was added to freshly picked leaves (6g), and each sample analyzed by GLC to yield the results of Table 2.

These results are not directly comparable to the extraction of intrafoliar chemicals; nevertheless, they do indicate the probable effects of the different

TABLE 2. RECOVERY OF SYNTHETIC FARNESOL FOLLOWING DIFFERENT LEAF TREATMENTS

Treatment	Added farnesol recovered (μg)	Recovery (%)
Fresh	54	>90[a]
Freeze dried	10	<20[a]
Oven dried	4.5	<10

[a]For all solvents used for extraction.

treatments on the recovery of farnesol and related chemicals. As substantial amounts of volatile substances like farnesol are lost by oven-drying, and to a lesser extent by freeze-drying, all subsequent GLC analyses were made from freshly picked leaves, using absolute methanol as a solvent.

More compounds were extracted from fresh than dried leaves, and the waxy extracts from fresh leaves gave serious trouble with blocked needles and partial injections when evaporated to the small volumes required. The Florisil column was advantageous in removing hydrocarbons and esters. The farnesol-containing ether fractions collected were cleaner and easier to inject into the GLC. Each step of the procedure was monitored by duplicate samples with added synthetic farnesol. None of this alcohol was lost in the column.

Gas–Liquid Chromatography. Fresh strawberry leaf extract yielded a compound (called compound A) of similar retention time to synthetic farnesol, both on 10% Silar 10CP and Carbowax 20 M columns (Figure 1). Contrary to the findings of Regev (1978), there were no consistent varietal differences as far as this compound was concerned. Sampling was continued throughout the season (September 1979–February 1980). In all cultivars, only trace amounts could be extracted from old, winter foliage. The compound increased in quantity with the first spring growth, and then remained steady through the season. The quantities of this compound in the fresh leaves of all four cultivars were far less than those reported for farnesol from oven-dried

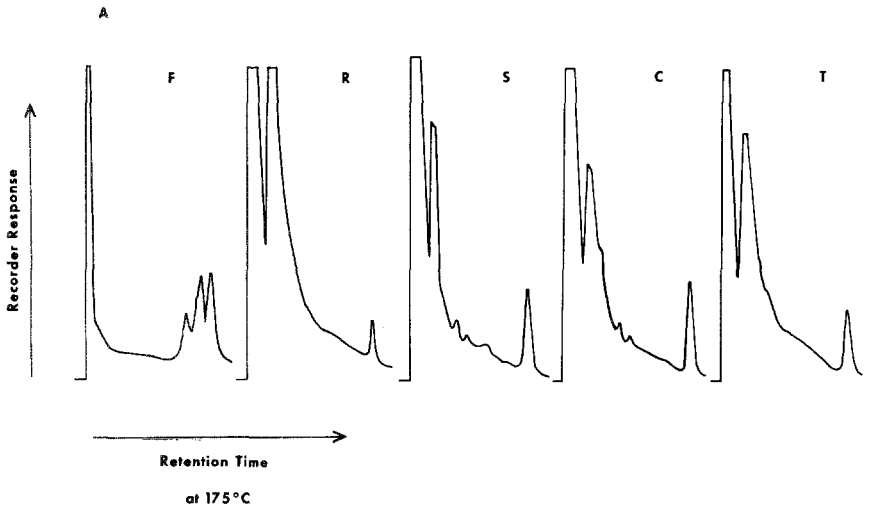

FIG. 1. (A) Gas–liquid chromatographs of 1 μl of farnesol standard (F) and 3 μl of extracts of fresh strawberry leaf varieties, Redgauntlet (R), Sequoia (S), Cambridge Favourite (C), and Tioga (T). The standard was run at 1/6 the sensitivity of the strawberry extracts. (B) Gas–liquid chromatographs of 1 μl farnesol standard (F) and 3 μl of an extract of fresh leaf from a hop variety, Sticklebract (H).

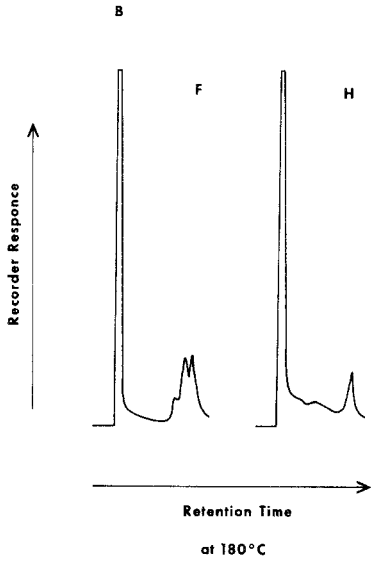

FIG. 1. Continued.

leaves by Regev (1978) (Table 3). Moreover, there was no correlation between the content of this chemical in the foliage and *T. urticae* populations on the leaves.

Whole plants of all cultivars were separated into older (outer), mature, and immature (inner) leaves, on one occasion in October 1979. These were treated as before, i.e., fresh leaf extraction with methanol, and the results, together with the corresponding mite counts, are shown in Table 4.

On the nonpolar OV-101 column, however, the results from the fresh strawberry leaf extract were quite different from those on the polar columns. Peak A, collected as a single entity from the 10% Silar 10CP column (Murray et al., 1972) was split on OV-101 into a number of compounds, none of which had the same retention time as synthetic farnesol.

GLC of the equivalent quantity of oven-dried leaf extract from Tioga and Sequoia yielded a slightly smaller peak (labeled compound B) than that from fresh leaves, with a similar retention time to synthetic farnesol on 10% Silar 10CP.

Fresh hop leaf analysis by GLC revealed very similar compounds (labeled compound C) to those in strawberry foliage, on both polar and nonpolar columns. Again, there was no correlation with mite populations on the leaves, as seen in Table 5.

Mass Spectrometry. The mass spectrum of authentic farnesol was obtained as a standard every time either hop or strawberry samples were being analyzed by GC-MS. All four peaks of synthetic farnesol collected had

TABLE 3. QUANTITIES OF COMPOUND A EXTRACTED FROM FRESH FOLIAGE, AND MITE POPULATIONS ON LEAVES OF STRAWBERRY CULTIVARS (1979–1980 SEASON)

			October 1979		December 1979		February 1980		Regev (1978)			
	Winter Foliage		Spring preharvest		Mid season harvest		End season late harvest		Kalansawa		Tira	
Variety	Chemical[a]	Mite[b]	Chemical	Mite	Chemical	Mite	Chemical	Mite	Chemical	Mite	Chemical	Mite
Redgauntlet	Trace	0	28.73	18.06 ± 10.00	23.65	648.00 ± 88.20	6.44	41.75 ± 12.62				
Sequoia	Trace	0.02 ± 0.02	20.13	1.31 ± 0.59	27.10	724.72 ± 84.12	7.37	18.35 ± 4.60	191.40 ± 62.63	31.50 ± 5.05	215.69 ± 3.14	4.21 ± 0.44
Cambridge Favourite	Trace	0.10 ± 0.10	22.05	3.47 ± 1.90	19.40	846.41 ± 70.54	7.59	80.39 ± 17.38				
Tioga	Trace	0.29 ± 0.17	23.00	10.22 ± 6.68	27.10	647.39 ± 123.73	3.67	26.52 ± 11.31	47.33 ± 7.03	6.23 ± 2.82	29.81 ± 3.45	1.16 ± 0.48

[a] μg chemical/g dry wt leaf (equivalent).
[b] Mean No. active mites/leaf ± SE.

TABLE 4. EFFECT OF LEAF AGE ON QUANTITY OF COMPOUND A EXTRACTED FROM, AND MITE POPULATIONS ON, STRAWBERRY LEAVES (OCTOBER 1979)

	Redgauntlet		Sequoia		Cambridge Favourite		Tioga	
Variety	Chemical[a]	Mite[b]	Chemical	Mite	Chemical	Mite	Chemical	Mite
Young leaves	17.13	7.81 ± 5.69	48.32	7.06 ± 5.53	19.23	4.00 ± 1.73	34.30	10.00 ± 3.18
Mature leaves	46.58	126.18 ± 68.84	37.28	18.18 ± 11.38	22.66	41.56 ± 15.61	49.75	46.00 ± 9.63
Older mature leaves	44.16	94.93 ± 33.09	NA[c]	73.18 ± 34.11	61.16	86.93 ± 31.33	46.96	293.65 ± 53.53

[a] μg chemical/g dry wt leaf (equivalent).
[b] Mean No. active mites/leaf ± SE.
[c] Not available.

TABLE 5. QUANTITIES OF COMPOUND C EXTRACTED FROM FRESH HOP FOILAGE AND MITE POPULATIONS ON LEAVES (FEBRUARY 1980)

Hop cultivar	Foliar chemical (μg/g dry wt leaf equivalent)	Mean No. mites per leaf ± SE
Extension 70-5-9	8.24	1058.50 ± 124.86
Extension 70-5-77	5.91	938.20 ± 221.29
Extension 71-2-62	19.75	742.79 ± 181.36
Green Bullet	13.42	341.20 ± 185.83
Superalpha	4.59	169.20 ± 73.80
Sticklebract	12.95	86.60 ± 41.72
Seedling No. 1	trace only	1379.50 ± 246.05
Seedling No. 2	11.70	1192.00 ± 196.00
Seedling No. 3	21.93	645.39 ± 157.42

identical mass spectra and are isomers of farnesol. The farnesol fragmentation pattern, with major molecular ions at *m/e* 204, 161, 119, 107, and 93, and base peak at *m/e* 69, was obtained every time, but none of the >100 spectra from strawberry or hop fractions showed this pattern.

The main peak with a farnesol retention time from oven-dried strawberry leaves (i.e., compound B) had a mass spectrum resembling a hydrocarbon, with significant fragments at *m/e* 57, 71, 85, 97, and 111.

With fresh leaf extracts of strawberry (i.e., compound A) all material from the relevant area of the 10% Silar 10CP column was collected and pooled from several runs. This fraction showed several peaks when passed through an OV-101 column, and mass spectra of all these peaks were obtained. With the larger peaks, spectra were run at the start, middle, and end of the peaks. No farnesol-type spectra were seen.

The equivalent fresh hop leaf extract (i.e., compound C) had a mass spectrum with the base peak at *m/e* 141, and no major ions corresponding to those of the farnesol spectrum. This was very similar to the mass spectra from one of the peaks which made up compound A in strawberry leaves.

Thin-Layer Chromatography. The ether extracts from fresh leaves of all strawberry cultivars, and from oven dried Tioga and Sequoia leaves, were run on TLC with the farnesol standard on each side of the plate. The synthetic farnesol standards each appeared as two spots close together (see Figure 2). These probably represented the *cis–trans*, and *trans–trans* isomers, as observed by Regev and Cone (1975a).

The fresh leaf ether fractions showed spots with similar but slightly higher R_f values than synthetic farnesol. They were closer to the minor isomer.

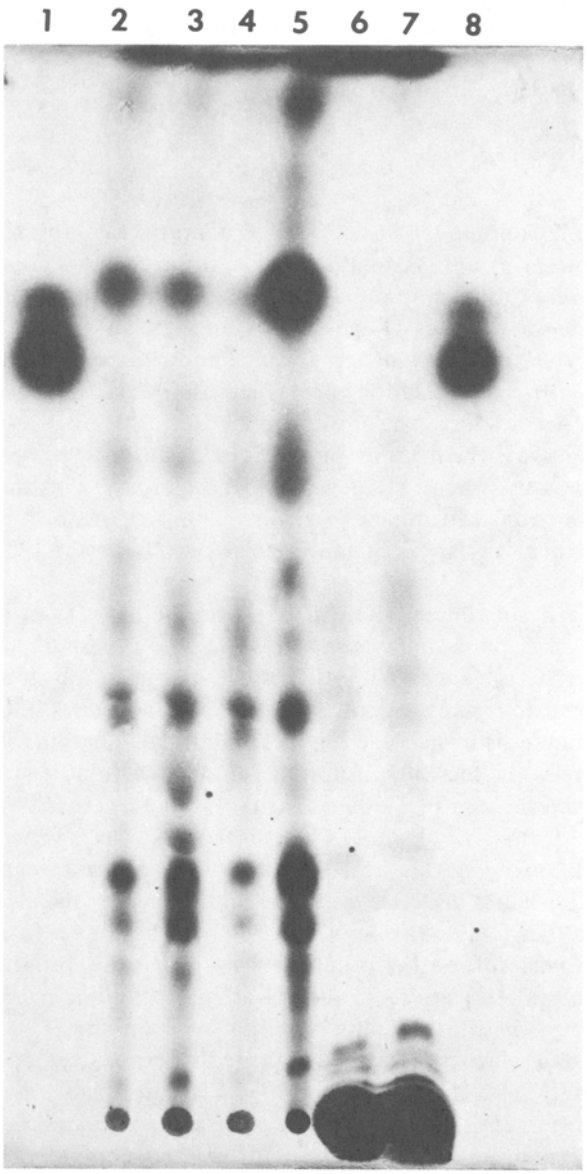

FIG. 2. Thin-layer chromatography plate showing: 1 and 8, farnesol standard; and extracts of fresh leaves from: 2, Tioga; 3, Sequoia; 4, Redgauntlet; 5, Cambridge Favourite; 6, oven-dried Tioga; and 7, Sequoia.

From the oven-dried leaves, only a trace was found with R_f values similar to farnesol (Figure 2). No quantitative analysis was possible because unequal amounts were spotted onto the plates.

DISCUSSION

Regev and Cone (1975a) identified one component of the *T. urticae* male sex attractant–arrestant system as the sesquiterpene alcohol, farnesol. Differences were observed in population development of two-spotted spider mite on five hop varieties (Regev and Cone, 1975b). From quantitative analyses, they submitted evidence of correlation between varietal farnesol levels, and *T. urticae* populations on the leaves. The most resistant cultivars were determined to have the lowest foliar farnesol levels, and the most susceptible cultivars the highest. The same relationship was observed for *T. cinnabarinus* on strawberry (Regev, 1978) and on roses. This was believed to result from the gonadotrophic and sex-attractant effects of farnesol on the mite, and further work investigated these effects (Regev and Cone 1976a,b; Regev, 1979).

Use of farnesol content as a "marker" tool in the selection of resistant varieties appeared potentially useful for application to mite management in New Zealand berry fruits. Although the hypothesis was interesting, the methods described by Regev and Cone (1975b) and Regev (1978) to prepare and extract materials from the leaves were modified. Heating the leaves for a long time causes the loss of substantial amounts of volatile substances like farnesol, as can be seen in Table 2. Farnesol stored naturally in leaf tissue would be lost less readily from the system than synthetic farnesol added to leaves. Even allowing for this, however, there was a large discrepancy between results from fresh and dried leaves in the preliminary trials. On this basis, it was expected that fresh strawberry leaves would be shown to contain large quantities of farnesol, on the order of 3–4 times those obtained by Regev (1978). Further modifications to the extraction technique included refining extracts through the Florisil column to improve handling.

At this stage, farnesol was still expected to be recovered, according to Regev's findings, and indeed, on polar GLC column packings, a peak was obtained with a retention time similar to one isomer of farnesol (see Figure 1). However, the amount of this compound was low compared with Regev's (1978) report. There were no consistent varietal differences, and no correlations with the seasonal pattern of mite development on the leaves (see Table 3).

More thorough examination of this compound, using nonpolar as well as polar columns for GLC and GC-MS, demonstrated that it was not farnesol. GC-MS analysis was performed on: (1) fresh leaf ether extract from the hop cultivar Sticklebract; (2) fresh leaf extract from all four strawberry cultivars;

and (3) oven-dried leaf ether extract from Tioga strawberry, and the synthetic farnesol isomers. These were obtained by the method of Murray et al. (1972). The hop material was also collected cleanly, but on the nonpolar OV-101 column; this compound had a totally different retention time to that of any of the farnesol isomers. Further the mass spectrum of this compound was not that of farnesol. This emphasizes that both polar and nonpolar columns are required for GLC analysis of even simple compounds, particularly where GLC analysis alone is being taken as proof.

Similarly, further analyses of strawberry extracts under the above conditions, i.e., multiple collections, OV-101 column, and mass spectrometry of all peaks obtained, could produce no traces resembling those of farnesol. Rather, the fresh leaf compound (compound A) in both hop and strawberry, was tentatively identified as another leaf alcohol, probably a $C_{14}H_{28}O$ chain; and the compound extracted from oven-dried (B) leaves was revealed as a hydrocarbon. Thus the compounds being measured from fresh and from oven-dried leaves, although having very similar retention times on the polar columns, are not the same. Neither one is farnesol. When leaves are heated, not only are many volatile substances lost, but also the whole leaf chemistry is altered, with oxidation of many compounds. Thus the so-called "farnesol" measured from oven-dried leaves (Regev, 1978) appears, in fact, to be a created hydrocarbon from a crude leaf extract.

CONCLUSIONS

Farnesol is acknowledged as an important component of the sex attractant of this mite, but it is unlikely that it derives it directly from the host plant. If it is present, it is certainly in trace quantities only, and is not detectable in any of the hop or strawberry cultivars tested above.

The evidence presented here contradicts the measurement of farnesol content as a valuable pest management tool to identify plant resistance to spider mites. It emphasizes the importance of detailed analysis of foliar chemical content. Chromatography techniques alone can lead to false assumptions, so that final characterization by mass spectrometry is most important. The season-long investigation was very profitable, as results on single occasions were sometimes misleading in the correlation of mite numbers and foliar chemicals. In fact, season-long, the mite populations on the four cultivars were not very different (Gunson, unpublished data). The important varietal differences seem to be in tolerance to damage and possibly timing of population development in spring, rather than any actual resistance factor. Investigation is continuing.

The strawberry cultivars investigated by Regev (1978) are very closely related (Dr. I.K. Lewis, personal communication). Fresno and Tioga are full

siblings, and one of their parents, Lassen, is twice a grandparent of Sequoia, while Aliso was derived from selfing one of the parents of Sequoia. Therefore they may be unlikely to have a wide variety of chemical content. Redgauntlet and Cambridge Favourite are less closely related, but quantities of chemicals extracted from all varieties were still very similar.

For the selection and breeding of resistant varieties, the most promising approach seems still to be that of bioassay, particularly in assessing host-plant acceptance (e.g., Dabrowski and Rodriguez, 1971, 1972; Rodriguez et al., 1976; Dabrowski and Bielak, 1978). These feeding experiments and the search for any key chemicals would be assisted by the development of good artificial diets for these mites. Screening of new varieties for field use will continue to depend, for the present, on biological measures of the populations on the leaves, along with assessment of damage done by these pests.

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MONOOXYGENASE ACTIVITIES OF FAT BODY AND GUT HOMOGENATES OF MONARCH BUTTERFLY LARVAE, *Danaus plexippus*, FED FOUR CARDENOLIDE-CONTAINING MILKWEEDS, *Asclepias* SPP.[1]

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Abstract—Monarch butterfly larvae were examined for NADPH-dependent monooxygenase activities. Midgut and fat body homogenates catalyzed aldrin epoxidation and *p*-chloro-*N*-methylaniline *N*-demethylation at consistently low rates compared to many other lepidopteran larvae. Homogenates from larvae collected from four different milkweeds (*Asclepias* spp.) with quite different cardenolide contents had similar levels of activity. There were no detectable variations in activity due to season or year of collection.

Key Words—Monarch butterfly, *Asclepias* spp., monooxygenase, induction, cardenolide, aldrin epoxidation, *p*-chloro-*N*-methylaniline demethylation, Lepidoptera, Danaidae.

INTRODUCTION

Monarch butterfly larvae *Danaus plexippus* (Lepidoptera: Danaidae) ingest complex mixtures of cardenolides, C_{23} steroid glycosides, when feeding upon milkweed, *Asclepias* spp. This larval–host plant interaction has been the subject of considerable research because sequestered cardenolides (Duffey,

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1980) may limit vertebrate predation (Parsons, 1965; Brower and van Zandt Brower, 1964; Roeske et al., 1976). Chemical and physical properties of butterfly cardenolides are determined by constituents of the larval host plant (Roeske et al., 1976) and perhaps larval cardenolide biotransformation (Reichstein et al., 1968; Seiber et al., 1980). These properties will determine the disposition and biological activity of cardenolides in both the insects and their predators.

Biotransformation may be a particularly important determinant of the chemical properties of sequestered cardenolides. Based upon their chromatographic behaviors, milkweed cardenolides represent a considerable range of polarities. Since butterfly extracts contain some cardenolides which are more polar than cardenolides in their food plants, it is likely that some milkweed cardenolides are metabolized in larvae to more polar derivatives.

Polar derivatives of secondary plant substances, including steroidal cardenolides, may be products of NADPH-dependent larval monooxygenase-catalyzed reactions (Krieger et al., 1971; Wilkinson and Brattsten, 1972). Steroids are among the known substrates of monooxygenases, and many substrates are inducers of monooxygenases, increasing their activity (Terriere and Yu Shyi, 1974; Brattsten, 1979).

In the present study, we measured aldrin epoxidation and *p*-chloro-*N*-methylaniline *N*-demethylation as indicators of oxidative metabolic capability of tissue homogenates of monarch butterfly larvae collected in different areas, years, times in the season, and from different milkweed species.

METHODS AND MATERIALS

Aldrin epoxidation and *p*-chloro-*N*-methylaniline *N*-demethylation are classic monooxygenase transformations and have been used frequently in comparative metabolic studies. These substrates are available in pure form, and the transformations result in one easily measured matabolite. Sufficient quantities of milkweed cardenolide substrates and metabolite standards for direct studies are lacking.

Four species of milkweed were fed to actively feeding ultimate instar larvae collected in the field from particular host plants to evaluate the potential of milkweeds as monooxygenase inducers. Some larvae were fed the same milkweed on which they were collected and others were transferred to milkweeds of higher or lower cardenolide content. Other collections were made from the same locale at different times in the season, as well as in different years to evaluate possible seasonal influences on monoxygenase activities. Additionally, larvae were collected from the same *Asclepias* species growing in different areas to assess influence of locale on monooxygenase activity.

Animals. Monarch larvae were collected in late summer as penultimate or ultimate instars in the Central Valley of California from *Asclepias fascicularis* Dcne., *A. eriocarpa* Benth., *A. speciosa* Torr., and *A. erosa* Torr. in 1976 and from *A. fascicularis* and *A. eriocarpa* in 1978. In the laboratory (26–32° C, 14 hr light–10 hr dark), larvae were provided with the complete aerial portion of fresh milkweed plants on which to feed. The bases of the plant stems were immersed in water and larvae fed ad libitum. In 1978, larvae were used within one day and so photoperiod was not regulated. When larvae were transferred to other food plants, the transfers were made during the intermolt period or before active feeding began in the ultimate instar.

Midgut and fat body of actively feeding late ultimate instar larvae were the enzyme sources for all the biochemical studies (Krieger and Wilkinson, 1969; Thongsinthusak and Krieger, 1974).

Chemicals. Glucose-6-phosphate disodium salt dihydrate, nicotinamide adenine dinucleotide phosphate sodium salt, reduced NADP (Calbiochem, San Diego, California), ethylene diamine tetraacetate, benzo[a]pyrene, and *p*-chloro-*N*-methylaniline hydrochloride were purchased. Aldrin and dieldrin were gifts of Shell Development Company (Modesto, California). Aldrin was recrystallized from methanol prior to use. *p*-Nitroanisole was synthesized in this laboratory (Trautman et al., 1979). Solvents were nanograde (Mallinckrodt Chemical Co., St. Louis, Missouri).

Homogenate Preparations. Fat body and gut homogenates were prepared in ice-cold reaction mixture using an all-glass tissue grinder. The larvae were cut longitudinally and the gut removed carefully with tweezers. The gut was laid on absorbent paper and incised longitudinally, rinsed in ice-cold 1.15% KCl to free the gut of its contents, blotted dry, and placed in the tissue grinder containing reaction mixture. The fat body was removed with tweezers, rinsed in ice-cold 1.15% KCl, and transferred to a separate ice-cold tissue grinder containing reaction mixture. Tissues were ground by hand until no large pieces remained. The homogenate contained larval tissue, either fat body or gut, and reaction mixture in a 2:3 v/v ratio. The reaction mixture contained 75 mg glucose-6-phosphate, 20 mg NADP, and 21 mg KCl in 100 ml 0.1 M phosphate buffer, pH 7.4, at 20° C.

Enzyme Assay. Epoxidation was assayed using 0.2 or 1.0 ml homogenate and 100 μg aldrin/ml (final). Homogenate was preincubated in open tubes for 4 minutes in a 30° C water bath shaker before addition of aldrin in methanol (25 μl). After 4 min, acetone (1.0 ml) was added with mixing to stop the reaction. Reagent blanks were prepared by substituting reaction mixture for homogenate. Hexane extracts (4.0 ml) of the reacted homogenates were then analyzed by electron capture gas chromatography using a Varian Aerograph Series 2100 equipped with a [^{3}H]scandium detector. The glass column was packed with 5% SE30 on Chromosorb W. The following conditions were employed: injector temperature, 260° C; column temperature, 220° C; detector

temperature, 280°C; N_2 flow rate, 40 ml/min. Dieldrin production (picograms/milligram protein/minute) was measured by comparison to a standard curve. Protein content was determined by the method of Lowry (Lowry et al., 1951).

In addition to the aldrin epoxidation assay, three other enzyme assays were performed. *p*-Nitroanisole (PNA) *O*-demethylation (Trautman et al., 1979), *p*-chloro-*N*-methylaniline (PCMA) *N*-demethylation (Kupfer and Bruggeman, 1966), and benzo[a]pyrene (BP) oxidation (Hayakawa and Undenfriend, 1973) were measured using minor modifications of described procedures. The epoxidation reaction mixture was also used for PNA *O*-demethylation and PCMA *N*-demethylation. The reaction mixture for BP oxidation contained 39.05 mg NADPH, 66.77 mg $MgCl_2$, and 4.62 mg EDTA in 100 ml 0.1 M phosphate buffer, pH 7.4, at 20°.

RESULTS

Preliminary studies were conducted to establish appropriate in vitro assay conditions using larvae collected from *Asclepias fascicularis*. Both fat body and gut were found to have low levels of aldrin epoxidase, PCMA *N*-demethylase, PNA *O*-demethylase, and BP oxidase activities. Radiometric procedures for BP and PNA oxidations were sufficient to detect activity but were too insensitive to give reliable rate measurements of low levels of oxidation. Aldrin epoxidation and PCMA *N*-demethylation were catalyzed by NADPH-dependent enzyme systems. Rates of product formation were proportional to time and incubate protein content. In most cases, gut homogenates were more active than corresponding fat body preparations. This has been observed in most Lepidoptera (Wilkinson and Brattsten, 1972; Krieger et al., 1971). More recent experiments using larger samples of monarch larvae have revealed that gut homogenate oxidation activity is greater than that of fat body homogenates ($P < 0.05$) (data not presented).

Oxidation activities of homogenates of the 1976 collections are shown in Table 1. In this table, activity is expressed in terms of the amount of product formed per tissue per unit time and as specific activity in terms of product formed per milligram protein per minute. The former unit may better represent larval oxidative capability because it represents total activity per insect. Both units provide an index of the relative in vitro activity of organ systems such as the fat body and gut.

Local environmental factors had no apparent influence upon oxidative metabolic activity. In 1976, larvae were taken from *A. eriocarpa* growing at Fresno and Woodland, California, and from *A. fascicularis* sites near Fresno and Sutter, California. The respective mean gut homogenates ($N = 2$) for aldrin epoxidase were 6.9, 9.3, 11.7, and 6.7 pmol dieldrin produced/mg

TABLE 1. ALDRIN EPOXIDASE AND PCMA *N*-DEMETHYLASE ACTIVITIES OF HOMOGENATES OF LARVAE TAKEN FROM FOUR DIFFERENT *Asclepias* SPP. (1976 SAMPLES)

		Homogenate activity[b]			
		Epoxidation		*N*-demethylation	
Host	Enzyme source (*N*)[a]	A	B	C	D
A. fascicularis	Gut (6)	555 ± 99[c]	11.6 ± 3.2	192 ± 29	586 ± 197
	Fat body (6)	359 ± 128	no data	no data	no data
A. eriocarpa	Gut (6)	370 ± 56	8.3 ± 1.7	149 ± 27	450 ± 79
	Fat body (6)	434 ± 106	12.5 ± 3.5	126 ± 49	479 ± 154
A. erosa	Gut (3)	458 ± 125	11.6 ± 2.4	114 ± 11	391 ± 11
	Fat body (3)	242 ± 27	8.0 ± 1.0	61 ± 9	472 ± 175
A. speciosa	Gut (5)	325 ± 24	12.8 ± 4.1	114 ± 40	517 ± 100
	Fat body (5)	212 ± 28	27.4 ± 13.6	76 ± 13	131 ± 61

[a] Number of homogenates tested.
[b] A, expressed as picomoles dieldrin per tissue per 10 minutes; B, expressed as picomoles dieldrin per mg protein per minute; C, expressed as nanomoles *p*-chloroaniline per tissue per 30 minutes; D, expressed as picomoles *p*-chloroaniline per mg protein per minute.
[c] $\bar{X}$ ± standard deviation.

TABLE 2. ALDRIN EPOXIDASE ACTIVITY OF HOMOGENATES OF LARVAE COLLECTED FROM TWO *Asclepias* SPP. GROWING AT SEVERAL LOCALES (1978 COLLECTIONS)

Host	Enzyme source	n^a	N^b	Collection site and date	Specific activity (pmol dieldrin/mg protein/min ± SE)
Asclepias eriocarpa	Gut	2	3	Woodland 7/28	14.5 ± 1.1
	Fat body	2	3		4.4 ± 1.0
	Gut	3	3	Woodland 8/30	5.1 ± 0.6
	Fat body	3	3		2.4 ± 0.4
	Gut	3	2	Woodland 9/11	8.5 ± 0.2
	Fat body	3	2		7.6 ± 0.1
Asclepias fascicularis	Gut	3	3	Sutter 8/4	6.6 ± 0.8
	Fat body	3	3		7.2 ± 0.9
	Gut	3	3	Sutter 8/15	12.6 ± 0.6
	Fat body	3	3		12.1 ± 1.7
	Gut	3	3	Winters 8/18	6.7 ± 0.4
	Fat body	3	3		2.4 ± 0.5
	Gut	3	3	Winters 8/29	6.5 ± 0.2
	Fat body	3	3		4.4 ± 0.4
	Gut	3	3	Woodland 9/8	8.5 ± 0.2
	Fat body	3	3		7.6 ± 0.1
	Gut	3	3	Sutter 9/14	7.9 ± 1.0
	Fat body	3	3		8.0 ± 0.7

[a]Number larval tissues per homogenate.
[b]Number homogenates tested.

protein/min. The mean *N*-demethylation activities were 485, 420, 543, and 589 pmol *p*-chloroaniline produced/mg protein/min. In the summer of 1978, larvae were collected on several different occasions from *A. eriocarpa* in Woodland and from *A. fascicularis* in Sutter, Woodland, and Winters, California. Gut and fat body homogenate activities for aldrin epoxidation ranged from 5.1 to 14.5 pmol dieldrin produced/mg protein/min (Table 2). Although caution must be exercised in the interpretation of these data due to the small sample size, no statistically significant differences in activity related to the various collection sites were observed.

Fat body and gut homogenates of larvae collected from the four milkweeds, *Asclepias fascicularis*, *A. eriocarpa*, *A. erosa*, and *A. speciosa*, had similar levels of aldrin epoxidase and PCMA *N*-demethylase activities (Table 1). These host plants represent a range of over an order of magnitude in total cardenolide content. *A. fascicularis*, *A. eriocarpa*, *A. erosa*, and *A. speciosa* average 0.17, 4.2, 5.6, and 0.9 mg cardenolide as digitoxin equivalents/g dried leaf (Brower et al., in preparation). The effect on oxidative

TABLE 3. ALDRIN EPOXIDASE AND PCMA *N*-DEMETHYLASE ACTIVITIES OF LARVAL GUT HOMOGENATES (FOOD PLANT STUDY)

Field host plant	Lab Host plant	N^a	n^b	*p*-Chloroaniline[c] (nmol/mg protein/min)	Dieldrin[c] (pmol/mg protein/min)
Asclepias fascicularis	*A. fascicularis*	2	6	0.566 ± 0.033	9.2 ± 3.51
A. fascicularis	*A. speciosa*	3	6	0.612 ± 0.063	15.4 ± 1.81
A. erosa	*A. erosa*	2	2	0.436 ± 0.074	13.04 ± 4.54
A. erosa	*A. speciosa*	4	5	0.544 ± 0.094	13.54 ± 4.31
A. speciosa	*A. speciosa*	1	1	0.228	5.59

[a]Number of experiments performed.
[b]Total number of homogenates tested.
[c]Plus/minus standard error of the mean where there was more than one experiment.

metabolism of transferring larvae from their field host plant to a milkweed of differing cardenolide content was evaluated. We measured aldrin epoxidation and PCMA *N*-demethylation activities (Table 3) of gut homogenates collected on *A. fascicularis* and fed on *A. fascicularis*, collected on *A. erosa* and fed on *A. erosa*, and collected on *A. erosa* and fed on *A. speciosa*. There were no statistically significant differences in oxidative metabolic capability related to food plant.

DISCUSSION

Monarch larvae have relatively low levels of monooxygenase activity (Krieger et al., 1971) as measured by PCMA *N*-demethylation and aldrin epoxidation in tissue homogenates of last instar larvae. Each oxidation was NADPH-dependent and product formation was proportional to time and incubate protein content. pH dependence was shown for PCMA *N*-demethylation. These properties are as expected for lepidopteran larval monooxygenase enzyme systems (Krieger and Wilkinson, 1969). Similar levels of activity were found in larvae collected from different locales and at different times in the season.

Brattsten (1979) measured aldrin epoxidation and PCMA *N*-demethylation in crude gut homogenates of armyworm larvae (*Spodoptera eridania*). The monooxygenase activities of armyworm gut depended upon what plant they had fed on in the preceding 24 hr. In related experiments, the gut homogenate *N*-demethylase activities were induced by plant chemicals incorporated into an artificial diet at levels varying from 0.1 to 0.26% in the diet. Brattsten reports an increase in *N*-demethylase activities from 61 to 212% over controls.

In our experiments, monarch larvae were consuming milkweeds which ranged in cardenolide content from 0.17 mg/g dried leaf to 5.6 mg/g dried leaf. This represents approximately 0.009% to 0.3% cardenolide in the diet (assuming the wet weight of a leaf is ca. 80% water). These concentrations did not induce *N*-demethylation or aldrin epoxidation activities in *D. plexippus* tissue homogenates.

The milkweed cardenolides represent a wide range of polarities and lipophilicities. Many are lipophilic enough to be substrates for monooxygenase enzymes. The relatively long residence time of these compounds in the larval tissues and the extent and doses to which the larvae are naturally exposed partially satisfy the criteria for a monooxygenase inducer. It is uncertain at this time whether cardenolides are substrates of the monooxygenase enzyme systems of the monarch larvae. Preliminary work in our laboratory has shown biotransformation of milkweed cardenolides in gut and fat body homogenates of monarch larvae (Marty et al., in preparation). In addition, monarch larvae dosed with particular cardenolides were found to

contain more polar cardenolide metabolites (Seiber et al., 1980). Experiments are ongoing to determine the roles of monooxygenase enzyme systems in cardenolide biotransformations. Increased knowledge of the fate of cardenolides in larval tissues, particularly with respect to their metabolism, will aid in elucidating roles of the monoxygenases in the chemical ecology of the milkweed–monarch butterfly interaction.

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Book Review

Controlled Release Technologies: Methods, Theory and Applications. Agis F. Kydonieus ed. Boca Raton, Florida: CRC Press, 1980. Vol. I, $74.95, 261 pp; Vol. II, $74.95, 273 pp.

Volume I discusses the fundamental concepts and theoretical background of controlled-release processes (Chapter 1) as well as four controlled-release technologies: monolithic, membrane, multilayered–laminated, and ultra-microporous systems. Volume II (Chapter 1 and 2) deals with erodible, bioerodible, soluble, hydrolyzable, and biodegradable devices; Chapter 3, retrograde chemical reaction systems while the remainder of the volume deals with microencapsulation systems (Chapters 4–8) and hollow fiber and miscellaneous controlled-release systems (Chapters 9–12).

These volumes should be of great value, particularly to those new to the field of controlled-release technology. Each chapter, written by experts, is well documented with references to the technical and patent literature.

There are, however, a few minor shortcomings in terms of some repetition and loss of some continuity as a result of having multiple contributors. Fick's Law, for example, and the following mathematical treatments as applied to membranes is essentially repeated twice in Volume I (pages 132 and 187). There are a number of polymers discussed in Chapters 2 and 3 that the reviewer would not consider typically biodegradable, such as the various esters derived from polymethacrylic acid. Chapters 2 and 3 in Volume II could well have been combined and made more concise. There are also a few minor technical/typographical errors: page 240 (Volume II), pyridine should not have been listed as a polymerizable monomer; page 243 (Volume II), "Organophosphorous insecticide" should read "Organophosphorus insecticide"; page 187 (Volume I), "Cm is the connection of diffusant . . ." should perhaps read "Cm is the concentration of diffusant . . ."; page 220 (Volume I), "carbolxylate" should read "carboxylate"; page 23 (Volume I), section under "Homogeneous Monolith," I could find nowhere in this section the meaning of "m" in equation (3).

The above notwithstanding, the reviewer strongly recommends these volumes as part of one's library on controlled-release technology.

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CASTOR SACS AND ANAL GLANDS OF THE NORTH AMERICAN BEAVER (*Castor canadensis*): Their Histology, Development, and Relationship to Scent Communication

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Abstract—Both sexes of beavers possess a pair of castor sacs and a pair of anal glands located in paired subcutaneous cavities between the pelvis and the base of the tail. The castor sacs are not glandular in the histological sense, hence references to these structures as preputial glands or castor glands are misnomers. The wall of the castor sacs is plicate and comprised of three distinct zones: an outer layer of vascular connective tissue, a two- to five-cell-thick layer of mitotic epithelial cells, and several densely packed layers of cornified epithelium which grade into more widely separated sheets toward the lumen. Monocultures of a gram-positive facultatively anaerobic bacterium were present in the lumen of all castor sac preparations. Differences in the frequency of castoreum deposition were not attributable to differences in the structure of the castor sacs. The anal glands of beavers are holocrine sebaceous glands. These glands develop more rapidly than the castor sacs. Anal gland tissue from embryos exhibited cellular characteristics associated with the production of sebum. Secretory activity was evident in all preparations. The relationship of castoreum and anal gland secretion to scent communication among beavers is discussed.

Key Words—*Castor canadensis*, castor sacs, anal glands, chemical communication.

INTRODUCTION

Both sexes of beavers (*Castor canadensis*) possess a pair of castor sacs and a pair of anal glands located in subcutaneous cavities between the pelvis and base of the tail (Svendsen, 1978). The contents of the castor sacs mix with urine to form castoreum. Beavers deposit this thin yellow liquid on mud

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mounds constructed within the home range of the family (Aleksiuk, 1968; Svendsen, 1980; Müller-Schwarze and Heckman, 1980; Butler and Butler, 1979).

The anal glands are located caudal to castor sacs (Svendsen, 1978). Ducts from the glands open on papillae lateral to the rectum and orifice leading to the vestibule of the castor sacs. Anal gland secretions vary in color from straw to light brown, are viscous and insoluble in water, and have a pungent odor.

The variety of terminology reflects the paucity in data concerning the histology of these structures. "Castor glands" (Brady and Svendsen, 1981; Butler and Butler, 1979; Svendsen, 1978) and "preputial glands" (Müller-Schwarze and Heckman, 1980; Wilsson, 1971) have been used synonymously with "castor sacs" and "oil sacs" (Müller-Schwarze and Heckman, 1980) with "anal glands." The purpose of this research was to describe the histology of the castor sacs and anal glands and test the following hypotheses: Both structures are true glands; both structures develop synchronously in beavers; and castor sacs and anal glands do not differ in structure or activity among beavers of different age or gender, or vary seasonally. These data will provide a baseline for assessing the role of castoreum and anal gland secretion in integrating the social behavior of beavers.

METHODS AND MATERIALS

The castor sacs and anal glands used for microscopic study were from beavers live-trapped in southeastern Ohio. Sexing and handling of these beavers followed the methods of Svendsen (1980). Animals were classified as adults, two-year-olds, yearlings, or kits.

Beavers were anesthetized with Nembutal (pentobarbital sodium, Abbott Laboratories). Both pairs of anal glands and castor sacs were removed surgically from 11 animals of different age and gender. These animals were subsequently sacrificed. Portions of the excised glands were fixed immediately in glutaraldehyde, buffered with phosphate to pH 7.1, and rinsed with buffered sucrose. Pieces of tissues were postfixed in buffered 1% osmium tetroxide, dehydrated in an ethanol series, and embedded in an Epon–Araldite mixture. Tissues were sectioned on a Reichert OmU2 microtome. Thick sections were transferred to clean microscope slides and stained with toluidine blue. Thin sections were contrasted with uranyl acetate and lead citrate and examined on a Siemens Elmiskop I electron microscope.

Paraffin sections of both anal glands and castor sacs from four beavers were prepared by fixing excised tissue in Carnoy's fixative for 20 hr at 4°C. Tissues were dehydrated in an ethanol series, cleared with three changes of benzene, and embedded in paraffin under a vacuum. Sections (10 μm) were cut and stained with either hematoxylin and eosin, azan, or trichrome stains

according to Humason (1967). One of the aforementioned females was pregnant. Castor sacs from three of the fetuses (approximately 11-weeks-old) were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Structures from the remaining fetus were excised and immediately immersed in liquid nitrogen. Sections of 15-μm thickness were cut on an American Optical Cryo-cut microtome, transferred to clean cover slips, and allowed to thaw. Tissues were stained with toluidine blue, dehydrated in an ethanol series, cleared with xylene, and mounted on clean microscope slides for microscopic examination.

RESULTS

Anatomy of Castor Sacs. The castor sacs have been reported to be diverticula of the urinary or reproductive tracts (Kacnelson and Orlova, 1954; Wilsson, 1971; Svendsen, 1978). Both the urinary and reproductive tracts of males and females terminate anterior to the vestibule that connects the castor sacs, thus the castor sacs cannot be derivatives of these tracts. Rather the castor sacs are epithelial pockets of the wall of the urogenital sinus. Sphincters are absent at the junction of the castor sacs and the sinus, thus the contents of the sacs can readily combine with urine to form castoreum. This arrangement necessitates the excretion of castoreum when beavers urinate.

Histology and Development of Castor Sacs. Three distinct layers of tissue comprised the wall of the castor sacs: a peripheral layer of vascularized connective tissue, a layer of germinative epithelium two to five cells thick, and a zone of cornified layers of epithelium lining the lumen (Figure 1a). Projections of the connective tissue and the corresponding flexure of epithelial and keratinized epithelial cells form papillae which jutted into the lumen of the sac (Figure 1a). The distance between adjacent layers of cornified cells increased progressively toward the lumen (Figure 1b). Ruptures in the innermost layers of cells were common. One species of bacterium was observed in the lumen of all preparations of the sacs (Figure 2). Subsequent culture and characterization showed this bacterium to be a gram-positive, facultatively anaerobic, pleomorphic cocci. This bacterium imparted a granular appearance to the area adjacent to the layers of cornified cells lining the lumen of the castor sacs. Ducts leading from the wall to the lumen of the sac were conspicuously absent. In addition, no glands were present that emptied into either the castor sacs or the vestibule connecting the sacs.

The castor sacs were poorly developed in prenatal beavers. Several layers of epithelial cells surrounded by connective tissue was the extent of development at 11–12 weeks gestation (Figure 3a). The walls of the sacs were beginning to convolute and a lumen was present, but the epithelial cells lining the lumen were not cornified (Figure 3b). The castor sacs of two stillborn

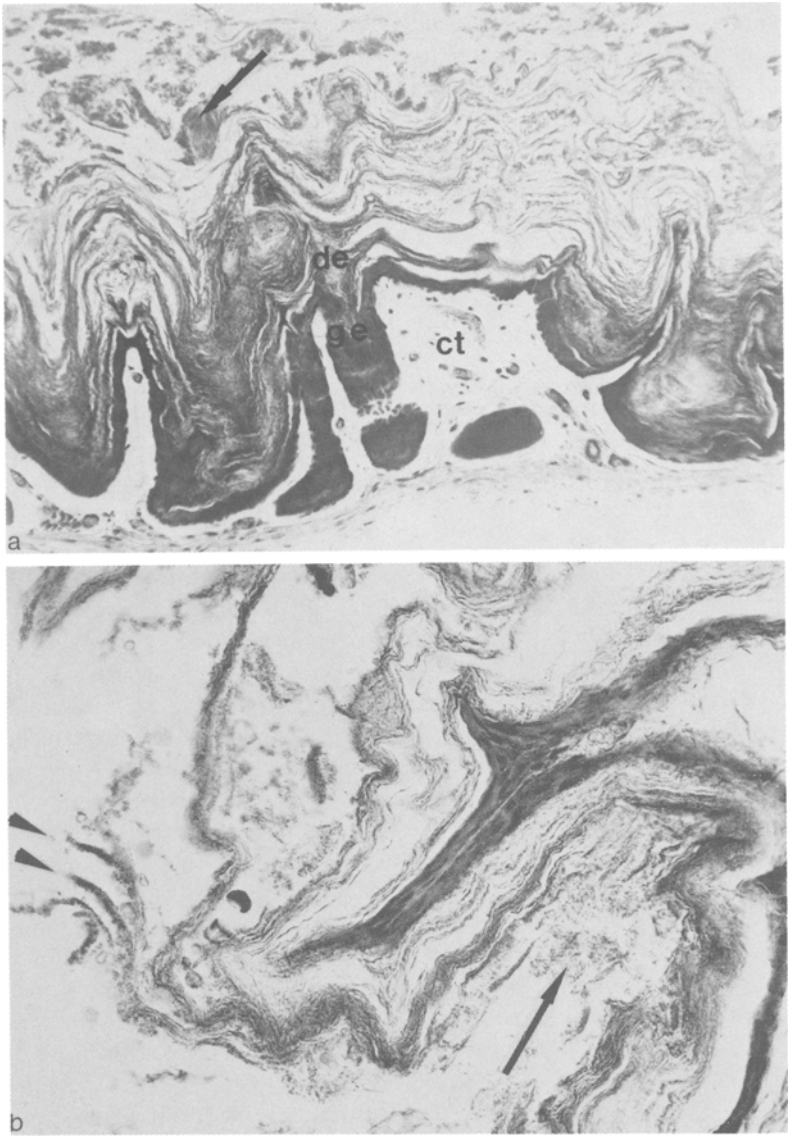

FIG. 1. (a) Section through the wall of the castor sacs showing stratification of tissue layers and plicate structure. ct, connective tissue; ge, germinative epithelium; de, densely packed cornified epithelium. Granularity (arrow) results from sloughing of cells and bacteria in the lumen of the sac. Adult female. Trichrome, × 275. (b) Section through the wall of the castor sac. Lumen of the sac is at the left of the photograph. Dark layer of cells is germinating epithelium. Ruptures in cornified layers are visible at left (arrowheads). Bacteria are responsible for the observed granularity (arrow). Adult female. Azan, × 900.

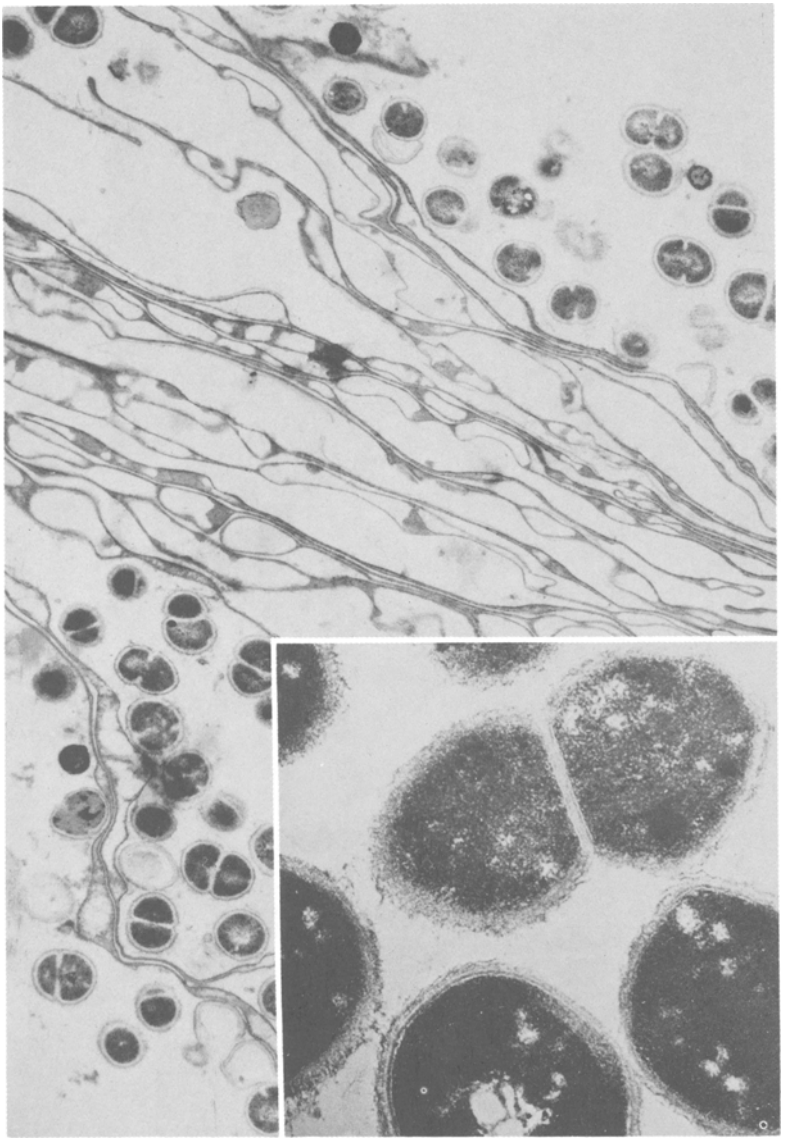

FIG. 2. Bacterium inhabiting the castor sacs. Cornified epithelial layers are evident traversing obliquely in the micrograph. Adult female. ×17,600. Inset: Dividing bacterium in lumen of the castor sac. Kit male. ×84,900.

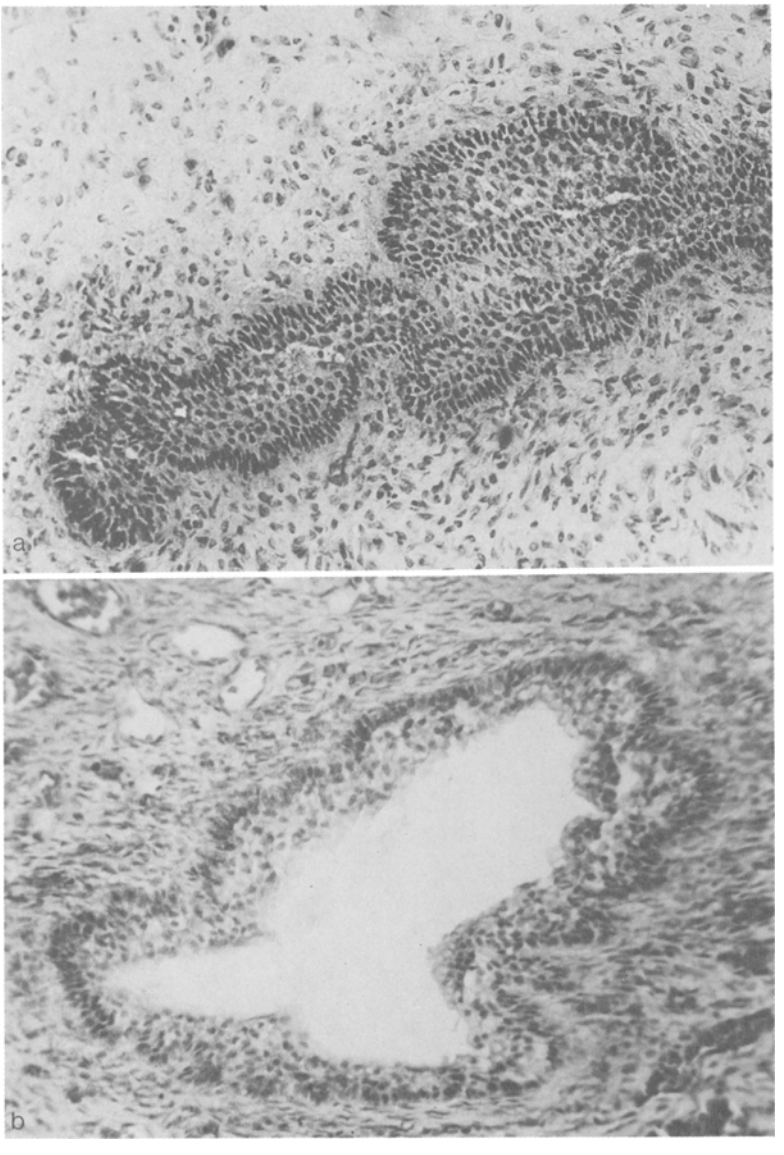

FIG. 3. Development of the castor sacs in 11-week-old embryonic beavers. (a) Castor sacs appear as several layers of undifferentiated cells surrounded by connective tissue. Male. Toluidine blue, ×250. (b) Lumen has formed but epithelial cells lining the lumen have not undergone keratinization. Male. Hematoxylin and eosin, ×250.

beavers were thin-walled and flaccid. The castor sacs of a 5-month-old kit were structurally indistinguishable from the sacs excised from adults.

Histology and Development of Anal glands. The anal glands are compound tubuloacinar sebaceous glands (Figure 4a). Maturation of cells occurred centripetally within the acini. Growth of immature, uninucleate cells at the periphery pushed adjacent cells to the center of each acinus. Cells at the center of the acini exhibited characteristics associated with production of sebum: proliferation of lipid, enlargement, and nuclear degeneration (Figure 4b). Secretory activity was visible in all preparations of anal glands and was independent of age or gender of the beaver, or the season in which the sample was collected.

The anal glands of beaver were well developed in embryonic beaver. Formation of lobes was well underway and mature sebaceous cells were visible in preparations (Figure 5a,b). The anal glands of kits at birth were relatively large and turgid with sebum.

DISCUSSION

Castor Sacs. Reference to the castor sacs as "castor glands" or "preputial glands" is inappropriate. The preputial glands found in males (clitoral glands in females) of several species of rodents are true holocrine sebaceous glands (Brown and Williams, 1972). Castor sacs of beavers are pouches lined with cornified epithelial cells. These sacs produce no secretion, hence are not glands in the strict histological sense. However, sloughed epidermal cells may contribute to the production of chemical signals in castoreum, and the castor sacs' function may be similar to that of a holocrine gland.

No differences in the structure of the castor sacs were evident in kits >5 months of age, yearlings, two-year olds, or adults. The absence of scent-mound construction by kit beavers (Hodgdon, 1978; Svendsen, unpublished data) is not attributable to immaturity of the castor sacs.

The frequency of scent-mound construction varies seasonally. Frequencies of scent-mounds constructed by different beaver families increased with increasing extralodge activity in late winter and early spring, peaked in May and early June, declined during July, and remained low throughout the remainder of the year (Svendsen, 1980). The castor sacs neither enlarge or regress on an annual cycle (Svendsen, 1978) nor do they vary seasonally in structure. In addition, exfoliation of cornified cells occurs at a constant rate (Goldschmidt and Klingman, 1974). The location of the castor sacs relative to the urethra makes it physically impossible for beavers to urinate without urine mixing with the contents of the castor sacs. We interpret this evidence to indicate that castoreum is being produced continually and is not correlated with the seasonal variation in scent-marking behavior. This system of scent

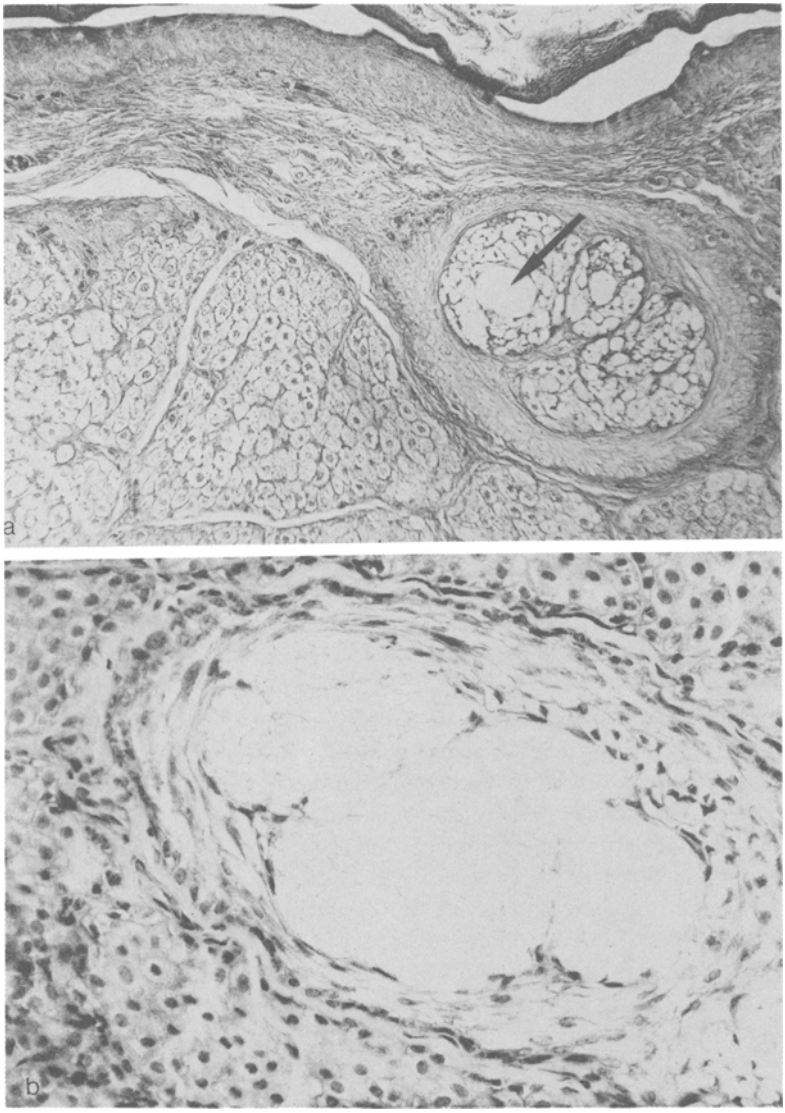

FIG. 4. (a) General structure of anal glands. Uninucleate cells compose each acinus. Secretory activity is visible at arrow. Kit male. Azan, ×275. (b) Maturation of sebaceous cells. Degeneration of nucleus, accumulation of lipid, and enlargement of cells are visible in center of photomicrograph. Adult female. Azan, ×530.

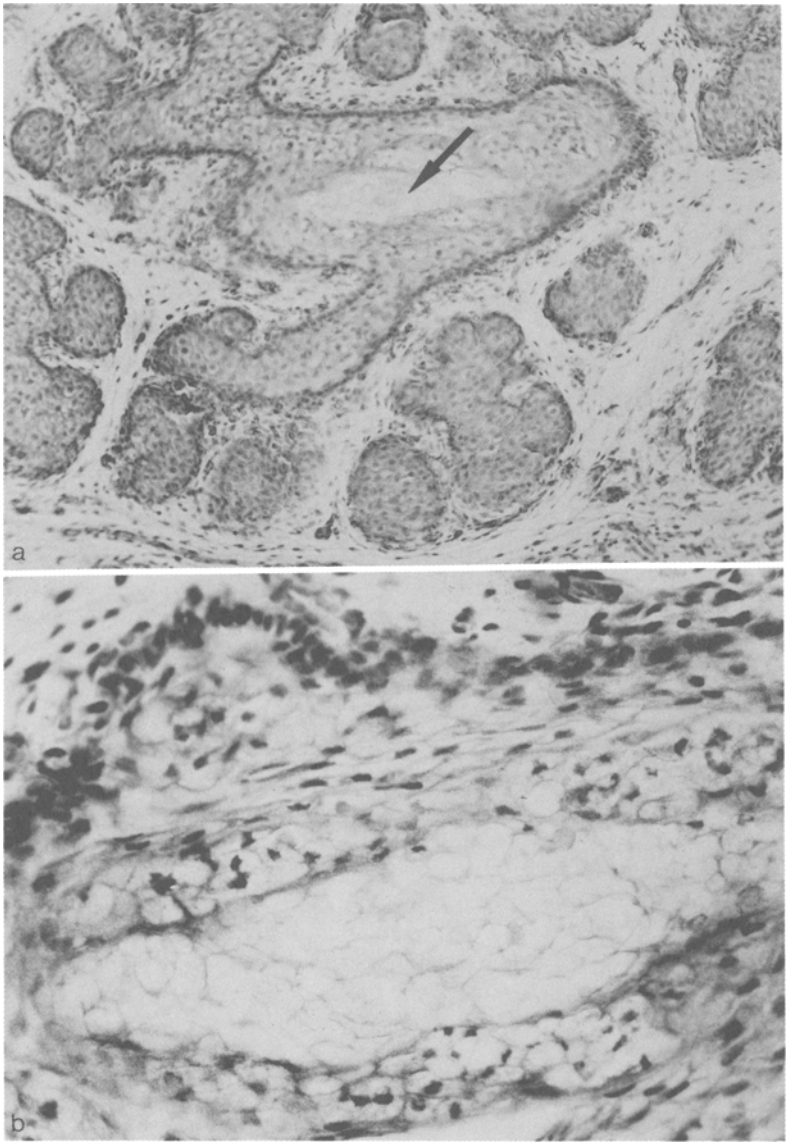

FIG. 5. Development of the anal glands in 11-week-old embryonic beavers. (a) Branching to form compound structure is evident. Cells at center of photograph (arrow) show changes associated with production of sebum. Male. Toluidine blue, ×250. (b) Mature sebaceous cells. Cells are anucleate and filled with lipid. Male. Hematoxylin and eosin, ×500.

production, which is continually "on", is modulated by the selection of the substrate for the deposition of castoreum. Castoreum that is produced during periods of low scent-marking activity is simply voided in the water.

Urine extracted from the bladder of beavers and applied to artificial scent-mounds elicited no response from conspecifics whereas addition of castoreum did (Svendsen and Walro, unpublished data). These data indicate that the castor sacs are essential for the production of chemical signals in castoreum. Whether urine is necessary to interact with the contents of the castor sacs to produce a signal or is simply a mechanism for transport to the exterior is not known.

This study is the first to demonstrate the presence of bacteria in the castor sacs. This monoculture of cocci appeared dark and crystalline in preparations stained with toluidine blue. This bacterium is likely the "calcium sediments" described by Kacnelson and Orlova (1954) in the castor sacs of river beavers (*C. fiber*). Bacteria are known to inhabit cavities and pouches of mammals, metabolize available substrate, and produce odoriferous compounds. These compounds are hypothesized to serve as chemical signals in several species of mammals (Albone et al., 1976). Whether the bacterium that inhabits the castor sacs is responsible for the chemical signals in castoreum or simply resides in the sac is not known. If the bacterium is responsible for the cues in castoreum, urine provides a more readily metabolizable substrate than the contents of keratinized cells lining the sacs.

The development of the castor sacs in *C. canadensis* parallels the development of the sacs in *C. fiber*. Wilsson (1971) reported that the castor sacs of European beavers about one month of age were empty and flaccid, but sacs of three-month-old animals had castoreum in them.

Anal Glands. The anal glands are true holocrine sebaceous glands. Differences in secretory activity of anal glands were not evident in histological preparations. All preparations of anal gland tissue exhibited secretory activity regardless of age, gender, or the time of year at which the sample was taken.

Data from captive animals and marked animals in the field suggest that beavers anoint themselves with anal gland secretion during bouts of autogrooming (Svendsen and Walro, in preparation). The secretion has a dual function. Anal gland secretion supplements the sebaceous glands of the skin in maintaining the water-repellent quality of the fur. Excision of these structures reduces the ability of the pelage to repel water (Svendsen and Walro, in preparation). Prohibition of autogrooming in *C. fiber* produces similar results (Wilsson, 1971). Both the glands that produce a secretion which waterproofs and maintains the fur and the motor patterns associated with application of the secretion would be expected to develop early in semiaquatic animal. Such is the case in beavers. Anal glands synthesize sebum prior to parturition, and a kit, on its first evening outside the lodge, displayed well-developed motor patterns of autogrooming with the forepaws (Svendsen and Walro, in

preparation). Similar results are reported for *C. fiber*. Motor patterns of autogrooming develop at four days postpartum in the European beaver—long before the kits began spending evenings outside the lodge (Wilsson, 1971). Preliminary experiments indicate that anal gland secretion is also a source of chemical signals in beavers (Svendsen and Walro, in preparation). Thus beaver, when they groom themselves, impart an odor or disseminate scent to other parts of their body.

This study relates the histology and development of both the castor sacs and anal glands to chemical communication among beavers. Isolation and production of chemical cues in castoreum and anal gland secretion are subjects of future investigation.

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CONSTITUENTS OF TEMPORAL GLAND SECRETION OF THE AFRICAN ELEPHANT, *Loxodonta africana*

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Abstract—Temporal gland secretion (TGS), obtained from 15 different mature African elephants in Kruger National Park was analyzed for volatile constituents. Only five volatile components were present. *p*-Cresol was present in all samples, but phenol was found as an appreciable component of only one sample and as trace amounts in six others. Three sesquiterpenes were identified, the latter two being new natural products: *E*-farnesol, farnesol hydrate (3,7,11-trimethyl-2,10-dodecadien-1,7-diol), and farnesol dihydrate (3,7,11-trimethyl-2-dodecen-1,7,11-triol). These sesquiterpenes represent the first isolated from mammals. Ten samples of TGS, serum, and saliva were assayed for cholesterol, urea, and proteins including several enzymes.

Key Words—African elephant, *Loxodonta africana*, temporal gland secretion, farnesol, sesquiterpenes, phenol, cresol, cholesterol, proteins, urea.

INTRODUCTION

The temporal gland is an organ uniquely possessed by African elephants (*Loxodonta africana*, Blumenbach) and Asiatic elephants (*Elephas maximus*, L.). The ancient mammoth (*Mammathus primigenius*, Blumenbach) also

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possessed temporal glands that probably were larger than those of modern elephants (Pocock, 1916), the glands of which weigh from 230 to 1590 g in bulls of various ages. The gland is located on the side of the head in the temporal fossa about midway between eye and ear, and opens to the surface by a duct near the center of its lower border.

Temporal glands of proboscids were first reported in 1734 (Perrault) and are well known as mixed (sebaceoapocrine) skin glands. The sebaceous part occurs superficially, whereas the apocrine part is deep and comprises most of the gland. The gland's secretory activity was reported to be related to the mating period and reproduction, but more recently this has been denied (Short, 1966; Sikes, 1971; Eisenberg et al., 1971). Current reports (Eisenberg et al., 1971; Sikes, 1971; McKay, 1973) propose this organ as a potential scent gland (as are most apocrine glands) which possibly has a dual function.

The opportunity to obtain biological data on the African elephant will not be available long, particularly if the dramatic increase in poaching of wild elephants fostered by skyrocketing ivory prices throughout the world continues. This could result in the African elephant being relegated to an endangered species, further reducing the opportunity to secure data from wild stock. Such data could be of taxonomic significance when compared to closely related species. Since the elephant is generally known to be comparable with man in growth rate, life span, and time of onset of puberty, studies of biochemistry could have some pertinence to humans. We believe that a compilation of constituents of temporal gland secretion from the African elephant will be of considerable value to scientists interested in elephants. Therefore, the twofold purpose of this report is to present both volatile and nonvolatile constituents of the African elephant's temporal gland secretion and to suggest functions for this secretion.

Buss et al. (1976) showed that the organic and inorganic biochemical parameters measured for temporal gland secretion were consistent within a limited range in all cases except protein and cholesterol levels. Additional measurements of total, free, and esterified cholesterol are reported for TGS, serum, and saliva for ten more recent samples in the current study. Protein studies include electrophoretic separation of lipoproteins, glycoproteins, and LDH (lactic dehydrogenase) isozymes.

Adams et al. (1978) reported the analysis of temporal gland secretion volatiles from the African elephant. These authors, who sampled only one or two elephants, found phenol, *m*-, and *p*-cresol to be the major volatile components. They also reported the presence of more than 40 components in all gas chromatographic traces and indicated that one of these may be indole.

We have analyzed the temporal gland secretion from a larger number of elephants (15) and find only five volatile components in this secretion.

METHODS AND MATERIALS

Sample Collection. All samples for this study, like those reported by Buss et al. (1976), came from elephants harvested for management purposes in Kruger National Park, South Africa. For volatiles analyzed by GC-MS (Table 2), samples were used from 15 elephants (8 females) from 9 to 60 years of age collected over a two-year period between April 1976 and June 1978 (Table 1). Samples from many of the same elephants, as well as samples from those reported in 1976, were used for the nonvolatiles analyzed. All elephants were stimulated into temporal glandular secretion by helicopter driving from where they were located to the nearest service road. There they were killed by overdoses of saturated succinyl choline chloride solution, eviscerated, and loaded for transport to an abattoir.

Temporal gland secretion was collected by insertion of a heavy-gauge blunt stainless-steel needle about 3 cm into the temporal duct. The needle was connected to a 50-ml glass syringe. As the secretion flowed, it was recovered into the syringe. The samples were sealed in glass vials, placed in a glass thermos with ice and then frozen (−20°C) within an hour and shipped on dry ice.

GC-MS Analysis of Volatiles. Thawed samples were extracted three times with Burdick and Jackson methylene chloride, the extracts dried over anhydrous sodium sulfate, and the solutions analyzed directly by combined gas chromatography–mass spectrometry on a Finnigan 3200 GC-MS. Columns (1.6 m × 1 mm) containing 3% OV-17 and 10% SP-1000 on Supelcoport 60/80 were temperature programed at 10°/min from 50 to 200° (300° for OV-17). Proton magnetic resonance spectra were obtained on a Nicolet NT-200 superconducting spectrometer utilizing 5-mm tubes. Preparative gas chromatography was accomplished on a Glowall 320 gas chromatograph equipped with an argon detector using a 1% OV-17 column (1.6 m × 5 mm) on Supelcoport 60/80.

Analysis of Nonvolatiles. Measurements and assays included protein (Lowry et al., 1951), total cholesterol (Allain et al., 1974; Witte et al., 1974), cholesterol esters (Webster, 1962), urea (Archibald, 1945), alkaline phosphatase (Bessey et al., 1946), amylase (Rinderknecht et al., 1971), LDH (Amador et al., 1965), and peroxidase (Matkovics et al., 1977). Enzymes were assayed in triplicate and expressed as μM/hr/mg protein. Polyacrylamide gel electrophoresis was by the methods of Gorovsky et al. (1970) and Wardi and Michos (1972) and included LDH isozyme visualization by the method of Storey (1977).

RESULTS

Volatiles. Only five compounds were detected, even at extremely high gain and elevated temperatures, in contrast to the 40 volatiles previously

TABLE 1. SAMPLE SIZES FROM 21 ELEPHANTS COLLECTED IN 1976–1978 FOR CHEMICAL ANALYSES

Groups	Number	Sex	Age	Date	Secretion[a]			Serum	Saliva	Temporal gland
					Amount (ml)	Odor	Color			
1976										
Family unit	1289	F	25	03/17/76	10					
Family unit	1303	F	60	04/07/76	15	++[c]	red-br	–[d]	–	–
Family unit	1316	F pl[b]	28	04/15/76	15	+	light	–	–	–
1977										
	1340	F l	26	08/15/77	2			+	+	–
Family unit of 9	1341	F l	38	08/15/77	5			+	–	–
Family unit of 9	1347	M	14	08/15/77	1.5			+	–	–
Family unit of 9	1348	F l	14	08/15/77	1.5			+	–	–
Family unit of 8	1349	F l	21	08/16/77	2.5			–	–	+
Family unit of 8	1350	F	9	08/17/77	–			+	+	+
Family unit of 10	1351	M	9	08/17/77	2.5					
Family unit of 10	1352	F	14	08/18/77	8					
Family unit of 10	1353	F	16	08/17/77	5					
Family unit of 10	1356	M	24	08/18/77	10					
Family unit of 10	1364	F	33		–			+	–	–
Family unit of 10	1402	F	36		–			+	–	–
1978										
Family unit	1477	F pl	25	04/25/78	4	+	light	–	–	–
Bull group of 2	1522	M	28	06/05/78	30		lgt-br	–	–	–
Bull group of 9	1523	M	26	06/07/78	15	+	brown	+	+	–
Bull group of 9	1524	M	26	06/08/78	30	++	light	+	+	–
Bull group of 5	1525	M	25	06/09/78	15	+	light	–	+	+
Bull group of 2	1526	M	25	06/10/78	4		brown	+	+	+

[a] Secretion flow commenced during helicopter drive.
[b] p: pregnant; l: lactating.
[c] +, noticeable odor; ++, strong odor.
[d] –, no material or observations received.

TABLE 2. VOLATILE COMPONENTS OF TEMPORAL GLAND SECRETION[a]

	Phenol	Cresol	Farnesol	Farnesol hydrate	Farnesol dihydrate
1289 F	–	m	–	–	–
1303 F	p	m	tr	tr	–
1316 F	tr	x	m	–	–
1341 F	–	m	x	–	–
1349 F	–	x	p	x	p
1352 F	tr	x	p	x	p
1353 F	–	m	x	p	p
1477 F	tr	x	x	m	p
1351 M		x	x	x	–
1356 M	tr	x	x	x	–
1522 M	–	x	m	x	–
1523 M	–	m	x	p	–
1524 M	tr	p	p	p	m
1525 M	tr	x	x	x	–
1526 M	–	p	x	x	p

[a]m = major (>50%); x = minor (20–49%); p = present (2–19%); tr = trace (<2%).

reported. *p*-Cresol was present in all samples and was the major component of five. However, phenol was an appreciable component of only one sample. Three compounds of higher molecular weight (Table 2) were also present, their relative amounts varying with the sample.

The presence of phenol and *p*-cresol was confirmed by comparison of their retention times (peak enhancement) and mass spectra with those of authentic samples and reported spectra (Stenhagen et al., 1974). *m*-Cresol did not separate from the para isomer on either the OV-17 or the 10% SP-1000 column. By comparison of mass spectra of known mixtures of meta- and para-cresol we found that the mixture contained no more than 10% of the meta isomer.

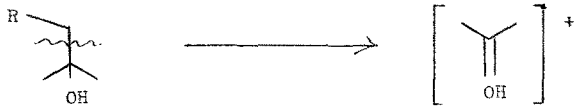
The other three components of the secretion posed a problem for structural analysis because they all exhibited the same apparent molecular ion at m/z 222, even though their elution temperatures spanned 40°. The large peak at m/z 69 suggested a terpene, and the apparent molecular weights suggested farnesol or farnesol derivatives. The compound which eluted first exhibited the following mass spectrum: m/z 222 (3%), 207 (2), 204 (3), 191 (10), 189 (5), 69 (100), 41 (80). Preparative gas chromatography afforded a sample which had the following proton nuclear magnetic resonance absorptions (200 MHz) δ 5.4 (1H, broad), 5.1 (2H, broad), 4.1 (2H,d), 2.0 (8H,m), 1.0–1.9 (complex). The proton NMR spectrum indicated the presence of three vinyl protons and an allylic CH_2 attached to an oxygen, suggesting farnesol itself. Comparison of the natural material with commercial farnesol, which is

a mixture of *E,E* and *Z,E* isomers (~2:1), indicated that the elephant natural material was all *E*-farnesol.

The remaining two terpenes had apparent molecular ions at *m/z* 222, yet eluted 20° and 40° higher than farnesol itself. Cyclic isomers of farnesol were discarded as possibilities, and synthetic farnesyl esters did not correspond. The fact that the FT-NMR spectrum of the second compound was very similar to that of farnesol suggested minor functional changes such as hydration of a double bond. Not enough of the third compound was available for FT-NMR.

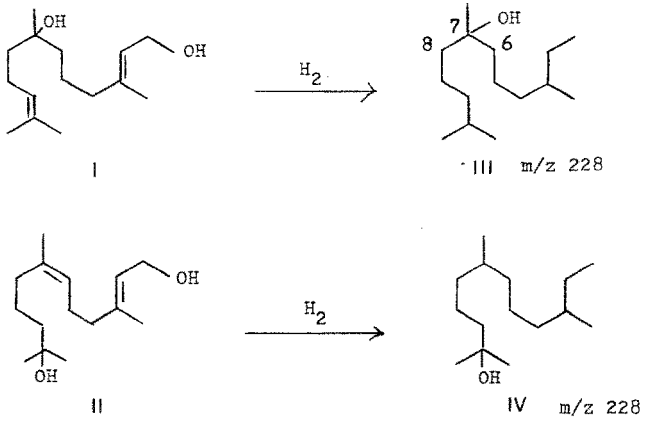
Hydrogenation of the second terpene with platinum oxide in ethanol gave a product with a base peak at *m/z* 69 and peaks at *m/z* 210 and 213 attributed to M-H_2O and M-CH_3 from a molecular ion of 228. To establish the presence of a hydroxy group the trimethylsilyl derivative [bis(trimethylsilyl)trifluoroacetamide, Applied Science Laboratories, Inc.] of both farnesol and the second terpene were prepared. GC-MS indicated the presence of the silylated hydroxyl group in farnesol (M-15 at *m/z* 279) and the presence of two silylated hydroxyl groups in the second terpene (M-15 at *m/z* 369). Thus, the second terpene appears to be a farnesol hydrate (I or II). Either of these would be in accord with the hydrogenation data which corresponds to hydrogenolysis of the allylic CH_2OH group.

Many saturated acyclic alcohols exhibit base peaks at *m/z* 69 (Ohashi and Nakyama, 1978). The absence of a significant peak at *m/z* 59 due to α-cleavage argued against structure IV.



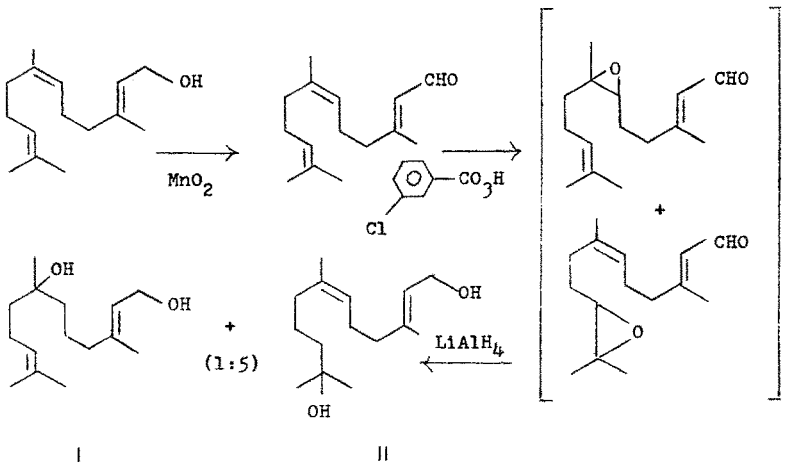
m/z 59

The hydrates (I and II) were synthesized from farnesol by Scheme 2 through the epoxides (Nilles et al., 1973). Separation of I from II (formed in the ratio of 1:5) was achieved by preparative gas chromatography. Compound I exhibited an identical retention time (peak enhancement) and the same mass spectrum as the natural product. Hydrogenation of I led to the same hydrogenolysis product as the natural material. This hydrogenolysis product III (*m/z* 228) showed large fragments at *m/z* 129 and *m/z* 143 attributed to cleavage at the C6–C7 and C7–C8 bonds respectively, while the hydrogenolysis product IV of II did not exhibit these cleavages and had a base peak at *m/z* 59 corresponding to α-cleavage. Since farnesol is completely *trans* (*E*) in the secretion, one might expect the double bond in I to be *trans*. This is confirmed by its synthesis from all-*trans*-farnesol, but the stereochemistry of the newly introduced hydroxy group is unknown.

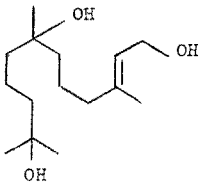


SCHEME 1.

The proton nuclear magnetic resonance spectra of I and II confirmed (chemical shifts of the methyl groups) that hydroxylation had taken place at the 7 and 11 positions, respectively. Thus, the structure of the second terpene is 3,7,11-trimethyl-2,10-dodecadien-1,7-diol (farnesol hydrate). The remaining terpene, which also exhibited an apparent molecular ion at m/z 222 and eluted 20° higher, appeared to be a dihydrate. The synthetic scheme for I and II was modified to prepare V by using excess meta-chloroperbenzoic acid. The presence of the allylic alcohol in I, II, and V was verified by reoxidation of each to its respective aldehyde with activated manganese dioxide.



SCHEME 2.



V

3,7,11-Trimethyl-2-dodecen-1,7,11-triol (V) exhibited identical retention times (peak enhancement) and the same mass spectrum as the third terpene in the elephant temporal secretion.

Nonvolatiles. Protein values for TGS, serum, and saliva are listed in Tables 3 and 4. TGS protein (1978 samples) ranged from 38 to 99 mg/ml (2.6-fold variation), with a mean of 51.4 mg/ml, higher than the 1976 mean of 42.4 mg/ml (Buss et al., 1976). Electrophoretic separation resolved 10 discrete protein bands. Serum, temporal gland tissue, and TGS all demonstrated the band at 5 cm (albumin region), but its width and intensity varied (Figure 1). Coomassie blue staining (left), Sudan black (middle), and PAS (right) resolved, respectively, proteins, lipoproteins, and glycoproteins in the TGS (Figure 2). PAS glycoprotein staining demonstrated four bands (Figure 2), and a similarity was noted between the temporal gland tissue extract and the TGS.

Urea values range from 2 to 40 mM/liter (mean 17.2) for 1974, 1975 TGS samples and TGS 1976–1978 values range from 1.4 to 8.5 mM/liter with a mean of 4.96 mM/liter (Table 3). Female–male values and seasonal means were not significantly different. Serum and saliva levels are listed in Table 4.

Alkaline phosphatase levels in TGS and serum were similar (Table 3). TGS acid phosphatase levels were variable. Amylase levels were lower in TGS than serum (Table 3). Serum and TGS peroxidase levels were high compared to other vertebrates; TGS values ranged between 0.072 and 0.44 μM, with variation among the TGS samples. The 1976–1978 samples had measurable LDH levels, albeit reduced somewhat by freeze–thawing. The serum levels were similar to established levels in human serum; TGS levels were about half serum levels. Figure 3 shows the LDH isozyme pattern of TGS and tissue removed from the wall of the temporal duct.

Total TGS cholesterol values were more variable (5–420 mg/100 ml) in the current samples (5 males, 4 females) (Table 3) than in the previous five male elephant TGS cholesterol levels measured (Buss et al., 1976; 12–70 mg/100 ml). Saliva values ranged between 10 and 98 mg/100 ml, averaging 42.4 mg/100 ml (Table 4) and serum cholesterol ranged from 49 to 205 mg/100 ml, with a mean of 94 mg/100 ml (Table 4). When serum and TGS were available from the same elephant, no correlation was apparent between serum and TGS levels.

TABLE 3. TEMPORAL GLAND SECRETION

Number	Sex	Age	Month	Protein (mg/ml)	Urea (mM/liter)	Acid phosphatase (μM/hr/mg protein)	Alkaline phosphatase (μM/hr/mg protein)	Amylase (Somogyi units/100 ml)	Peroxidase (μM/hr/mg)	LDH (μM/hr/mg)	Cholesterol (mg/100 ml)	
											Total	Esters (%)
1976												
1303	F	60	April	49	5.1	–	–	–	–	–	5	75
1316	F	28	April	99	4.85	6.7	1.40	211	0.072	–	48	77
1977												
1340	F	26	August	63	1.4	–	1.63	–	0.097	0.036	–	–
1349	F	21	August	38	6.1	–	1.99	–	–	–	65	79
1364	F	32		–	–	13.0	2.25	–	–	0.029	74	79
1978												
1477	F	25	April	48	8.5	–	–	–	–	–	420	76
1523	M	26	June	46	7.3	–	–	–	0.44	–	200	75
1524	M	26	June	55	2.7	–	–	171	–	–	25	78
1525	M	25	June	60	4.9	12.03	–	–	–	0.029	45	–
1526	M	25	June	50	3.8	–	–	–	0.122	–	50	–
N				9	9	3	4	2	4	3	9	
Range				38–99	1.4–8.5	6.7–13.0	1.40–2.25				5–420	
Mean				51.4	4.96[a]						103	77

[a]April mean = 6.15; June mean = 4.6; August mean = 3.7.

TABLE 4. ANALYSIS OF SERUM AND SALIVA

	Protein (mg/ml)		Urea (mM/liter)		Acid phosphatase (μM/hr/mg protein)		Alkaline Phosphatase (μM/hr/mg protein)		Amylase (Somogyi units/100 ml)		Peroxidase (μM/hr/mg)		LDH (μM/hr/mg)		Cholesterol (mg/100 ml)			
															Total		Esters (%)	
Number	Serum	Saliva	Serum	Saliva	Serum	Saliva	Serum	Saliva	Serum	Saliva	Serum	Saliva	Serum	Saliva	Serum	Saliva	Serum	Saliva
1976																		
1303	76	–	–	–	–	–	–	–	–	–	–	–	–	–	125	–	51	–
1316	97	–	2.7	–	2.8	–	1.27	–	–	–	0.073	–	0.0557	–	98	–	56	–
1977																		
1340	–	–	6.7	–	–	–	–	–	–	–	–	–	–	–	80	98	68	–
1349	100	–	–	5.1	–	–	–	–	–	–	–	–	–	–	75	–	–	–
1350	–	42	8.5	–	–	–	–	–	–	–	–	–	–	–	75	60	–	51
1364	105	–	–	–	6.3	–	1.70	–	355	116	0.069	–	0.159	0.078	205	–	77	–
1978																		
1477	–	–	–	–	–	–	–	–	–	–	–	–	–	–	68	–	–	–
1523	88	–	2.8	4.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1524	100	28	3.1	1.4	–	–	–	–	401	125	–	–	–	–	49	15	–	–
1525	80	33	3.9	2.9	–	–	–	5.14	–	–	–	0.86	–	–	79	29	–	–
1526	100	37	3.6	3.3	–	4.0	–	3.51	–	–	–	–	–	–	86	10	76	–
N	8	4	7	5											10	5		
Range	76–105	28–42	2.7–8.5	1.4–5.1											49–205	10–98		
Mean	93.3	35	4.47	3.46											94	42.4	65	

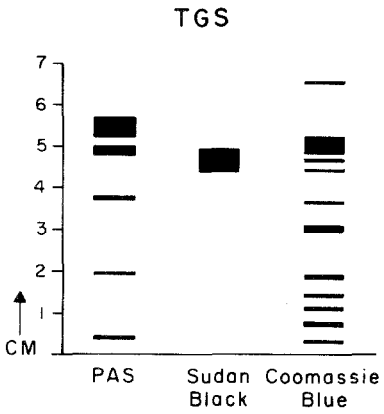


FIG. 1. PAGE (5% gel): 200 μg protein/gel: TGS. Right to left: Coomassie blue stain, Sudan black stain, PAS.

DISCUSSION

The forty components in all GC traces reported previously are in marked contrast to our total of five volatile components from the African elephant temporal gland secretion (both samplings were obtained from wild African elephants). Although the diol I and the triol V are stable in our GC-MS interface, their dehydration (and/or rearrangement) during chromatography and/or at the interface of the GC-MS may account for some of the complexity observed by previous workers. All compounds are stable under the GC-MS analysis conditions and the analyses of individual samples are reproducible.

There appears to be no particular correlation between either sex or age and composition of the secretion. Cresol is the major component in more of the females than in the males. Traces of phenol are found occasionally in males as well as females, but the three sesquiterpenes appear invariably in

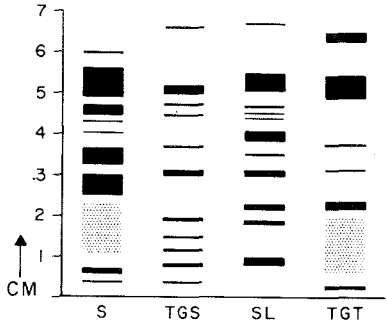


FIG. 2. PAGE (7½% gel): Coomassie blue stain: 150 μg protein/gel. Left to right: serum, TGS, saliva, temporal gland tissue (TGT).

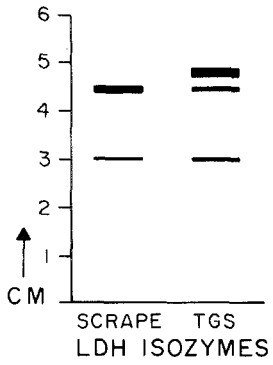


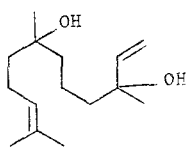
FIG. 3. PAGE (8% gel): LDH isozyme stain: 250 μg protein TGT, left: TGS, right.

both. Farnesol hydrate seems to be more prevalent in males than females. The samples which had appreciable odor were high in either phenol and/or cresol, as anticipated.

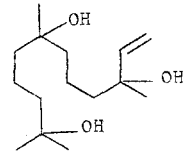
Cresol has been measured in guinea pig perineal secretions (Wellington et al., 1979), functioning as a germicide, and is degraded by bacterial decomposition involving peroxidase enzymes. Possible degradation of cresol in this manner in elephant temporal gland secretion may result in cresol differences among individuals. Phenol, ortho- and para-cresols have been identified as major components of moose tarsal gland secretion (Dombalagian, 1979) where large variations have also been noted.

Farnesol has been found in the bumble bee (Stein, 1963) and is used as a sex attractant by the spider mite (Regev and Cone, 1975). Farnesyl acetate has been found in the Dufour's gland of ants (Bergstrom and Lofquist, 1972) and other farnesyl esters have been identified as major components in bee Dufour's gland secretion (Fernandes et al., 1981, and references therein). In these bees, as well as the elephant, all of the farnesol (ester) is of the *trans* (*E*) form. In insects, sex attractants can be dependent on the correct mixture of *E* and *Z* isomers (Klun et al., 1973).

This is the first report of sesquiterpenes in mammals and the diol I and the triol V are new natural products. A similar diol VI and triol VII related to nerolidol have been reported from caparrapi oil (Castillo et al., 1966). Squalene, a triterpene, has been reported in the springbok (*Antidorcas*



VI



VII

marsupialis) (Burger et al., 1978) and the brown hyena (*Hyaena brunnea*) (Lamb, 1980).

The nonvolatile components measured demonstrated: high protein levels in both the serum and TGS; reconfirmation of the previously measured high protein levels in TGS; a wide range of cholesterol values in TGS; some similarities of protein electrophoretic patterns of serum and TGS; a broad range of serum urea levels, 2.7–8.5 mM/liter [these values are more divergent than previously reported (Brown et al., 1978)]; and individual differences in enzyme levels which may result from microbiological contamination, especially within the temporal gland duct and fossa. Variation in peroxidase in the TGS may occur from the ability of the enzyme to degrade cresol (Critchlow and Dunford, 1972).

Field observations of Estes reported by Buss et al. (1976, p. 447) point out that among temporal glands dissected from adult bulls "sticks, leaves and various other small artifacts of the environment have been found in the temporal pore, suggesting that this part of the body is rubbed, or somehow comes in contact with various objects." Reference is made to Figure 17, a "photograph showing accumulated debris in the main duct of the temporal gland." Such debris was never found in the temporal-gland duct of females. Therefore, since farnesol hydrate seems more prevalent in males than females, we suggest that this ingredient is involved in scent marking by adult male African elephants.

Since degradation of cresol by bacterial decomposition might result in cresol variation among individual elephants, we also suggest that such variation in cresol aids in helping recognize individuals. We are testing phenol, cresol, farnesol, and the two hydrates, as well as mixtures of these components, to establish their role (if any) in elephant communication.

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TOXICITY STUDIES OF ANALOGS OF 2-TRIDECANONE, A NATURALLY OCCURRING TOXICANT FROM A WILD TOMATO[1]

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Abstract—Toxicity bioassays of the naturally occurring insecticide 2-tridecanone and a group of structural analogs against the tomato fruitworm (*Heliothis zea*) reveal a significant influence of chain length on toxicity of methyl ketones. This effect may be due to the differential ability of these compounds to penetrate lipid barriers and reach the active site, since a close relationship is seen between toxicity and lipophilicity. Congeners of chain lenths 15 and over are less active than predicted by this relationship, possibly due to steric effects.

Key Words—2-Tridecanone, structure-activity relationships, *Lycopersicon hirsutum* f. *glabratum* C.M. Mull, *Heliothis zea* (Boddie), *Lepidoptera*, *Noctuidae*, resistance.

INTRODUCTION

The wild tomato *Lycopersicon hirsutum* f. *glabratum* C.M. Mull has been reported to be resistant to a number of important arthropod pests of the cultivated tomato, *L. esculentum* Mill. (Kennedy and Henderson, 1978, and references cited therein). Kennedy and Yamamoto (1979) determined that resistance in this wild species to the tobacco hornworm, *Manduca sexta* (L.), results from larval contact with a toxic factor in the exudate of glandular trichomes found on the aerial parts of the plant. This exudate also exhibited contact toxicity to larvae of the tomato fruitworm, *Heliothis zea* (Boddie).

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Williams et al. (1980) isolated the toxic component of the exudate and identified it as 2-tridecanone, a simple methyl ketone. This report was described as the first positive identification of a nonalkaloid chemical defense against insects in plants of the genus *Lycopersicon*.

The present investigation examines the relationship between structure and activity of 2-tridecanone and congeners varying in chain length, carbonyl position, and other characteristics.

METHODS AND MATERIALS

The compounds selected for study were 2-pentanone, 2-heptanone, 2-nonanone, 2-decanone, 2-undecanone, 2-dodecanone, 2-tridecanone, 3-tridecanone, 7-tridecanone, 2-tetradecanone, 2-pentadecanone, 3-hexadecanone, 2-nonadecanone, and *n*-tridecane (Pfaltz and Bauer, 375 Fairfield Ave., Stamford, Connecticut 06902); 5-tridecanone and 2-tridecanol (Chemical Samples Co., 4692 Kenny Rd., Columbus, Ohio 43221); and *n*-tridecanal (ICN/K&K Labs, 111 Express St., Plainview, New York 11803). Each compound was dissolved in chloroform to a concentration of 10 mg/ml.

Specified amounts of each sample were pipetted onto filter paper disks (5.5 cm in diameter) in large glass Petri dish covers. Approximately 0.25 ml of solution was required to saturate the paper, so a few drops of chloroform were added to each disk receiving less than this amount to ensure uniform distribution over the paper surface. If a disk was to receive more than 0.5 ml of a solution, uniform distribution was obtained by pipetting the solution onto the paper 0.5 ml at a time and allowing the chloroform to evaporate between administrations. Control disks were prepared by treatment with 0.25 ml of pure chloroform. The solvent was allowed to evaporate entirely from all disks before use in any bioassay.

Newly hatched first instar *Heliothis zea* larvae were obtained from a laboratory colony maintained on a synthetic diet (Burton, 1970) at North Carolina State University. This colony is replenished annually with field-collected moths. Each treated and control disk was placed in the bottom of a 60 × 15-mm plastic Petri dish and 20 newly hatched larvae were transferred to the paper surface. All dishes were sealed with Parafilm® to prevent the escape of larvae and vapors. The Petri dishes were covered and placed in the dark at 27°C for 6 hr. Larvae were then transferred from the dishes to small, paper-sealed plastic cups containing the synthetic diet (Burton, 1970). These were returned to the dark 27°C room for an additional 18 hr to allow any recovery or further mortality to occur. The number of living and dead larvae was recorded during examination under a stereoscopic dissecting microscope. Larvae which failed to respond in any manner to gentle prodding with an insect pin were classified as dead.

All of the compounds were screened at rates of 210.5 and 420.9 $\mu g/cm^2$ of treated filter paper surface (= 5 and 10 mg/disk) to determine their relative activities against *H. zea*. Those exhibiting significant activity were selected for bioassay at a series of rates from 42.1 to 420.9 $\mu g/cm^2$ in increments of 42.1 μg. By this method an approximate LC_{50} range was determined, and those compounds of special interest (i.e., those varying in carbonyl position or having chain lengths between 9 and 15) were bioassayed in this range at a series of rates in 10.5-μg increments. These results allowed us to narrow the LC_{50} range considerably, and a final bioassay was conducted with these selected compounds in a 2.1-μg incremented series of rates within this restricted range. The final bioassay was duplicated and the resulting mortality data averaged at each concentration. LC_{50} values and their corresponding 95% confidence limits were calculated by the logarithmic transformation and chi-square method of Litchfield and Wilcoxon (1949). The LC_{50}s of two commerical insecticides, malathion and methomyl, were also determined.

RESULTS

The results of the initial screening test are compiled in Table 1. Compounds were classified as having high, moderate, low, or no activity according to the criteria presented in the table footnotes. Larval mortality ranged from 0 to 10% on the control disks during this initial screening.

LC_{50} values (Table 2) were determined for compounds exhibiting high and moderate toxicity, excluding *n*-tridecane. The moderate activity of this alkane is apparently the result of its solvency on the protective waxes of the

TABLE 1. RELATIVE TOXICITIES OF 2-TRIDECANONE AND STRUCTURAL ANALOGS TO FIRST INSTAR *H. zea* LARVAE BASED ON SCREENING BY FILTER PAPER BIOASSAY AT 210.5 AND 420.9 $\mu G/CM^2$

High activity[a]	Moderate activity[b]	Low activity[c]	Inactive[d]
2-decanone	2-nonanone	2-heptanone	2-pentanone
2-undecanone	2-tetradecanone	2-pentadecanone	3-hexadecanone
2-dodecanone	n-tridecane		2-nonadecanone
2-tridecanone	2-tridecanol		n-tridecanal
3-tridecanone			
5-tridecanone			
7-tridecanone			

[a]Caused 90–100% mortality at 210.5 $\mu g/cm^2$.
[b]Caused 30–90% mortality at 210.5 $\mu g/cm^2$ or 90–100% mortality at 420.9 $\mu g/cm^2$.
[c]Caused 0–30% mortality at 210.5 $\mu g/cm^2$ or 30–90% mortality at 420.9 $\mu g/cm^2$.
[d]Caused less than 30% mortality at 420.9 $\mu g/cm^2$.

TABLE 2. LC_{50} VALUES DETERMINED FOR 2-TRIDECANONE, CERTAIN ANALOGS, AND TWO COMMERICAL INSECTICIDES BY BIOASSAY ON FILTER PAPER AGAINST *H. zea*[a]

Compound	$LC_{50}(\mu g/cm^2)$	95% confidence interval
2-Nonanone	245.18	236, 255
2-Decanone	99.33	93.4, 106
2-Undecanone	64.19	63.3, 65.1
2-Dodecanone	25.36	24.5, 26.3
2-Tridecanone	17.05	15.9, 18.3
3-Tridecanone	16.31	15.3, 17.4
5-Tridecanone	19.15	18.3, 20.0
7-Tridecanone	19.36	17.6, 21.3
2-Tetradecanone	26.41	18.7, 37.3
2-Pentadecanone	578.75	552, 607
2-Tridecanol	76.82	63.7, 92.7
Malathion	4.08	3.07, 5.43
Methomyl	2.01	1.73, 2.34

[a]LC_{50} values and 95% confidence intervals calculated by method of Litchfield and Wilcoxon (1949).

insect cuticle. At both screening rates the dead larvae were severely desiccated, a condition not apparent in larvae killed by the other compounds. For this reason, *n*-tridecane was omitted from further study.

DISCUSSION

Methyl-2-ketones exhibit a marked relationship between chain length and toxicity to *H. zea* larvae. The data suggest that the activity of such a compound is contingent upon its lipophilicity, since this trait is also influenced to a great extent by the number of carbon atoms which constitute the molecular chain. We therefore plotted the logarithm of the octanol–water partition coefficient (log P) for each compound against the logarithm of its toxicity (log $1/LC_{50}$), following the example of Hansch (1969) and Martin (1978). The resulting plot resembles a parabola, and an equation was fit to the data by curvilinear regression (Figure 1). A parabola of this type has been explained by assuming that the toxicity of a congener depends upon the probability that it will reach the site of action. This probability depends upon both the total distance a congener must travel across lipid barriers such as the cuticle and cell membrane to reach the active site, and the ability of the congener to pass through these barriers. The latter is determined by the lipophilicity of the compound as measured by P, so the probability of reaching the active site depends heavily upon this value (Martin, 1978; McFarland,

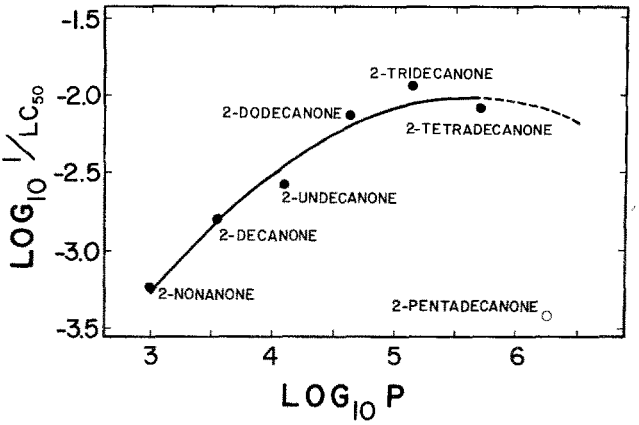

FIG. 1. Relationship between toxicity (= $1/LC_{50}$ in nmol/cm^2) and lipophilicity (as indicated by the partition coefficient, P) of methyl-2-ketones. For 2-nonanone through 2-tetradecanone, $r^2 = 0.97$.

1970). For methyl-2-ketones of chain length 9–14 this regression curve explains 97% of the variation in toxicity among congeners as a function of P. Inclusion of 2-pentadecanone in the model lowers r^2 from 0.97 to 0.78 (Figure 1). The peculiarity exhibited by 2-pentadecanone may result from its inability to "fit" the site of action properly because of its size. If this is the case, steric effects supercede hydrophobic effects in determining toxicity when the molecule exceeds 14 carbon atoms in length.

This model predicts that the position of the carbonyl carbon in the tridecanone molecule ought not affect its toxicity since it does not alter the value of P. Indeed, 2-, 3-, 5-, and 7-tridecanone do not differ significantly in this respect (Table 2).

The moderate activity of 2-tridecanol may result from its oxidation to 2-tridecanone by the alcohol dehydrogenase enzymes of *H. zea*. However, we have not yet determined whether these enzymes act upon 2-tridecanol in fruitworm larvae, so this hypothesis remains speculative. It does appear, nevertheless, that penetration of these compounds is more important in toxicity differences than is metabolism of a compound once it has entered the cell. Preliminary studies with the synergist piperonyl butoxide have resulted in no effect on 2-tridecanone toxicity, indicating that the microsomal mixed-function oxidase system of *H. zea* is not involved in the metabolism of this ketone.

The essential oil of *L. hirsutum* f. *glabratum* is also known to contain 2-heptanone, 2-undecanone, and 2-dodecanone; however, in insect-resistant accessions such as Plant Introduction (PI) 134417 2-tridecanone forms by far the bulk of the oil composition (Soost et al., 1968). It is interesting from an

evolutionary viewpoint that the most prevalent of several toxic ketones in the oil is that which is most toxic to insects. There is evidently significant adaptive value attached to the production of large quantities of 2-tridecanone. The model in Figure 1 predicts that 2-tetradecanone should be about as toxic as 2-tridecanone, although the former is absent from the essential oil. Presumably the plant would be required to expend more of its resources were it to base its defense upon 2-tetradecanone, while 2-tridecanone would be equally effective as an insecticide with less expenditure required for its biosynthesis.

Also of interest is the observation (Kennedy et al., 1981) that the production of 2-tridecanone is under photoperiodic control in PI 134417. Exposure to long days led to a two-fold increase in the 2-tridecanone content of leaflets over that seen in plants grown under short days. Luckwill (1943) reported that flowering in *L. hirsutum* f. *glabratum* is photoperiodically controlled and occurs in response to short daylengths. It thus appears not only that the plant produces a mixture of essential oils that is richest in the most toxic of several ketones present, but that it also may be able to allocate its resources either to reproduction or defense, depending upon need during a particular season by using photoperiod as an environmental cue.

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RESPONSE OF THE ICHNEUMONID PARASITE *Nemeritis canescens* TO KAIROMONES FROM THE FLOUR MOTH, *Ephestia kuehniella*

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Abstract—2-Acylcyclohexane-1,3-diones, a novel class of compounds in the larval mandibular glands of *Ephestia*(=*Anagasta*) *kuehniella* Zeller, act as kairomones. Their relative activities in eliciting oviposition responses from the larval parasite *Nemeritis*(=*Venturia*) *canescens* (Grav.) are reported.

Key Words—Kairomones, 2-acylcyclohexane-1,3-diones, *Ephestia keuhniella* Zeller, (syn. *Anagasta kuehniella* Zeller), Lepidoptera, Pyralidae, *Nemeritis canescens* (Grav.) (syn. *Venturia canescens* (Grav.)), *Ephestia cautella* (Wlk.), *Ephestia elutella* (Hubner), Hymenoptera, Ichneumonidae, *Plodia interpunctella* (Hubner), oviposition.

INTRODUCTION

Corbet (1971) showed that the parasite *Nemeritis*(=*Venturia*) *canescens* (Grav.) probes with its ovipositor in response to droplets of a secretion from the mandibular glands of larvae of *Ephestia*(=*Anagasta*) *kuehniella* Zeller and that the intensity of the response depends on the amount of the secretion. A component of the secretion that caused *Nemeritis* to probe was shown to be present also in the larval mandibular glands of *Ephestia cautella* (Wlk.), *Ephestia elutella* (Hubner), and *Plodia interpunctella* (Hubner) (Mudd and Corbet, 1973), and was identified as 4-hydroxy-2-oleoylcyclohexane-1,3-dione together with 2-oleoylcyclohexane-1,3-dione (Mudd, 1978). Mossadegh (1980) confirmed the presence of the former compound in the mandibular glands of *P. interpunctella* and found another unidentified

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compound with an empirical formula of $C_{22}H_{38}O_3$, which is therefore almost certainly 2-palmitoylcyclohexane-1,3-dione. Subsequently several more 2-acylcyclohexane-1,3-diones (see Figure 1) were identified (Mudd, 1981) in the mandibular gland of *E. kuehniella*.

Here we report experiments to determine the relative activities, as kairomones, of the 2-acylcyclohexane-1,3-dione components found in *E. kuehniella*.

METHODS AND MATERIALS

The structures of the components tested and the approximate amounts present in the mandibular glands of *E. kuehniella* larvae are shown in Figure

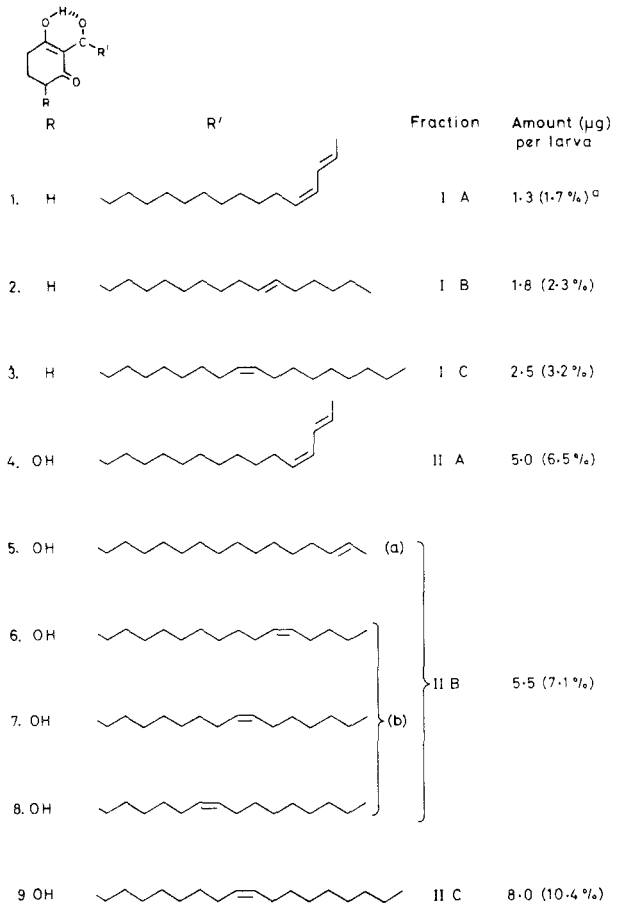

FIG. 1. Approximate percentages of the mandibular gland contents.

TABLE 1. NUMBER OF OVIPOSITION MOVEMENTS[a] MADE IN RESPONSE TO COMPONENTS[b] AT DIFFERENT CONCENTRATIONS

Day	Component	Control[c]	10	1	0.1	0.01	0.001	SED
1	9	1.192	1.620	1.027	0.513	0.130	–[d]	0.077
2	3	1.056	0.339	0.050	–	–	–	0.154
3	4	0.879	1.693	1.045	0.520	–	–	0.125
4	1	1.179	1.715	1.201	0.763	0.289	–	0.156
5	2	0.863	0.280	–	–	–	–	0.181
6	5	0.687	0.100	–	–	–	–	0.324

[a]Means of log (number of movements + 1).
[b]See Figure 1.
[c]Whole mandibular gland extract at 10 μg.
[d]–, No movements recorded at this dose.

1. The purification and structure determination of the components has been reported (Mudd, 1981). Isolation of the active components from extracts of dissected glands by high-pressure liquid chromatography (HPLC) was monitored by UV absorption and the oviposition response of *Nemeritis*. Initially, components 5, 6, 7, and 8 (fraction IIB, Figure 1) eluted as a single HPLC peak from both silica and reverse-phase columns. Repeated high-resolution chromatography was required to separate even small amounts of fractions IIB(a) and IIB(b) since fraction IIB components differ only in double bond position. Component 5 [IIB(a)] was relatively inactive (Table 1), and a preliminary experiment showed that fraction IIB(b) was also of low activity, so the unseparated fraction IIB was used in the experiment (Table 2) comparing the relative activities of the 2-acylcyclohexane-1,3-dione components of the mandibular gland.

TABLE 2. RELATIVE ACTIVITY OF COMPONENTS (10 μg) AS KAIROMONES

Component[a]	Mean of $\log_{10}$(number of movements + 1)
4	2.073
1	1.807
9	1.578
2	0.447
3	0.360
IIB	0.201
SED	0.1250

[a]See Figure 1.

Investigation of Factors Affecting Bioassay

The response of *Nemeritis* was observed when doses of components were inserted into an arena containing the insects.

A number of elements in the behavioral sequence leading to an oviposition movement could be distinguished in the response of *Nemeritis* to these components: (1) increased locomotion; (2) circling the treated cover slip; (3) circling with antennae in contact with cover slip; (4) raising of abdomen and unsheathing of ovipositor; and (5) jabbing the cover slip with the ovipositor, followed by returning it to the resting position.

With the more active compounds, this sequence was often compressed and some of the steps omitted so only full oviposition movements were scored, one "movement" lasting from the time when the ovipositor changed from the resting position (pointing horizontally to the rear) to the ovipositing mode (thrust forward and downward with abdomen raised) until its return to the resting position. Blank cover slips (ether only) consistently failed to induce any response.

Freshly dosed cover slips were used for each test to avoid possible interference from marker pheromones of *Nemeritis* or chemical degradation of components. Individual Nemeritis varied considerably in the amount of a particular component required to cause oviposition. To obtain reasonably consistent results, 12 or more insects per arena were required.

The number of oviposition movements is influenced by a number of factors investigated in preliminary experiments below.

Dimensions of Arena. The components appear to be detectable by *Nemeritis* over only a few centimeters; therefore in a larger arena they were less likely to find the component. Also, since *Nemeritis* are positively phototaxic, they normally spend a high proportion of their time on the roof (with overhead illumination) of the arena, so it was important to have a low roof from which they could detect the stimulus. However, with too small an arena, collisions between insects interfered with attempts at oviposition. This was more important for the less active components since *Nemeritis* was than more easily deterred from oviposition attempts.

Duration of Oviposition Period. For moderately active compounds at the levels tested (see below), most oviposition movements were made in the first 2–3 min, but for the most active components oviposition attempts continued unabated for more than 10 min. So that each bioassay could be prepared and completed within one working day (thus avoiding day-to-day variations), a test period of 5 min was chosen.

Interval between Bioassays. To avoid habituation, brief exposure to chemical stimuli and long intervals between exposures are desirable. However, because of day-to-day variations in the responsiveness of *Nemeritis*, it was preferable to complete the bioassays of components within an afternoon.

A bioassay design allowing 30 min between successive exposures to the components proved adequate in practice.

Temperature. The insects became noticeably less active below 20°C but between 24° and 28°C no significant difference was observed. The bioassays were done at 26 ± 1°C.

Bioassay Procedure

Based upon the preliminary experiments above, the following bioassay design was adopted.

Twelve newly emerged *Nemeritis* were confined in an arena (1 cm high and 15 cm diam) with a muslin-covered top and a glass base plate throughout each bioassay. They were fed on a 50% honey solution in dental rolls placed on the muslin roof of the arena. In such conditions the *Nemeritis* could be kept alive and fully responsive for more than two weeks. Doses of components in ether (10μl) were applied to the center of the cover slips (18 mm diam) from a 10-μl syringe held vertically and in contact with the cover slip to produce circular spots of approximately equal area. After evaporation of the ether, the cover slips, on a filter paper, were slid into the arena.

Each pure component (1, 2, 3, 4, 5, and 9, Figure 1) of the secretion was tested for 5 min at five levels (10, 1, 0.1, 0.01, 0.001 μg/cover slip) with a control consisting of the whole mandibular gland extract (10 μg), making six treatments in all (Table 1). One component was tested each day on the six consecutive days from emergence of the *Nemeritis* on day 1. In a final assay (Table 2), six components (1, 2, 3, 4, 9, Fraction IIB, Figure 1) were tested, each at the 10-μg level, after two days rest, on day 8.

Each test used a Latin-square design, with six replicate groups of 12 insects forming the rows and six time periods the columns, to enable any time-dependent changes in responsiveness to be eliminated. The groups were tested in the same order during each time period without a break between periods, so each group had 30 min to recover between periods. Because the response of a particular group might depend on its previous treatment, the design ensured that each treatment was preceded once by each other treatment to enable any such carry-over effects to be eliminated; however, none were detected.

RESULTS AND DISCUSSION

The components (Figure 1) vary greatly in their activity as kairomones (Table 1). The day-to-day variation in the responsiveness of *Nemeritis* was indicated by their response to the whole mandibular gland extract (10 μg) used as a control, so the bioassay scores could be adjusted and the relative activities

of the components at the 10-μg level calculated. They were in the same order and of magnitudes comparable to those observed in the final experiment on day 8 where all components were tested at 10 μg (Table 2). The responses on day 8 were as large as those in the first tests (even on day 1), showing that there was no deterioration of response in the final bioassay (Table 2).

At the highest level tested (10 μg), the response of *Nemeritis* to component 4 was so intense that it was difficult to count the number of oviposition movements. The insects crowded around the dosed cover slips and occasionally broke away in balls of fighting groups, apparently attacking each other with ovipositors and mandibles. Also, with component 4 the oviposition movements ("jabbing sessions") were very prolonged, and the ovipositor returned relatively less often to its normal resting position. For these reasons, the activity of component 4 in Table 2 may be an underestimate.

The order of activity of the components was 4, 1, 9, 2, 3, 5 (or IIB), and the most active component (4) caused over a 100 times as many oviposition movements as the least active components (IIB) at the same concentration (Table 2).

There was no evidence of decreasing responsiveness at high concentrations in these experiments (Table 1); activity increased with concentration up to the highest level tested (10 μg). However, in a preliminary experiment with component 9, there appeared to be a leveling out of *Nemeritis'* response above 10 μg and, at 100 μg, contamination of *Nemeritis* caused grooming which interfered with oviposition behavior.

Structure–Activity Effects

Comparison of the activities of components 4 and 9, which differ only in their side chains, indicates that the terminal *Z*,*E*-conjugated diene side chain contributes more effectively than the oleoyl side chain to the activity of the components as kairomones. This is confirmed by a similar comparison of the activities of components 1 and 3.

The oleoyl side chain also contributes to the activity of these compounds because 9 is much more active than the hydroxylated components of IIB (Table 2), but it is not by itself sufficient to confer high activity since 3 is relatively inactive.

The most active component, 4-hydroxy-2-(*Z*,*E*)-12',14'-hexadecadienoylcyclohexane-1,3-dione (4) and the next most active (1) differ only in the group at C-4 (OH or H). Similarly, with components 3 and 9 having the 2-oleoyl side chain, the 4-hydroxy compound (9) is much more active than the unhydroxylated compound (3), which confirms the importance of the C-4 hydroxy group to the activity of these compounds.

The specificity of the kairomonal activity therefore seems mainly attributable to the novel terminal diene and 4-hydroxy groups in these 2-acylcyclohexane-1,3-dones, which are new natural products from insects.

Despite their widespread occurrence (Vinson, 1976), very few kairomones of Lepidoptera have been identified compared with their sex pheromones. Like the hydrocarbon kairomones of *Heliothis zea* (Jones et al., 1971) and *Heliothis virescens* (Vinson et al., 1975) and the heptanoic acid kairomone of *Phthorimaea opeculella* (Hendry et al., 1973), compounds examined here are active in the nanogram range and are thus considerably less active than lepidopteran sex pheromones which are often active in the picogram range. The low volatility of the kairomones of *E. kuehniella*, caused by their polarity and high molecular weight, results in detectability over a much shorter range than is the case with sex pheromones. This may be important biologically insofar as a more precise location of larvae in the substrate by both the moth and parasite is possible.

Vinson (1976) and Blum (1977) pointed out that the release of a kairomone by a host, which enables parasites or predators to locate it, would be expected to be selected against unless it was functionally important to the host. This is supported by the work of Corbet (1971, 1973a,b), which showed that the mandibular gland secretion also regulates the population density of *Ephestia* by its effect upon larval dispersal and the oviposition behavior of the moth. Some synthetic and natural 2-acylcyclohexane-1,3-diones (derived from plants) show insecticidal, fungicidal, or antibiotic properties (see Mudd, 1981), so it is also possible that the compounds have a protective purpose.

Kairomones have been shown to increase the efficiency of parasitization by entomophagous insects in the field (Lewis et al., 1972; Gross et al., 1975) and, of those examined here, the *Z,E*-conjugated dienes (4 and 1) and the 4-hydroxy-2-oleoylcyclohexane-1,3-dione (9) appear to be potentially the most useful.

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ALLELOPATHIC POTENTIAL OF *Erica vagans*, *Calluna vulgaris*, AND *Daboecia cantabrica*

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Abstract—In laboratory tests the allelopathic potential of *Erica vagans*, *Calluna vulgaris*, and *Daboecia cantabrica* was determined. Aqueous extracts of flowers of *D. cantabrica* and leaves of *C. vulgaris* inhibit root and hypocotyl growth of red clover, the former causing 51% inhibition of germination. Intact aerial parts of the Ericaceae here studied drastically reduced the growth of red clover and 100% inhibition of germination was caused by flowers of *D. cantabrica*. Inhibition of aqueous extracts remains after chromatographic separation, and two well-defined inhibition zones may be observed. Hydrosoluble organic compounds (phenol-like compounds) could probably be responsible for the inhibitions detected.

Key Words—Allelopathy, Ericaceae, *Erica vagans*, *Calluna vulgaris*, *Daboecia cantabrica*, red clover inhibition.

INTRODUCTION

In previous papers we have reported the possible allelopathic effects of different Ericaceae upon herbaceous species in Galicia (northwest Spain) (Ballester et al., 1977, 1979). In other regions the Ericaceae have also been thought to exhibit allelopathic properties that favor their expanding invasion (Chou and Muller, 1972). The inhibitory effects observed are due, at least in part, to the presence of phenolic compounds not only in the plants themselves but also in the soil beneath (Ballester et al., 1972; Carballeira and Cuervo, 1980).

The present paper studies the possible allelopathic effects of *Erica vagans* L., *Calluna vulgaris* (L.) Hull, and *Daboecia cantabrica* (Hudson) C.Koch. Of

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the three, *C. vulgaris* is the most widely distributed in this region, covering the more regressive heathlands, *E. vagans* and *D. cantabrica* normally are found only in very wet soils.

METHODS AND MATERIALS

Erica vagans, *C. vulgaris*, and *D. cantabrica* were collected in full flower at different sites in Galicia and immediately brought to the laboratory, where leaves, stems, and flowers were separated. Three different bioassays were carried out to determine the biological activity of these Ericaceae members.

In one test 20 g of intact leaves, stems, or flowers (fresh weight) were extracted with 100 ml distilled water for 24 hr in the dark at room temperature. Fifty red clover seeds (*Trifolium pratense* L.) were sown on Whatman No. 1 filter paper in 110 × 15-mm Petri dishes and 4 ml of the filtered extract added (equivalent to 0.8 g fresh weight). Distilled water was used in the control.

In another test, 5 g of intact fresh leaves, stems, or flowers were laid on the bottom of a 110 × 15-mm Petri dish beneath Whatman No. 1 filter paper, 5 ml of water were added, and 50 red clover seeds sown. The control had shredded Parafilm instead of plant material (McPherson and Muller, 1969).

In the last test, 4 ml (equivalent to 0.8 g fresh weight) of aqueous extracts, obtained as above, were concentrated under reduced pressure and spotted on 3 × 57-cm Whatman No. 1 paper strips, the chromatograms being developed with isopropanol–ammonia–water (10:1:1). Ten pieces, 3 × 3 cm, were cut from the chromatograms and used as seed beds, these being moistened with 1.5 ml distilled water and placed in 40 × 40-mm Petri dishes. In each dish 15 red clover seeds were sown (Chou and Muller, 1972).

In all tests red clover seeds were incubated in the dark at 24–25°C for 72 hr, the number of germinated seeds counted, and the lengths of the root and hypocotyl measured. The ionic strengths of the filtered aqueous extracts of leaves, stems, and flowers were measured at 22°C using a Philips PW 9504 conductivity meter. Aqueous solutions of 0.01 M KCl and NaCl (Merck analytical reagent grade) were used as standards.

The bioassays were repeated three times, three replications being set up in each repetition. The standard error and/or the least significant difference at the 5% confidence level were determined.

RESULTS AND DISCUSSION

Aqueous extracts of the aerial parts of the three Ericaceae studied were significantly inhibitory to the growth of the root and hypocotyl of red clover. The most marked effect was exhibited by the extracts of the flowers of *D.*

TABLE 1. EFFECT OF AQUEOUS EXTRACTS OF LEAVES, STEMS, AND FLOWERS ON ROOT AND HYPOCOTYL GROWTH OF RED CLOVER (MEAN ± SE)

	N[a]	Root (mm)	Hypocotyl (mm)
Erica vagans			
Leaves	98 (65)[b]	7.4 ± 0.5	14.1 ± 1.2
Stems	107 (71)	6.8 ± 0.4	16.9 ± 2.3
Flowers	94 (62)	6.5 ± 0.3	13.8 ± 1.6
Calluna vulgaris			
Leaves	89 (59)	4.3 ± 0.2	7.4 ± 0.6
Stems	102 (68)	9.4 ± 0.7	13.6 ± 1.9
Flowers	102 (68)	7.1 ± 0.3	12.8 ± 1.4
Daboecia cantabrica			
Leaves	97 (64)	6.5 ± 0.3	11.9 ± 0.9
Stems	107 (71)	10.8 ± 0.9	13.8 ± 1.3
Flowers	62 (41)	4.5 ± 0.2	5.9 ± 0.6
Control	120 (80)	10.8 ± 0.9	16.6 ± 1.3
LSD (5%)	7.5%	2.1	2.4

[a]Number of roots and hypocotyls measured.
[b]Percentage of germinated seeds.

cantabrica, which even inhibited germination by 51% of control (Table 1). None of the Ericaceae hitherto studied under the same conditions described in this paper have displayed such a strong inhibitory effect (Ballester et al., 1977, 1979), similar levels of inhibition having been achieved by methanolic extracts of *E. australis* (Carballeira, 1980). The inhibition of growth caused by the leaf extracts of *C. vulgaris* is also striking, although germination is less affected in this case as compared to *D. cantabrica* (Table 1). Flower and leaf extracts usually give rise to greater inhibition than stem extracts, which is paralleled by the greater content of phenolic compounds in the former than in the latter.

Since conductivity of extracts is low (Table 2) (a minimum of 143 μmho in the stem extract of *E. vagans* and a maximum of 268 μmho in the leaf ex-

TABLE 2. CONDUCTIVITIES[a] OF AQUEOUS EXTRACTS, IN μMHO (MEAN ± SE)

	Leaves	Stems	Flowers
Erica vagans	168 ± 36	143 ± 49	210 ± 25
Calluna vulgaris	268 ± 49	154 ± 36	196 ± 36
Daboecia cantabrica	208 ± 25	187 ± 32	197 ± 28

[a]Standard solutions 0.01 M: KCl, 1250 ± 25; NaCl, 1098 ± 25.

tract of *C. vulgaris*), it seems unlikely that the inhibition observed is due to the content of ionic salts, especially when the germination and growth of red clover seeds under the same conditions are unaffected by 0.01 M solutions of KCl and NaCl with much higher conductivities (1250 μmho and 1098 μmho, respectively). Moreover, the conductivities of the extracts of the Ericaceae studied in this paper are less than those found for *E. australis* and *E. arborea* (Ballester et al., 1979); their inhibitory effect upon red clover is significantly greater.

When leaves, stems, and flowers of the three Ericaceae here studied are placed in direct contact with red clover seeds in McPherson and Muller's (1969) test, the most striking results (Table 3) are produced by *D. cantabrica* flowers (100% inhibition of germination) followed by *C. vulgaris* leaves (67% inhibition of germination). The inhibition of root and hypocotyl growth in the seeds which germinate is also significant and, contrary to the other results obtained in this and the previous bioassay, *C. vulgaris* leaves and stems have a greater inhibitory effect upon hypocotyls than upon roots.

When the aqueous extracts of these Ericaceae are separated chromatographically (Figure 1), no individual zone of the chromatogram is found to inhibit germination. The highest rates of growth inhibition are produced by certain sections of the chromatograms of the flower extracts of *C. vulgaris* and *D. cantabrica*. In both there is an inhibition zone at R_f 0.2–0.4, and in *D. cantabrica* another appears at R_f 0.6–0.7. The chromatograms of leaf and

TABLE 3. EFFECT OF INTACT LEAVES, STEMS, AND FLOWERS ON ROOT AND HYPOCOTYL GROWTH OF RED CLOVER (MEAN ± SE)

	N[a]	Root (mm)	Hypocotyl (mm)
Erica vagans			
Leaves	65 (43)[b]	4.4 ± 0.3	7.5 ± 0.7
Stems	88 (58)	6.5 ± 0.4	7.1 ± 0.4
Flowers	93 (62)	4.9 ± 0.3	7.1 ± 0.2
Calluna vulgaris			
Leaves	42 (28)	7.4 ± 1.5	4.7 ± 0.7
Stems	81 (54)	7.0 ± 0.5	6.7 ± 0.6
Flowers	91 (60)	6.6 ± 0.5	6.6 ± 0.7
Daboecia cantabrica			
Leaves	64 (42)	5.4 ± 0.3	8.0 ± 0.5
Stems	63 (42)	5.0 ± 0.3	8.3 ± 0.7
Flowers	– (0)	–	–
Control	127 (85)	12.7 ± 1.6	23.7 ± 2.4
LSD (5%)	8.3%	5.4	5.0

[a]Number of roots and hypocotyls measured.
[b]Percentage of germinated seeds.

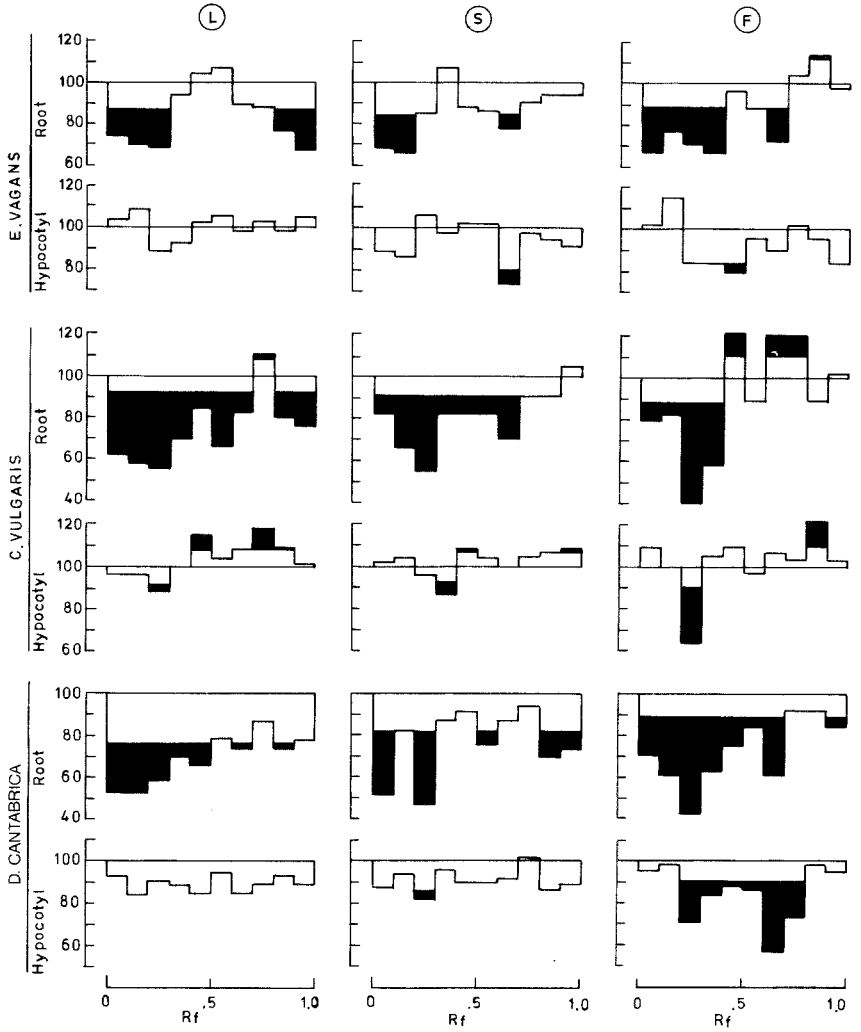

FIG. 1. Effects of aqueous extracts of leaves (L), stems (S) and flowers (F) of *E. vagans*, *C. vulgaris*, and *D. cantabrica* on root and hypocotyl growth of red clover after chromatographic separation. Chromatograms were developed in isopropanol–ammonia–water (10:1:1). Results are given as percent of control. Shaded areas differ significantly from the control at 0.05 level.

stem extracts exhibit inhibition zones with respect to red clover root growth, but their effect upon the hypocotyls is much less pronounced.

The inhibitions observed, although significant, are less than when the whole extracts are tested, presumably because the combined action of different inhibitors present in the whole extracts is stronger than that produced after chromatographic separation.

In our earlier research (Ballester et al., 1977, 1979) the greatest inhibitory effects appeared in chromatograms at the same R_f values as in the present work, suggesting the presence of inhibitors common to all the different Ericaceae studied so far. Ionic inhibition effects having been ruled out, the high levels of hydrosoluble phenolic compounds found in the Ericaceae lead one to suspect that these may be the agents responsible for the inhibitory effects observed. Fifteen different phenolic compounds (phenols, phenolic and cinnamic acids, coumarins, and flavonols) have been identified in *E. vagans* (Arines, 1973), *C. vulgaris* (Mantilla, 1974), and *D. cantabrica* (unpublished). A powerful unidentified inhibitor has also been detected in the roots of *E. vagans* (Arines, 1973) and *C. vulgaris* (Mantilla, 1974), as well as in the roots and associated soil of *E. australis* (Carballeira and Cuervo, 1980). In the case of *E. vagans* and *C. vulgaris* this root-borne agent could presumably act to reinforce the allelopathic potential of the aerial parts. On the other hand some of the compounds identified have also been detected in the soil under heather (Ballester et al., 1972), having probably been introduced via the allelopathic mechanisms typical of humid-zone plants (Rice, 1974).

Although the results described in this paper do not allow one to conclude that the Ericaceae exert any definite allelopathic action under field conditions, there nevertheless seems to be little doubt that the inhibitory potential of the hydrosoluble compounds they contain, in conjunction with other phenomena, could favor the progressive expansion of this family at the expense of herbaceous species.

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PHEROMONE HYDROLYSIS BY CUTICULAR AND INTERIOR ESTERASES OF THE ANTENNAE, LEGS, AND WINGS OF THE CABBAGE LOOPER MOTH, *Trichoplusia ni* (HÜBNER)[1]

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Abstract—Examination was made of the hydrolytic activities of esterases obtained from the antennae, legs, and wings of 3-day-old cabbage looper moths, *Trichoplusia ni* (Hübner), by elution and by homogenation of those appendages. Pheromone hydrolysis in 1-min assays was monitored by use of tritium-labeled (*Z*)-7-dodecen-1-ol acetate and thin-layer chromatography to separate the reaction products. Listed according to the activities of the esterases obtained by homogenation, the organs were antennae > legs > wings. In contrast, the order according to the activities of the eluted esterases was wings > legs > antennae. Also, the eluted enzymes were less active than the esterases obtained by homogenization. The relatively high pheromone-hydrolyzing activity present in homogenized antennae suggests that the esterases originated inside the antennae and lends support to the hypothesis proposed in earlier investigations that pheromone-inactivating enzymes may play an important role in the olfactory process, possibly by clearing pheromone from the vicinity of the olfactory receptors. The esterases detected on the cuticle, on the other hand, may function by preventing surface accumulation of pheromone. The higher measured esterase activity in homogenates of prothoracic legs than of mesothoracic or metathoracic legs suggests that the prothoracic legs, which are used to clean the antennae of debris, may function by removing and degrading pheromone from the surface of antennae.

Key Words—Antennae, esterases, pheromone degradation, cuticle, olfaction, cabbage looper, *Trichoplusia ni*, Lepidoptera, Noctuidae.

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

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INTRODUCTION

During the past several years many insect pheromones have been identified for practical use in insect control. However, as pointed out by Roelofs (1980), the successful use of pheromones in insect control also requires ". . . in depth physiological and behavioral studies of insects in conjunction with the chemical analyses." One aspect of insect physiology that has received little attention but that may play an important role in the insect's perception of and behavioral response to pheromones is enzymes that degrade the pheromone. Schneider (1970) first proposed that such enzymes may serve to clear the pheromone from the surface and/or interior of the antennae after transduction and also to clear the body surface of pheromone, which might otherwise desorb and interfere with communication of the insect.

In the cabbage looper, *Trichoplusia ni* (Hübner), pheromone-hydrolyzing esterases that can be isolated from the chemosensory hairs on the antennae appear to originate in the inner structure of the antennae, possibly as membrane-bound esterases (Ferkovich et al., 1980). These enzymes have a considerably higher activity per unit of protein than esterases isolated from tissues which lack chemosensory sensilla.

That pheromone-catabolizing enzymes are also localized on the cuticle of the insect has been deduced from in vivo studies with the silk moth, *Bombyx mori* (L.) (Kasang and Kaissling, 1972), and the cabbage looper, *T. ni* (Mayer, 1975). In these studies, the insect was first exposed to the pheromone; then, the antennae and legs were extracted with various solvents to yield the pheromone and its breakdown products. It was assumed that the apolar solvents, such as pentane, extracted only the nonpolar compounds in the outer layers of the cuticle, and that the polar solvent extracted pheromone and metabolites from the inner layers.

If indeed pheromone-degrading enzymes are present on the surface of the cuticle, the following questions arise: can these enzymes be isolated in an active form? And, if so, are their activities comparable to those of esterases found inside the antennae? We now report a study to compare the activities of esterases eluted from the antennae and other appendages with those of esterases obtained by homogenization of those appendages.

METHODS AND MATERIALS

Chemicals. Tritiated (*Z*)-7-dodecen-1-ol acetate (200 mCi/mmol) was prepared by treating a benzene solution of (*Z*)-7-dodecen-1-ol (0.149 mmol) with an approximately two-fold molar excess of [^{3}H]acetic anhydride (400 mCi/mmol; New England Nuclear) in the presence of a little pyridine. After standing at room temperature 2 days, the mixture was partitioned between cyclohexane and 7% aqueous sodium bicarbonate. The organic phase was

dried and transferred to a silica gel SEP-PAK®. The SEP-PAK was eluted with 2 ml *n*-hexane (eluate was discarded), then with 5 ml benzene containing 1% ethyl acetate (v/v). Radioactivity in the benzene eluate was measured by scintillation counting and was found to contain 21.0 mCi of labeled pheromone (70.5% recovery). The labeled pheromone was then shown to be ≥99% pure by gas chromatography (1.8 m × 6 mm Dexsil® 300 on 100–200 mesh Supelcoport®).

Insect Ringer's solution was prepared according to Ephrussi and Beadle (1936).

Elution of Esterases. Ringer's solution was used to elute esterases from the antennae, legs, and wings of 3-day-old adult cabbage looper moths, and elution time was 12 hr at 22°C. The antennae of each live adult were held in a capillary tube containing ≈60 μl of the solution. For each sex, the antennal eluates from 120 insects were combined, dialyzed against Ringer's solution, and concentrated with 500 μl with a ProDi Mem® (10,000 mol wt cut-off membrane) apparatus. The prothoracic, mesothoracic, and metathoracic legs on one side of the body of each insect were held in 100 μl of solution, and the wings of each insect were held in 100 μl of solution. For each sex, the combined eluants from the legs of 51 insects and from the wings of 102 insects were centrifuged at 20,000 *g* for 30 min, dialyzed, and concentrated to 500 μl as described above.

Homogenization of Tissues. Male and female antennae (80 pairs per sex), legs (12 pair each of prothoracic, mesothoracic, and metathoracic (per sex), and wings (12 pairs) were dissected from 3-day-old adult moths and homogenized in a solution of 0.5 M sucrose and 0.05 M Tris HCl (pH 7.5). The homogenates were sonicated for 30 sec (Biosonic III, minimum intensity) and centrifuged at 20,000 *g* for 30 min at 4°C; then the supernatants were dialyzed against Ringer's solution and concentrated by the procedure described above.

For one experiment, insects were examined as to the relative pheromone-hydrolyzing activities in their mesothoracic and metathoracic legs. Thus, legs of each type were excised and homogenized separately. Enzyme concentrates were prepared from the homogenates as described above and were assayed for protein content and pheromone-hydrolyzing activity.

Protein Assay. The supernatants were assayed for protein by the Bio-Rad® procedure (Anonymous, 1979), with bovine gamma-globulin as standard.

Pheromone Assay. The assay for hydrolysis of radiolabeled pheromone was as previously described (Ferkovich et al., 1980). Briefly, preliminary tests were conducted to determine the conditions for a linear rate of hydrolysis in the assay mixture. In these tests, conducted at 22°C, the amount of protein (enzyme) was kept between 5 and 15 μg, but the concentration of pheromone was varied from 2.0×10^{-6} to 1.5×10^{-5} M, and reaction time, from 1 to 5 min. Then, specific activity was determined under linear-rate conditions on the basis of four replicate 1-min assays.

RESULTS AND DISCUSSION

The esterases obtained by homogenization of male and female antennae and legs (Figure 1A,B) had a higher specific activity than the corresponding eluted enzymes (Figure 1C,D). The activity of the esterases isolated from the wings by homogenization was similar to that obtained by elution.

During 1 min of assay, the actual amounts of product formed per microgram of antennal protein obtained from males to females, respectively, were 6.3 and 4.9 pmol for the homogenation-derived enzymes, and 0.2 and 0.1 pmol for the eluted enzymes. Ferkovich et al. (1980) reported that the esterases they isolated from chemosensory sensilla by fracturing the tips of the hairs on *T. ni* antennae had been weakly membrane bound (extrinsic membrane proteins) and could be released from the hairs by homogenization. In our study the antennal esterases isolated by homogenization were more active in hydrolyzing the pheromone than those isolated by elution. Thus, the esterases within the antenna are probably involved in degrading the pheromone and/or other nonpheromone chemicals at or near the membrane receptor sites. On the other hand, the esterases on the surface of the antennae probably facilitate pheromone deposition because of their low activity. Otherwise, the pheromone would be rapidly degraded on the antennal surface, and the probability of pheromone molecules reaching nerve-membrane receptor sites within the antenna would be lower. The higher cuticular esterase activity on legs and wings than on antennae suggests that esterases serve to keep the cuticle of legs and wings free of pheromone. This suggestion becomes more meaningful in light of the high amount of pheromone released per female. One female cabbage looper moth releases an average of 812 ng of pheromone per evening (Bjostad et al., 1980). Although adsorption of released pheromone onto the epicuticle of insects within the vicinity of calling females has not been shown, it is tempting to speculate that such adsorption does occur and is followed by hydrolysis of the pheromone by the cuticular esterases reported in this paper. If the pheromone were continually desorbed from the bodies of insects after mating was effected, males might be erroneously attracted to nonreceptive females or possibly to other males releasing pheromone from their body surfaces (Schneider, 1970).

What was the source of the esterase eluted in Ringer's solution from the cuticle of the antennae, legs, and wings? One possibility is the enzymes originated in the ducts or pore canals in the integument. Esterase(s) have been detected in the pore canals and in the endocuticle during moulting in larvae of *Calopodes ethlius* Stoll and are thought to be involved in the synthesis and maintenance of the epidermal wax layer or in some other cuticular process (Locke, 1959, 1974). Another possibility is that the esterases were leached from the subcuticular layers of the organs. Ahmad (1970) detected carboxylesterase activity histochemically in the subcuticular (epidermal) layer of the

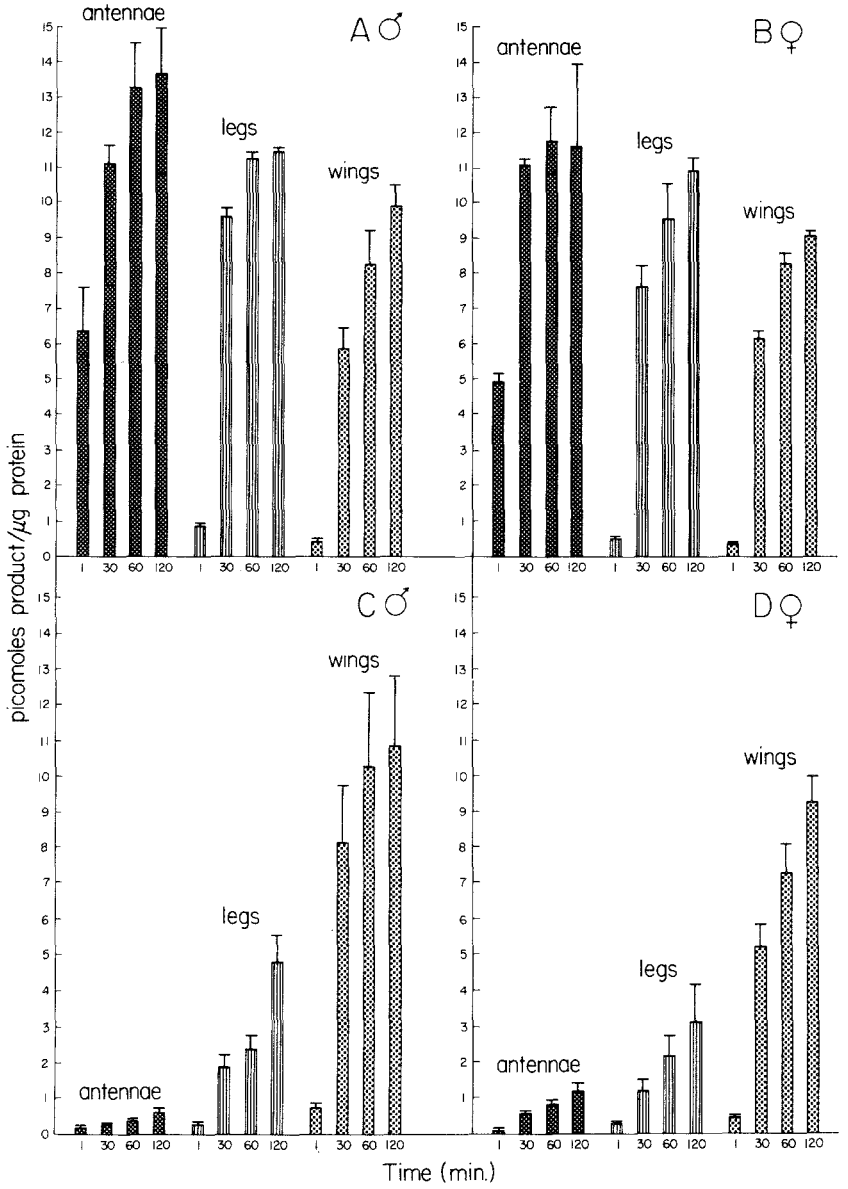

FIG. 1. Time courses of pheromone hydrolysis by esterases of *T. ni* male and female antennae, legs, and wings. (A and B) Homogenates as sources of pheromone-hydrolyzing esterase; (C and D) esterase eluted from the surfaces of antennae, legs, and wings into Ringer's solution for 12 hr. Each bar represents the standard deviation of the mean of four replications.

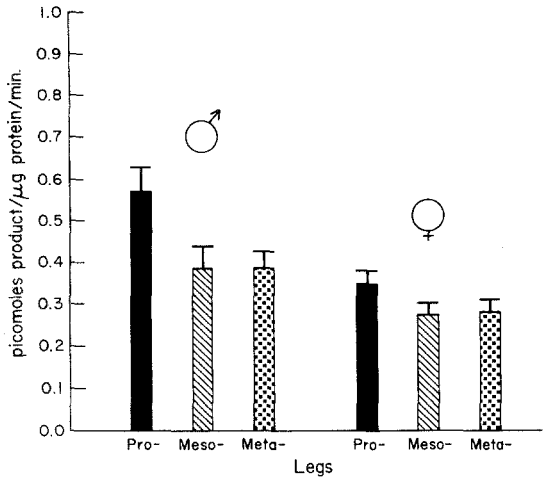

FIG. 2. Hydrolysis of pheromone by esterases obtained from homogenates of *T. ni* pro-, meso-, and metathoracic legs. Each bar represents the standard deviation of the mean of four replicates.

abdominal body wall of adult *Musca domestica* L. However, Kapin and Ahmad (1980) reported that in the integument of 5th instar gypsy moth larvae, the cuticle was the greater source of integumental esterase than the epidermis.

Although relatively low in esterase activity per unit of protein, the eluate of antennae contained considerably more total protein (6.4 and 3.4 μg protein/pair, male and female, respectively) than that of legs (1.4 and 2.6 μg protein/pair, male and female, respectively) or wings (2.2 and 3.2 μg protein/pair, male and female, respectively). If protein were released from the pore tubules in the olfactory sensilla, it would tend to dilute the cuticular esterase eluted from the epicuticle. This possibility becomes even more plausible when the reports of Riddiford (1970) and Seabrook (1977) are considered. Riddiford (1970) reported that a ^{3}H-labeled pheromone was bound by protein(s) in the antennae of saturniid moths, *Antheraea* spp., and that holding the organs in Ringer's solution for 30 min resulted in elution of the bound product. The proteins, which could be resynthesized by the antennae after the elution, were thought to originate in the pore tubule system of the olfactory sensilla. Whether the proteins were receptors or enzymes, however, was not clarified. Also, Seabrook (1977) indicated that the pore tubules in an olfactory sensillum of the eastern spruce budworm, *Choristoneura fumiferana* (Clemens), contained a proteinaceous fluid. Scanning electron micrographs of the olfactory trichodium of this insect showed that the sensillum was coated with a material that could be digested with protease. He concluded that the material removed was a protein that was extruded through the pores in the sensillum.

Considering the low specific activity of esterases eluted from the antennae of *T. ni*, the question arises as to how the insect removes the pheromone from the antennal surface. Although it has not been demonstrated that the cabbage looper moth cleans its antennae of pheromone during grooming behavior, many moths of Noctuidae do groom their antennae to keep them free of debris (Callahan and Carlysle, 1971). Thus, we considered the possibility that the prothoracic legs, which have a special antenna-grooming organ called a comb or epiphysis, have more esterase activity than the mesothoracic or metathoracic legs, which are not used in grooming. Figure 2 shows that the pheromone-degrading activity in the supernatant (20,000 *g*) from a homogenate of the prothoracic legs was higher than that of the mesothoracic or metathoracic legs. These data distinctly raise the possibility that the prothoracic legs are used to remove and degrade pheromone from the antennae during grooming. However, the data do not suggest the origin of the esterases—whether cuticular surface or interior tissue (i.e., muscle).

We noted that the specific activity of eluted esterases was higher for wings than for legs or antennae but are uncertain as to the significance of this finding. The females of *T. ni* have a relatively high nightly release rate of pheromone per individual (Bjostad et al., 1980). Possibly, therefore, the wings, because of their larger surface area, adsorb more pheromone during calling than other parts of the insect and require a more efficient means of degrading the pheromone.

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SOCIOCHEMICAL ALTERATION OF HONEYBEE HOARDING BEHAVIOR

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Abstract—Hoarding experiments were conducted with honeybees (*Apis mellifera* L.) in cages containing comb treated with either 2-heptanone, isopentyl acetate, citral, or geraniol. 2-Heptanone increased hoarding rates; isopentyl acetate decreased hoarding rates; citral and geraniol had no observed effect.

Key Words—*Apis mellifera*, Hymenoptera, Apidae, 2-heptanone, isopentyl acetate, citral, geraniol, hoarding.

INTRODUCTION

Empty comb in a honeybee (*Apis mellifera* L.) nest plays a major role in influencing the nectar-harvesting activities of honeybees. Substantially increased amounts of empty comb in the nests of colonies during strong nectar flows significantly increase the amount of honey that they store (Rinderer and Baxter, 1978a). Similarly, increased amounts of empty comb result in increased rates of sucrose solution hoarded by caged bees in laboratory experiments (Rinderer and Baxter, 1979, 1980). The stimuli through which comb influences nectar-harvesting are chemical (Rinderer, 1981), and single pheromones often have multiple functions in social insect chemocommunication systems (Blum, 1977). Consequently, knowledge of any effects of known worker honeybee sociochemicals on the hoarding behavior of bees is desirable.

This paper reports the effects of four sociochemicals on the hoarding behavior of bees in laboratory hoarding cages. These small cages (Kulinčević et al., 1973) house bees with a piece of empty comb and feeders containing sucrose solution, water, and pollen substitutes. Bees remove sucrose solution from the feeder and hoard it in the comb (Free and Williams, 1972; Kulinčević

and Rothenbuhler, 1973). Differential amounts of sucrose solution hoarded in these cages are related to differential amounts of honey stored by field colonies. This is true for differences arising primarily from either genotype (Kulinčević et al., 1974; Rothenbuhler et al., 1979) or environment (Rinderer and Baxter, 1978a, 1979; Rinderer et al., 1979).

METHODS AND MATERIALS

In preparation for the experiments, pieces of comb (46.25 cm^2) were placed in desiccators with an atmosphere saturated with either 2-heptanone (2-H), isopentyl acetate (IPA), citral, or geraniol. In each instance, combs increased in weight by ca. 4 g during exposure to volatile chemicals. IPA and 2-H are components of alarm pheromones from honeybee stings and mandibular glands, respectively, which mediate defensive behavior (Boch et al., 1970); geraniol and citral are components of an attractant pheromone from Nasonov glands that mediates orientation and aggregation (see reviews by Blum, 1977; Wilson, 1971). Comb was kept in desiccators for four days and then placed in hoarding cages. Within 4 hr, bees were added to the cages and experimental studies began.

For evaluation of each sociochemical, combs of emerging adult worker bees were obtained from the brood nests of seven colonies. Different colonies were used for evaluating each chemical. The combs of emerging bees were held in an incubator (35°C and 50% relative humidity) until the bees were 0–24 hr old. Groups of 30 bees (Rinderer and Baxter, 1978b) from each colony were then placed into eight cages containing comb treated with the appropriate sociochemical and eight cages containing untreated comb. Each group of cages was placed in a separate incubator (35°C and 50% relative humidity).

Each cage was inspected daily for seven days; the amount of sucrose solution removed from the feeders was measured, and all feeders were replenished.

RESULTS

An inspection of Table 1 reveals that two of the four chemicals, 2-H and IPA, significantly influenced hoarding behavior; 2-H increased hoarding ($P < 0.001$) while IPA decreased it ($P < 0.003$). In all experiments, differences were significant in hoarding rates between colonies. No interactions occurred between the factors of chemical treatment and colony source, which indicates that bees from all colonies responded in similar ways to the chemicals.

TABLE 1. HOARDING RESPONSE (ML OF SUCROSE SOLUTION) OF BEES CAGED WITH COMB TREATED WITH SOCIOCHEMICALS

Sociochemical treatment	$\bar{X} \pm SE$[a]	Analysis of variance			
		Source of variation	*df*	*F*	*P*<
2-Heptanone	15.7 ± 0.3	T[b]	1	16.44	0.001
Control	12.5 ± 0.2	C	6	17.04	0.001
		I	6	1.7	NS
Isopentyl acetate	13.7 ± 0.2	T	1	9.20	0.003
Control	16.2 ± 0.3	C	6	8.81	0.001
		I	6	1.27	NS
Citral	10.3 ± 0.1	T	1	0.86	NS
Control	10.7 ± 0.2	C	6	8.26	0.001
		I	6	1.12	NS
Geraniol	14.8 ± 0.3	T	1	0.42	NS
Control	15.4 ± 0.3	C	6	15.34	0.001
		I	6	0.51	NS

[a]Each mean is calculated from 8 replicate measurements from each of 7 colonies.
[b]T = treatment; C = colonies; I = interaction.

DISCUSSION

The presence of two compounds resulted in a change in hoarding and the presence of the other two did not, even though all four compounds are known to be pheromones that are perceived by honeybees. Also, of the two chemicals that did affect hoarding, one increased and one decreased it. Therefore, the results are unlikely to be caused by general chemical irritation, nor are the two responses likely to arise from a similar effect since the responses were directionally different. Rather, the effect of each chemical is likely to have its origins in a separate adaptive feature of the natural history of bees.

Bees in hoarding cages exhibit a defensive response under brief exposure to IPA vapors (Collins and Rothenbuhler, 1978). In the experiment reported here bees were continually exposed to IPA vapors and continually exposed to the hoarding stimuli provided by comb. Limited olfactory habituation coupled with the role of IPA in defense probably underlie the reduction of hoarding by bees continually exposed to IPA. The chemical tends to draw bees away from other activities into defense and, with some efficiency, continues to do so.

In a natural setting, IPA plays a major role in colony-defensive episodes. During such episodes, large numbers of bees may be temporarily drawn from other activities into defense, at least in part by the action of IPA (Maschwitz, 1964; Boch et al., 1962). Bees in a colony, while providing each other with

chemical stimuli, which release a defensive response, apparently do not provide each other with stimuli which terminate that response. Rather, termination has its beginnings when the intruder ceases to provide appropriate stimuli. Included among these stimuli is the presence of IPA released from stings delivered to the intruder.

When presented with 2-H, bees exhibit defensive responses similar to those elicited by IPA under both laboratory (Collins, 1980) and field conditions (Boch and Shearer, 1971). 2-H is regarded as an alarm pheromone that is secondary in function to IPA (Boch et al., 1970), even though 2-H is produced in quantities ranging to 40 μg (Boch and Shearer, 1967), whereas IPA is produced in quantities ranging to 5 μg (Boch and Shearer, 1966). This differential production suggests that 2-H has additional roles in which it functions as a primary behavioral mediator. In large quantities, 2-H repels bees (Simpson, 1966). This response occurs in the natural history of honeybees, since foraging bees apparently label exhausted food sources with 2-H that marks them as "empty" (Nuñez, 1967). While this function of 2-H explains, at least in part, the presence of large amounts of 2-H in foraging bees, it does not satisfactorily explain the presence of large amounts of 2-H in guard bees (Crewe and Hastings, 1976).

Perhaps 2-H released during defensive episodes by guard bees not only functions as a releaser pheromone with a secondary alarm role, but also functions as a primer pheromone which physiologically alters the bees and thereby increases the intensity of nectar-harvesting activities by foragers. Nectar foraging is regulated chemically through the action of volatiles from empty comb (Rinderer, 1981). Similar laboratory hoarding responses are evoked by both 2-H and comb volatiles. If this effect by 2-H occurs in the field, it would serve to increase the intensity of nectar-harvesting after a defensive episode and thereby stimulate a colony to regain losses incurred through the actions of intruders.

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SOUTHERN PINE BEETLE: Olfactory Receptor and Behavior Discrimination of Enantiomers of the Attractant Pheromone Frontalin[1]

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Abstract—In laboratory and field bioassays, the response of *Dendroctonus frontalis* was significantly greater to the mixture of (1*S*,5*R*)-(−)-frontalin and *alpha*-pinene than to (1*R*,5*S*)-(+)-frontalin and *alpha*-pinene. Electrophysiological studies revealed that antennal olfactory receptor cells were significantly more responsive to (1*S*,5*R*)-(−)-frontalin than to (1*R*,5*S*)-(+)-frontalin. Both enantiomers stimulated the same olfactory cells which suggests that each cell possesses at least two types of enantiomer-specific acceptors.

Key Words—Enantiomers, bark beetle, pheromone, *Dendroctonus frontalis*, Coleoptera, Scolytidae, southern pine beetle, electrophysiology, olfaction.

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INTRODUCTION

Frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane) was isolated and identified from hindguts of male western pine beetles (*Dendroctonus brevicomis*) (Kinzer *et al.* 1969). Subsequently, it was also isolated from hindguts of female southern pine beetle, *D. frontalis* (Pitman et al., 1969), volatiles either collected on Porapak Q® by aeration of both naturally and artificially infested host tree material (Stewart et al., 1977; J.R. West, personal communication) or from frass of boring females. Racemic frontalin elicited response at the antennal receptor cell level from *D. frontalis* (Payne, 1971). By itself, racemic frontalin was slightly attractive to flying beetles in the field and to walking beetles in a laboratory bioassay but was synergized in combination with host volatiles (α-pinene, oleoresin, or turpentine) (Kinzer et al., 1969; Payne et al., 1976, 1978a; McCarty et al., 1980). Frontalin occurred in a 15:85 mixture of the (+) and (−) enantiomers in the volatile compounds collected on Porapak Q by aeration of females boring in pine bolts (Stewart et al., 1977).

Behavioral response to pheromone enantiomers has been reported for several insect species (Iwaki et al., 1974; Borden et al., 1976, 1980; Hedden et al., 1976; Klimetzek et al., 1976; Vité et al., 1976a,b; Wood et al., 1976; Yamada et al., 1976; Harring and Mori, 1977; Krawielitzki et al., 1977; Miller et al., 1977; Birch et al., 1980, Lanier et al., 1980; Levinson and Mori, 1980; Mustaparta et al., 1980). However, electrophysiological data on response to pheromone enantiomers has only been reported for the gypsy moth, *Lymantria dispar,* and the beetles *Ips pini, I. paraconfusus, I. typographus,* and *Trogoderma granarium* (Yamada et al., 1976; Miller et al., 1977; Levinson and Mori, 1980; Mustaparta et al., 1980; Dickens, 1981).

The availability of optically pure enantiomers of frontalin (Mori, 1975) enabled us to conduct laboratory and field experiments on *D. frontalis* to elucidate response to the compounds at the olfactory receptor cell level and to determine their influence on behavior.

METHODS AND MATERIALS

Behavioral bioassays were measured in the laboratory using positive responses of walking beetles to an odor source (Payne et al., 1976). Data were obtained for male and female beetles in response to 1:4 mixtures of racemic (±)-frontalin (F), 15% (+) F and 85% (−)F, (+)F, and (−)F each together with D-α-pinene delivered at 10 μg/min in nanograde pentane via a power-driven microsyringe into a 1 liter/min airflow. α-Piene was delivered at 40μg/min in the mixtures. A pentane control was delivered at 1 μl/min. Racemic frontalin was 99% chemically pure via GLC. Optical purity of the frontalin enantiomers and D-α-pinene was $\geqslant$98% via chiral shift NMR and optical rotation, respectively. Beetles were assayed in groups of five, one sex at a time.

A positive response was recorded when an individual beetle walked upwind to the attractant source.

Field tests were conducted in a mixed pine–hardwood forest in the Sam Houston National Forest, Texas. The enantiomers were released undiluted from glass planchets (3 × 21 mm) at 0.5 mg/hr (Payne et al., 1978a). α-Pinene was eluted from a separate glass container (1 dram vial 33 mm high) at 2 mg/hr. The elution containers were placed on the center shaft of a four-vaned, sticky wing trap (Payne et al., 1978b). Traps were placed ca. 10 m apart within an active *D. frontalis* infestation (Payne et al., 1978b). Beetles were removed from traps and trap positions were rotated every 30 min per replicate (Payne et al., 1978b).

Antennal olfactory responses were measured through the use of electroantennograms (EAGs) and single-cell recordings (Schneider, 1957; Boeckh, 1962; Dickens and Payne, 1977). EAGs were recorded from five male and five female beetles in response to the following series of treatments: (1) 200 μg (±)F, (2) 200 μg 15% (+)F (30 μg) and 85% (−)F (170 μg), (3) 200 μg (+)F, (4) 200 μg (−)F, and (5) 2μl pentane control. The differential adaptation technique was used to provide insight on specificity at the acceptor level (Payne and Dickens, 1976). Each EAG preparation was exposed to one of the above treatments until completely adapted; that is, no response resulted in a second stimulation by the same material. Within milliseconds the preparation was then exposed to a second treatment. The presence or absence of response, or the degree of response to stimulation by the second material, was a measure of the relative interaction of the stimuli with the same acceptors. Single-cell responses, from three male beetles to 100 μg of (+)F, (−)F, and (±)F, were recorded to provide additional determination of specificity at the cellular and acceptor levels. Compounds were prepared in nanograde pentane, placed on filter paper, and delivered at 1 liter/min airflow via a solenoid-operated valve (Payne, 1975).

RESULTS AND DISCUSSION

In laboratory assays both sexes were attracted to all treatments (Table 1). The response of males to all treatments containing (−)F were significantly greater than to treatments containing (+)F. There were no significant differences between treatments for females.

Under field conditions all treatments were attractive to flying beetles of both sexes (Table 2). Both sexes responded significantly more to treatments containing (±)F and (−)F than to the treatment with (+)F. There were no differences in the levels of response between treatments with (±)F and the naturally occurring mixture 15% (+)F plus 85% (−)F. Furthermore, there were no significant differences in the sex ratios of responding beetles between any of the treatments.

TABLE 1. RESPONSE OF *Dendroctonus frontalis* TO ENANTIOMERS OF FRONTALIN (F) ALONE AND WITH D-ALPHA-PINENE (α-P) IN A LABORATORY BIOASSAY

Treatment	$\bar{X}$ % response[a] (±SE)/replicate[b]	
	Females	Males
(±)F + α–P	53 ± 13a	72 ± 9a
15%(+) + 85%(–)F + α–P	53 ± 10a	63 ± 6a
(+)F + α–P	50 ± 10a	48 ± 5b
(–)F + α–P	50 ± 7a	78 ± 7a
Pentane (control)	8 ± 4b	6 ± 2c

[a]Means followed by same letter per sex are not significantly different, $P \leq 0.05$, Duncan's new multiple range test.
[b]7 replicates at 4 females each, 10 replicates at 5 males each.

In other scolytid species where both enantiomers are produced, optimal attraction results only when both enantiomers are present. For example, in the ambrosia beetle, *Gnathotrichus sulcatus*, the attractant pheromone sulcatol (6-methyl-5-hepten-2-ol) is produced as 65% *S*-(+) and 35% *R*-(–) (Byrne et al., 1974). The enantiomers are essentially inactive alone, but the naturally occurring and racemic mixtures elicit a synergistic response (Borden et al., 1976).

In *I. pini* the principal pheromone ipsdienol, 2-methyl-6-methylene-2,7-

TABLE 2. FIELD RESPONSE OF *Dendroctonus frontalis* TO TRAPS BAITED WITH ENANTIOMERS OF FRONTALIN (F) PLUS D-ALPHA-PINENE (α-P), SAM HOUSTON NATIONAL FOREST, TEXAS, JULY 1977

Treatment	$\bar{X}$ No. caught[a] (±SE)/replicate[b]	Sex ratio (F/M)
June 20 - July 1, 1977		
(±)F + α–P	12 ± 2a	0.96
(+)F + α–P	6 ± 1b	0.91
(–)F + α–P	11 ± 2a	0.98
Blank wingtrap	3 ± 1c	0.87
July 7, 1977		
(±)F + α–P	25 ± 3a	0.94
15% (+)F + 85% (–)F + α–P	26 ± 3a	0.95
Blank wingtrap	6 ± 1b	0.88

[a]Means followed by same letters within days are not significantly different, $P \leq 0.05$, Duncan's new multiple range test.
[b]22 replicates June 20 - July 1; 6 replicates July 7.

octadien-4-ol (Silverstein et al., 1966), is produced in a 65:35 ratio of the (+) and (−) enantiomers by the eastern beetle population (Stewart, 1975). In the field the beetles were optimally attracted to a mixture of the two enantiomers (Lanier et al., 1980). Response to the (+) enantiomer was greater than to the (−) enantiomer, but considerably less than to the mixture of the two. In contrast, although *D. frontalis* produces both enantiomers and was attracted to each alone, optimal attraction was attributed to (−)F. This would appear to be of adaptive significance to the beetle since (−)F is produced in considerably greater amounts than (+)F (i.e., 85:15). Where considerably less difference exists between the amounts of the enantiomers produced by a species, both enantiomers must be present for optimal response, even though one enantiomer may be more attractive than the other.

Supportive data for discrimination of the enantiomers by *D. frontalis* were obtained from antennal olfactory receptor cells. Single-cell data showed that both (+)F and (−)F elicited response from the same olfactory cell; however, the spike frequency elicited by (−)F was consistently greater than the frequency elicited by (+)F (Figure 1, Table 3). In addition, responses to stimuli containing (−)F adapted within 300–400 msec, while a prolonged tonic

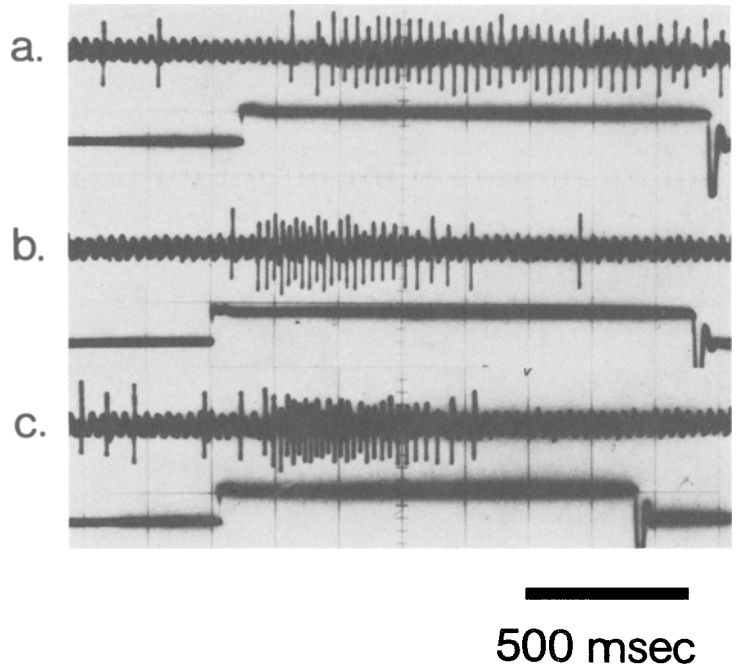

FIG. 1. Single-cell responses of a *D. frontalis* male to (+)F (a), (−)F (b), and (±)F (c). Displaced lower horizontal bar represents stimulus duration.

TABLE 3. SINGLE-CELL IMPULSES PER FIRST 300 MSEC TO 100 μg FRONTALIN ENANTIOMERS AND RACEMIC FRONTALIN

Replicate	Stimulus		
	(+)F	(−)F	(±)F
1	12	20	23
2	12	16	20
3	8	10	19

response was elicited by (+)F alone. The absence of significant differences in EAGs to (−)F and the mixtures of enantiomers adds further evidence that the (−) and (+) enantiomers stimulate the same olfactory cells.

The presence of separate acceptors for (−)F and (+)F was suggested by the fact that the frequency of impulses elicited by (+)F was consistently greater than to either (−)F or (+)F alone. If the enantiomers stimulated the same acceptors, with (+)F being less effective, response to (±)F intermediate to the response to (−)F and to (+)F would have been expected. The differential adaptation experiments provided further support for the presence acceptors specific for each enantiomer alone (Figure 2). Adaption to (±)F prevented response to (−)F as well as (+)F. The reverse was not true. (±)F elicited response, although small, from both (−)F- and (+)F-adapted antennae, indicating the presence of some acceptors specific for the (+) and the (−) forms, respectively. In addition, based upon response intensity, EAGs

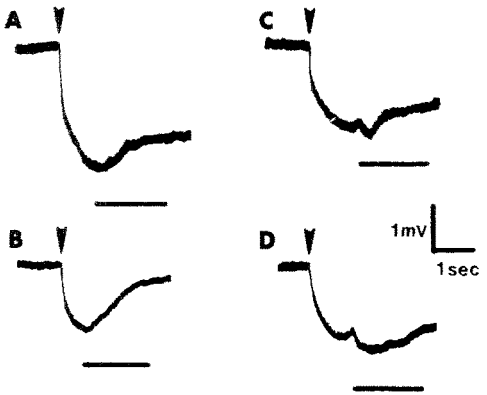

FIG. 2. Differential adaptation EAGs of a male *D. frontalis* antenna adapted to (±)F and exposed to (−)F (A) or (+)F (B) and an antenna adapted to (−)F (C) or (+)F (D) before exposure to (±)F. Arrow = onset of adapting material. Horizontal bar = second material.

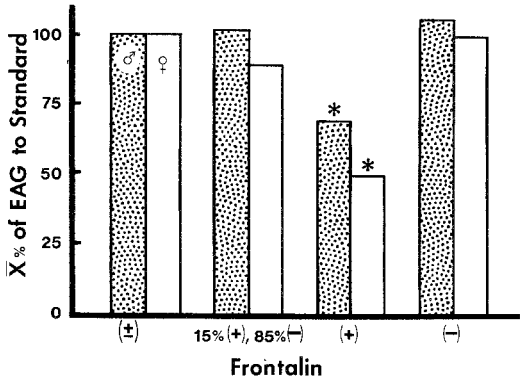

FIG. 3. Mean percent EAGs of *D. frontalis* to frontalin enantiomers: * = significantly different from all treatments/sex, 1-way ANOVA. Standard is frontalin.

indicated the presence of significantly more acceptors for (−)F than for (+)F (Figure 3).

These results support the hypothesis that enantiomer-bound information can be transferred to the insect via common receptor cells, as well as chiral specific acceptors. Borden et al. (1976) proposed a similar explanation for the interaction of enantiomers in *G. sulcatus*, although electrophysiological data were not available for the species. Chiral specificity has also been shown in *D. frontalis* at the single-cell level in the frequency of spikes elicited in response to enantiomers of a structurally related pheromone *exo*-brevicomin (*exo*-7-ethyl-5-methyl-6-,8-dioxabicyclo [3.2.1] octane) (Silverstein et al., 1968; Dickens and Payne, 1977).

We conclude that *D. frontalis* possesses chiral specificity at the antennal olfactory receptor and behavioral levels in its ability to respond to (+)F and (−)F. The level of response to the enantiomers appears directly correlated to the relative amounts in which they are produced. That is, both sexes were most responsive to (−)F, the predominant enantiomer, in field tests and at the receptor level. We have no explanation for the lack of correlation for the response of females in the laboratory bioassay.

The lack of a significant difference in the field response of *D. frontalis* to (±)F and the most active enantiomer, (−)F, is economically important from a pest-management standpoint. That is, the costs of potential frontalin-based suppression tactics (Richerson et al., 1980) would be significantly greater, and possibly prohibitive, if it were critical to have a single enantiomer synthesized.

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STRUCTURAL SIGNIFICANCE OF THE ALKYL SUBSTITUENT AT THE C-4 OF (+)-*trans*-VERBENYL ACETATE IN SEX PHEROMONAL ACTIVITY OF THE AMERICAN COCKROACH[1]

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Abstract—The significance of the alkyl group at the C-4 of (+)-*trans*-verbenyl acetate, which is the sex pheromone mimic of the American cockroach, was investigated. Seven alcohols possessing an ethyl, propyl, or dimethyl group at this position of the 6,6-dimethylbicyclo[3.1.1]heptane skeleton were synthesized and evaluated by behavioral assay. All of the alcohols were inactive, while three of four acetates of the 2α-alcohols induced sexual behavior in male cockroaches at the 0.02 or 0.5 mg dosage level, either of which is many orders of magnitude higher than the threshold level of the natural sex pheromones (10^{-8} mg). Among the acetates, the compounds with a methyl group or an α-oriented ethyl group at C-4 showed the highest activity. The results are discussed in terms of spatial requirements of the molecules for interactions with the receptor.

Key Words—Sex pheromone mimics, American cockroach, *Periplaneta americana*, [1*R*-(1α,2α,4α,5α)]-6,6-dimethyl-4-ethylbicyclo[3.1.1]heptan-2-yl acetate.

INTRODUCTION

Our discovery of the sex pheromone mimic of the American cockroach (*Periplaneta americana* L.), (+)-*trans*-verbenyl acetate (**1**) (Nishino et al., 1977), stimulated us to elucidate the structural requirements for the pheromonal activity, since **1** is structurally quite different from either periplanone A

[1]Studies on the sex pheromone mimic of the American cockroach, (+)-*trans*-verbenyl acetate. Part VIII. For Part VII, see *Comp. Biochem. Physiol.*, 70A: 229–234 (1981).
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or periplanone B, the natural sex pheromones of the animal (Talman et al., 1978; Persoons et al., 1979; Adams et al., 1979).

For this purpose, many analogs of **1** were synthesized (Nishino and Takayanagi, 1979a,b, 1980, 1981; Takayanagi and Nishino, 1980). Several important structural factors for pheromonal activity were previously elucidated from studies of the structure–activity relationships (Nishino and Takayanagi, 1981). The size of the alkyl group of the α-oriented ester group at C-2 was revealed to be an important factor together with the carbonyl oxygen atom of the ester group, which acts as an acceptor of a proton from the receptor site. The methyl group at the C-4 of **1** was also concluded to be a critical factor for spatial orientations of the molecule into the receptor space.

Since the size of the alkyl group at the C-4 was assumed to influence the pheromonal activity, novel analogs with elongated or multisubstituted alkyl moieties at this position were synthesized and bioassayed. As the stereostructures of the molecules were anticipated to be essential for interpreting interactions of the molecules with the receptor, conformational analysis for the original alcohols of the active acetates was carried out using the PMR method previously employed (Nishino and Takayanagi, 1979b,c, 1980).

METHODS AND MATERIALS

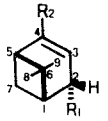
Instrumentation

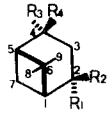
Specific rotations were measured in benzene at 25° C with an Union Automatic Polarimeter PM-201. Infrared (IR) spectra were recorded on a JASCO IRA-1 as liquid film (liquid sample) or KBr pellet (crystal). Gas chromatographic analysis (GC) was carried out with a Shimadzu 4BM-PF using a 2-m × 3-mm column packed with 3% OV-225 at 110° C flowing N_2 gas (40 ml/min.). Proton magnetic resonance (PMR) spectra were taken in deuterochloroform ($CDCl_3$) containing a little TMS on a Hitachi R-24 (60 MHz). Chemical shifts are expressed in ppm from the TMS peak, and coupling constants (J) in Hz. Low resolution mass spectra (MS) were taken at 70 eV on a Shimadzu GCMS-7000 with the same column used in GC analysis.

Synthesis

Structures of the target analogs are shown in Figure 1, together with several analogs previously synthesized, and synthetic schemes are shown in A, B, and C of Figure 2. Purity of the analogs was estimated to be more than 98% by GC analysis.

Oxidation of (+)-trans-*Verbenyl Acetate* (***1***) *with Selenium Dioxide.* A mixture of **1** ($[\alpha]_D$ +147°, Nishino and Takayanagi, 1979a) (3.0 g, 15 mmol) and selenium dioxide (3.0 g, 27 mmol) in dioxane (10 ml) was stirred at 80° C





1: R_1=OCOCH$_3$, R_2=CH$_3$
2: R_1=OCOH, R_2=CH$_3$
3: R_1=OCOCH$_2$CH$_3$, R_2=CH$_3$
4: R_1=OCOCH$_2$CH$_2$CH$_3$, R_2=CH$_3$
5a: R_1=OH, R_2=CH$_2$CH$_3$
5b: R_1=OCOCH$_3$, R_2=CH$_2$CH$_3$

6a: R_1=OH, R_2=R_4=H, R_3=CH$_3$
6b: R_1=OCOCH$_3$, R_2=R_4=H, R_3=CH$_3$
7a: R_1=OH, R_2=R_4=H, R_3=CH$_2$CH$_3$
7b: R_1=OCOCH$_3$, R_2=R_4=H, R_3=CH$_2$CH$_3$
8: R_1=R_4=H, R_2=OH, R_3=CH$_2$CH$_3$
9a: R_1=OH, R_2=R_4=H, R_3=CH$_2$CH$_2$CH$_3$
9b: R_1=OCOCH$_3$, R_2=R_4=H, R_3=CH$_2$CH$_2$CH$_3$
10: R_1=R_4=H, R_2=OH, R_3=CH$_2$CH$_2$CH$_3$
11a: R_1=OH, R_2=H, R_3=R_4=CH$_3$
11b: R_1=OCOCH$_3$, R_2=H, R_3=R_4=CH$_3$
12: R_1=H, R_2=OH, R_3=R_4=CH$_3$

FIG. 1. Structures of (+)-*trans*-verbenyl acetate and its analogs. Synthesis methods of **1–4**, **6a**, and **6b** and their activities were reported previously. All other analogs were synthesized and bioassayed in the present work.

for 4 hr. After the solvent was removed, the resulting residue was dissolved in ether (50 ml), and the insoluble material was filtered off and washed with ether. The combined filtrates were washed with saturated sodium bicarbonate solution, water, and brine; dried; and concentrated to give an oily residue. The distillate (83–86° C/0.07 mm Hg) of the residue was chromatographed over silica gel [*n*-hexane (C_6H_{14})-ethyl acetate (EtOAc), 8:1] and gave a mixture of **13** and its epimer (10:1). Recrystalization (from C_6H_{14}) of the mixture afforded pure **13** (1.7 g, 53% yield), mp 55.5–56° C; $[\alpha]_D$ + 218.4° (c = 0.5); IR: 1725, 1670, 1615, 1240 cm^{-1}; PMR: 0.85 [3H, singlet (s), CH_3(9)], 1.34 [1H, doublet (d), J = 9, H(7α)], 1.39 [3H, s, CH_3(8)], 2.07 (3H, s, OCOCH$_3$), 2.91 [1H, broad triplet (bt), J = 5, H(5)], 5.60 [1H, t, J = 3, H(2)], 6.58 [1H, multiplet (m), H(3)], 9.57 (1H, s, CHO); retention time (t_R, min) on GC: 5.3.

[*1*R-(*1*α,*2*α,*5*α)]-*6,6-Dimethyl-4-hydroxymethylbicyclo*[*3.1.1*]*hept-3-ene-2-yl Acetate* (**14**). After excess sodium borohydride was added dropwise to a solution of **13** (649 mg) in methanol (10 ml) at 0° C, the reaction was continued at 0° C for 2 hr. The solvent was removed, and the residue was dissolved in ether and washed with water and brine. The ether layer was concentrated to give an oil. Silica gel chromatography of the oil (C_6H_{14}–EtOAc, 4:1) afforded **14** (647 mg, quantitative), $[\alpha]_D$+159.6° (c = 1.0); IR: 3400, 1725 cm^{-1}; PMR: 0.91 [3H, s, CH_3(9)], 1.34 [3H, s, CH_3(8)], 2.01 (3H, s, OCOCH$_3$), 4.05 [2H, broad singlet (bs), C$\underline{H_2}$OH], 5.40 [1H, m, H(2)], 5.55 [1H, m, H(3)]; MS: *m/e*

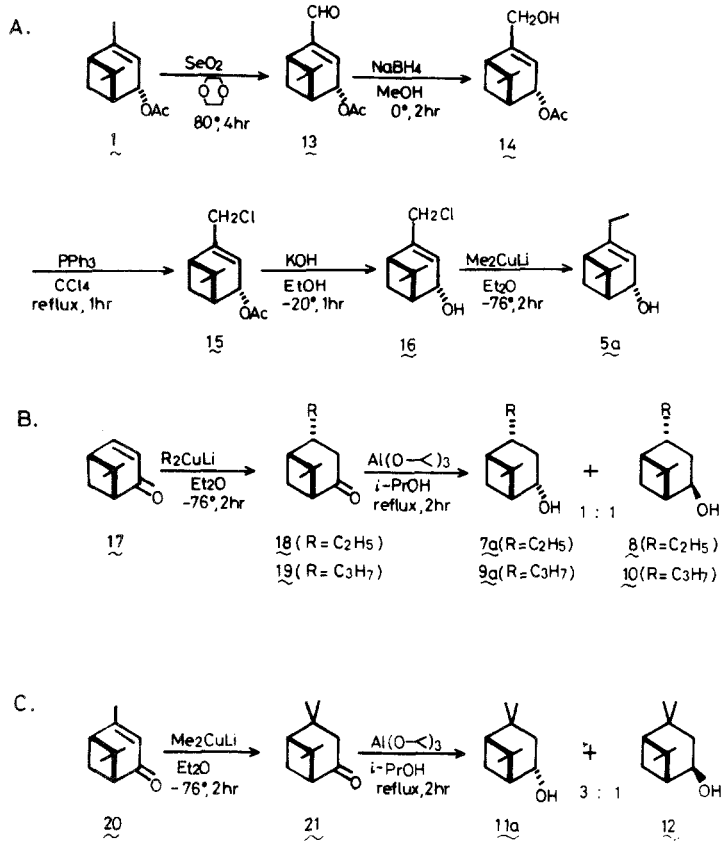


FIG. 2. Synthetic routes to the targeted alcohols.

179 (M^+-CH_2OH), 168, 150 (M^+-CH_3CO_2H), 135, 119 [base peak (B^+)], 107, 91; t_R: 10.2.

[*1*R-(*1α,2α,5α*)]-*6,6-Dimethyl-4-chloromethylbicyclo*[*3.1.1*]*hept-3-ene-2-yl Acetate* (**15**). A mixture of **14** (573 mg, 2.7 mmol) and triphenyl phosphine (968 mg, 3.7 mmol) in carbon tetrachloride (2 ml) was refluxed for 1 hr with stirring. Termination of the reaction was determined by TLC analysis. The reaction mixture was diluted with ether. The resulting precipitate was filtered off and washed with ether. The combined filtrates were washed and concentrated. The residual oil was chromatographed over silica gel (C_6H_{14}–EtOAc, 7:1) to obtain chloride (**15**) (523 mg, 85%), $[\alpha]_D$ +175.1° (c = 1.0); IR: 1740, 1255 cm^{-1}; PMR: 0.91 [3H, s, CH_3(9)], 1.35 [3H, s, CH_3(8)], 2.00 (3H, s, $OCOCH_3$), 3.97 (2H, s, CH_2Cl), 5.38 [1H, m, H(2)], 5.65 [1H, m, H(3)]; MS: m/e 168 and 170 (M^+-CH_3CO_2H), 153, 133 (160 and 170-Cl), 119 (B^+), 107, 91; t_R: 26.9

Hydrolysis of **15**. Compound **15** (452 mg) was hydrolyzed in a solution of potassium hydroxide (200 mg) in ethanol (2 ml) (−20° C, 1 hr) to give **16** (347 mg, quantitative), mp 38–38.5° C; $[\alpha]_D$+53.8° (c = 1.0); IR: 3320, 1650 cm^{-1}; PMR: 0.87 [3H, s, CH_3(9)], 1.37 [3H, s, CH_3(8)], 3.99 (2H, s, CH_2Cl), 4.32 [1H, m, H(2)], 5.69 [1H, m, H(3)].

[*1* R-(1α,2α,5α)]-*6,6-Dimethyl-4-ethylbicyclo*[*3.1.1*]*hept-3-ene-2-ol* (**5a**). To a stirred solution of lithium dimethylcuprate [prepared from a suspension of cuprous iodide (570 mg, 3.0 mmol) in ether (5 ml) and a solution (12 ml) of 0.5 M methyllithium in ether], a mixture of **16** (186 mg, 1 mmol) and ether (2 ml) was added at −76° C under argon gas atmosphere. The mixture was further stirred for 2 hr without the cooling bath and poured into saturated ammonium chloride solution. The precipitate was filtered off and washed with ether repeatedly. The combined ether layer was concentrated to give a residue. Silica gel chromatography of the residue (C_6H_{14}–EtOAc, 5:1) gave **5a** (147 mg, 89%); $[\alpha]_D$+101.0° (c = 1.0); IR: 3320, 1650 cm^{-1}; PMR: 0.84 [3H, s, CH_3(9)], 0.95 [3H, t, J = 7, $CH_2C\underline{H}_3$], 1.32 [3H, s, CH_3(8)], 4.28 [1H, m, H(2)], 5.30 [1H, m, H(3)]; MS: m/e 166 (M^+, $C_{11}H_{18}O$), 151 (M^+-CH_3), 148 (M^+-H_2O), 137, 123, 119 (B^+), 108, 105, 94, 91; t_R: 10.5.

Alkylation of (+)-*Apoverbenone* (**17**). An ether solution of lithium diethylcuprate [prepared from 1.2 M ether solution (22 ml, 26.5 mmol) of ethyllithium, cuprous iodide (2.5 g), and ether (10 ml)] was added to an ether solution (10 ml) of **17** ($[\alpha]_D$+283.2°, 1.2 g, 7.4 mmol) (Nishino and Takayanagi, 1979a) at −76° C in argon gas. The following procedures were similar to those for obtaining **5a**. Purification of the final ether extract by silica gel chromatography (C_6H_{14}–EtOAc, 10:1) afforded **18** (1.1 g, 89%). For **19**, a similar method was employed using lithium dipropylcuprate as the starting material. **18**, $[\alpha]_D$+18.0° (c = 0.5); IR: 1705 cm^{-1}; PMR: 0.81 [3H, s, CH_3(9)], 0.91 (3H, t, J = 7, $CH_2C\underline{H}_3$), 1.31 [3H, s, CH_3(8)]; MS; *m/e* 166 (M^+, $C_{11}H_{18}O$), 151 (M^+-CH_3), 137 (M^+-C_2H_5), 123, 109, 95, 83(B^+); t_R: 9.7. **19**, $[\alpha]_D$+20.7° (c = 1.0); IR: 1705 cm^{-1}; PMR: 0.84 [3H, s, CH_3(9)], 0.93 (3H, t, J = 5.5, $CH_2CH_2C\underline{H}_3$), 1.33 [3H, s, CH_3(8)]; MS: *m/e* 180 (M^+, $C_{12}H_{20}O$), 165 (M^+-CH_3), 151 (M^+-C_2H_5), 137 (M^+-C_3H_7), 122 (137-CH_3), 109, 95, 83(B^+); t_R: 15.2.

[*1* R-(*1α,2α,4α,5α*)]-*6,6-Dimethyl-4-ethylbicyclo*[*3.1.1*]*heptan-2-ol* (*7a*) *and Its 2β Isomer* (**8**). By the usual Meerwein-Ponndorf reduction [aluminum foil (250 mg), isopropanol (7 ml) and mercuric chloride (10 mg)], **18** (1.0 g) gave a mixture of **7a** and **8**. Silica gel chromatography (C_6H_{14}–EtOAc, 5:1) of the mixture yielded **7a** (430 mg) and **8** (444 mg) separately. **7a**, mp 46.5° C; $[\alpha]_D$ + 6.5° (c = 0.5); IR: 3280 cm^{-1}; PMR: 0.76 [3H, s, CH_3(9)], 0.83 (3H, t, J = 7, $CH_2C\underline{H}_3$), 1.22 [3H, s, CH_3(8)], 4.09 [1H, bt, J = 7, H(2)]; MS: *m/e* 153 (M^+-CH_3), 150 (M^+-H_2O), 139 (M^+-C_2H_5), 135, 121, 107, 85(B^+); t_R: 8.3. **8** mp 68° C; $[\alpha]_D$−27.9° (c = 0.4); IR: 3240 cm^{-1}; PMR: 0.94 (3H, t, J = 6, $CH_2C\underline{H}_3$), 1.03 [3H, s, CH_3(9)], 1.20 [3H, s, CH_3(8)], 4.22 [1H, m, H(2)]; MS:

m/e 168 (M^+, $C_{11}H_{20}O$), 153 (M^+-CH_3), 139 (M^+-C_2H_5), 135, 121, 111, 107, 97, 85(B^+); t_R: 7.1.

[*1R*-(*1α,2α,4α,5α*)]-*6,6-Dimethyl-4-propylbicyclo*[*3.1.1*]*heptan-2-ol* (**9a**) *and Its 2β Isomer* (**10**). A procedure similar to the above was performed on propylketone **19** (1.1 g) to give **9a** (435 mg) and **10** (440 mg). **9a**, mp 56–56.5° C; $[\alpha]_D$+2.3° (*c* = 0.9); IR: 3240 cm^{-1}; PMR: 0.79 [3H, s, CH_3(9)], 0.87 (3H, t, *J* = 6.5), 1.23 [3H, s, CH_3(8)], 4.14 [1H, m, H(2)]; MS: *m/e* 167 (M^+-CH_3), 164 (M^+-H_2O), 139 (M^+-C_3H_7), 125, 121, 111, 95, 85(B^+); t_R: 13.3. **10**, $[\alpha]_D$−14.7° (*c* = 1.0); IR: 3240 cm^{-1}; PMR: 0.87 (3H, t, *J* = 5.5, CH_2CH_2-$C\underline{H}_3$), 1.08 [3H, s, CH_3(9)], 1.40 [3H, s, CH_3(8)], 4.23 [1H, m, H(2)]; MS: *m/e* 182 (M^+, $C_{12}H_{22}O$), 167 (M^+-CH_3), 164 (M^+-H_2O), 139 (M^+-C_3H_7), 121, 111, 95, 85(B^+); t_R: 11.1.

Methylation of (+)-*Verbenone* (**20**). (+)-Verbenone (**20**) ($[\alpha]_D$+236.8°; Nishino and Takayanagi, 1979a) afforded dimethylketone (**21**) after treatment with lithium dimethylcuprate according to the method described for obtaining **5a**. **21**, $[\alpha]_D$+35.6° (*c* = 1.0); IR: 1705 cm^{-1}; PMR: singlet signals at 1.02, 1.08, 1.19, 1.37 (3H each, 4 *tert*-CH_3); MS: *m/e* 166 (M^+, $C_{11}H_{18}O$), 151 (M^+-15), 83(B^+); t_R: 10.2.

[*1R*-(*1α,2α,5α*)]-*4,4,6,6-Tetramethylbicyclo*[*3.1.1*]*heptan-2-ol* (**11a**) *and Its 2β Isomer* (**12**). Meerwein-Ponndorf reduction of dimethylketone (**21**) (900 mg) gave a mixture of **11a** and **12**. Silica gel chromatography of the mixture (C_6H_{14}–EtOAc, 5:1) afforded each isomer separately (**11a**:**12** = 590 mg: 198 mg = 3:1). **11a**, mp 91.5–92.5° C; $[\alpha]_D$+9.1° (*c* = 0.4); IR: 3280 cm^{-1}; PMR: singlet signals at 0.98, 1.00, and 1.26 (3H, 6H, and 3H, respectively, 4 *tert*-CH_3), 4.23 [1H, m, H(2)]; MS: *m/e* 153 (M^+-CH_3), 135 (153-H_2O), 125, 124, 111, 109, 107, 85(B^+); t_R: 6.6. **12**, mp 75.5–76° C; $[\alpha]_D$−8.0° (*c* = 0.5); IR: 3300 cm^{-1}; PMR: singlet signals at 0.96, 1.08, 1.21, 1.27 (3H each, 4*tert*-CH_3), 4.19 [1H, m, H(2)]; MS: *m/e* 153 (M^+-CH_3), 135 (153-H_2O), 124, 109, 107, 97, 85(B^+); t_R: 5.9.

Acetates. Only the alcohols possessing a 2α-hydroxyl group (**5a**, **7a**, **9a**, and **11a**) were converted to the corresponding acetates (**5b**, **7b**, **9b**, and **11b**) with acetic anhydride in pyridine, since several similar types of 2α-acetates were active, but all 2β-acetates were found to be inactive in previous work (Nishino and Takayanagi, 1981). **5b**, $[\alpha]_D$+120.5° (*c* = 0.4); IR: 1725, 1240 cm^{-1}; PMR: 0.90 [3H, s, CH_3(9)], 0.98 (3H, t, *J* = 7, $CH_2C\underline{H}_3$), 1.35 [3H, s, CH_3(8)], 2.01 (3H, s, $OCOCH_3$), 5.31 [2H, m, H(2) and H(3)]; MS: *m/e* 148 (M^+-CH_3CO_2H), 133, 119(B^+), 105, 91; t_R: 8.2.

7b, $[\alpha]_D$+11.1° (*c* = 0.5); IR: 1730, 1250 cm^{-1}; PMR: 0.84 [3H, s, CH_3(9)], 0.96 (3H, t, *J* = 7, CH_2CH_3), 1.24 [3H, s, CH_3(8)], 1.96 (3H, s, $OCOCH_3$), 5.13 [1H, bt, *J* = 7, H(2)]; MS: *m/e* 210 (M^+, $C_{13}H_{22}O_2$), 150 (M^+-CH_3CO_2H), 121(B^+); t_R: 9.2.

9b, $[\alpha]_D$+10.8° (*c* = 1.0); IR: 1730, 1250 cm^{-1}; PMR: 0.83 [3H, s, CH_3(9)], 1.23 [3H, s, CH_3(8)], 1.96 (3H, s, $OCOCH_3$), 5.12 [1H, bt, *J* = 7.5,

H(2)]; MS: *m/e* 181 (M^+-C_3H_7), 164 (M^+-CH_3CO_2H), 149 (164-CH_3), 135, 121(B^+), 107, 93, 85; t_R: 14.3.

11b $[\alpha]_D$+8.0° (*c* = 0.5); IR: 1720, 1245 cm^{-1}; PMR: 1.03 (9H, s, 3 *tert*-CH_3), 1.26 (3H, s, 1 *tert*-CH_3), 1.99 (3H, s, $OCOCH_3$), 5.20 [1H, m, H(2)]; MS: *m/e* 150 (M^+-CH_3CO_2H), 135 (150-CH_3), 107(B^+), 85; t_R: 7.6.

Behavioral Assay

The assay method, including sample preparation, followed Nishino and Takayanagi (1981). One milligram of the target alcohol was assayed first, and then the acetates at the same dosage. When typical sexual display of males (Roth and Willis, 1952) was observed at the 1-mg level of a compound, 0.5, 0.1, 0.05, and 0.02 mg of the compound were prepared and bioassayed in this order. For the behavioral assay, a group of 25 males isolated from females for at least 1 month was employed. The number of individuals displaying sexual behavior was counted within 3 min.

Conformational Analysis

The method involves the measurement of coupling constants from PMR spectra taken after addition of the chemical shift reagent Eu(dpm)$_3$ (Nishino and Takayanagi, 1979c). A solution of 0.1 mmol of an alcohol having an α-hydroxyl group at C-2 (**7a**, **9a**, or **11a**) in 0.3 ml of $CDCl_3$ containing a little TMS was made up in a 4-mm measuring tube. To the solution, 0.04 mmol of the shift reagent were added and spectra were taken. When no distinct separation of the proton signals was obtained, 0.01 mmol portions of the reagent were added successively. In **7a** and **9a**, the addition of 0.06 mmol of the reagent was sufficient for separation of the signals, whereas 0.05 mmol was sufficient in **11a**. After coupling constants were measured for the well-separated signals, the constants were transformed to dihedral angles through the Tori equations, $J = 12.4 \times \cos^2\theta$ ($0° \leqq \theta \leqq 90°$) and $14.3 \times \cos^2\theta$ ($90° \leqq \theta \leqq 180°$) (Kuriyama et al., 1963). Conformational inspection was carried out using the Dreiding model.

RESULTS

Assay Results

In the behavioral assay, all of the alcohols and the ester possessing a propyl group (**9b**) were inactive to males at the dosage of 1 mg. Among the active acetates, **7b** induced activity at 0.02 mg dosage, while **5b** and **11b** were active at 0.5 mg as shown in Table 1, and these are compared with the activity of the previously found mimics (**1**–**3** and **6b**) (Nishino and Takayanagi, 1981).

The cockroach responses induced by 0.5 mg of **5b** and **11b** were quite similar to that of the weak mimic, (+)-*trans*-verbenyl formate (**2**) (Nishino and

TABLE 1. COMPARISON OF SEX PHEROMONAL ACTIVITY OF PHEROMONE MIMICS IN THE AMERICAN COCKROACH

Mimic	Amount (mg)	Number of repetitions	Activity[a]
(+)-*trans*-Verbenyl acetate (**1**)[b]	0.02	20	9 ± 3
(+)-*trans*-Verbenyl formate (**2**)[b]	0.5	8	5 ± 3
(+)-*trans*-Verbenyl propionate (**3**)[b]	0.02	20	15 ± 4
(+)-Verbanyl acetate (**6b**)[b]	0.02	20	15 ± 6
5b	0.5	10	6 ± 2
7b	0.02	20	11 ± 4
11b	0.5	10	5 ± 3

[a]Average number of cockroaches showing typical sexual display within 3 min in a group containing 25 males ± SD.
[b]The data shown here were reported in Nishino and Takayanagi (1981).

Takayanagi, 1981). However, the induction period for sexual response was somewhat longer (1.5–2 min) as compared with the induction period (30–45 sec) observed for 0.02 mg of **7b**. In **5b** and **11b**, the sexual behavior persisted for about 1 min, but lasted for at least 3 min with **7b**, after elimination of the sample from the testing cage.

Conformations

As shown in Table 2, protons on the 6,6-dimethylbicyclo[3.1.1]-heptane ring resonated with different chemical shift values, exhibiting resolved

TABLE 2. CHEMICAL SHIFTS AND SPLITTING PATTERNS OF DIFFERENT PROTONS OF SATURATED ALCOHOLS AFTER ADDITION OF $Eu(dpm)_3$ (IN $CDCl_3$, AT 60 MHz)

Alcohol	Relative ratio alcohol/$Eu(dmp)_3$	Protons,[a] chemical shifts (ppm), and splitting patterns[b]						
7a	1/0.6	H(2)	H(3α)	H(1)	H(3β)	H(7α)	H(7β)	H(5)
		17.07	10.72	10.03	8.23	8.22	5.61	4.28
		m	ddd	ddd	ddd	d	ddd	m
9a	1/0.6	H(2)	H(3α)	H(1)	H(3β)	H(7α)	H(7β)	H(5)
		17.95	11.22	10.43	8.55	8.54	5.78	4.34
		m	ddd	ddd	ddd	d	ddd	ddd
11a	1/0.5	H(2)	H(3α)	H(1)	H(7α)	H(3β)	H(7β)	H(5)
		11.75	6.97	6.54	5.83	5.03	4.25	3.12
		m	dd	ddd	d	dd	ddd	dd

[a]For numbering, see Figure 1.
[b]d = doublet, dd = double doublet, ddd = double double doublet, m = multiplet.

TABLE 3. COUPLING CONSTANTS (J) BETWEEN PROTONS OF ALCOHOLS

Alcohol	$J_{1,2\beta}$	$J_{1,5}$	$J_{1,7\beta}$	$J_{2\beta,3\alpha}$	$J_{2\beta,3\beta}$	$J_{3\alpha,3\beta}$	$J_{3\alpha,4\beta}$	$J_{3\beta,4\beta}$	$J_{4\beta,5}$	$J_{5,7\beta}$	$J_{7\alpha,7\beta}$
(+)-*trans*-Verbenol[a]	3.0	5.1	5.2	$J_{2,3}$ = 3.0						5.2	8.7
5a	3.0	5.0	5.0	$J_{2,3}$ = 3.0						5.0	8.5
6a[a]	1.2	5.0	5.0	6.8	7.7	15.5	8.4	7.7	0.6	5.0	9.5
7a	1.5	5.0	5.0	6.5	8.0	15.0	8.5	8.0	?[b]	6.0	10.5
9a	1.5	5.0	5.0	6.5	8.0	15.0	8.0	8.0	?[b]	5.0	10.5
11a	2.5	5.0	5.0	3.5	9.0	16.0				6.0	10.5

[a]The data were obtained with a Varian Associates FT-80A Fourier transform NMR system equipped with a 32K computer memory (Nishino and Takayanagi, 1979c).
[b]The J value was impossible to read.

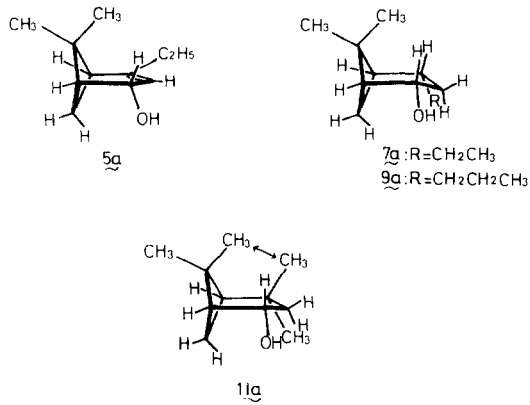


FIG. 3. Conformation of alcohols. Alcohol **5a** takes the rigid form, Y-shaped conformation, while **7a** and **9a** the bridged-chair conformation. Spatial interaction between 9- and 4β-methyls causes the half bridged-chair conformation to **11a**.

splitting patterns in the PMR spectra after addition of 0.5 or 0.6 molar ratio of $Eu(dpm)_3$ to the substrate alcohol. Coupling constants of the signals could be obtained from the splitting peaks (Table 3). The conformations of the alcohols are illustrated, as shown in Figure 3 with the Dreiding model, by transformation of the coupling constants to dihedral angles using the Tori equations.

Compounds possessing a double bond in the C-1–C-5 fragment such as **5a**, take the so-called "Y-shaped" conformation (Nishino and Takayanagi, 1979c). The bridged-chair conformation is assigned to **7a** and **9a**. In this conformation, the cyclohexane ring, which is made up of the C-1 to C-6 moiety, takes a chair form (C-7 is regarded as a bridging group). Dimethyl alcohol **11a** is presented as a half bridged-chair conformation which may be formed by the repulsion between 9- and 4β-methyls.

Since the acetate group at C-2 and the methyl group at C-4 have been revealed to be necessary for sex pheromonal activity in the verbenyl acetate mimics (Nishino and Takayanagi, 1981), locations of these two groups in the receptor space may be a key point in interpreting interactions with active sites of the receptor. To understand this, the Newman projections on C-1–C-2 (A) and C-5–C-4 (B) of the three types (I, II, and III) of conformation are shown in Figure 4.

In the Y-shaped compounds (I, *trans*-verbenol and **5a**), the hydroxyl group inclines at 60° below a plane made up of C-1, C-2, C-4, C-5, and the alkyl group (R) deviates from C-4 on the same level as the plane. In the bridged-chair conformation (II) such as **6a**, **7a**, and **9a**, the hydroxyl group shifts up to the plane (48°), while the R group shifts down at 43° from the plane as compared with the Y-shaped compounds. The hydroxyl group in the

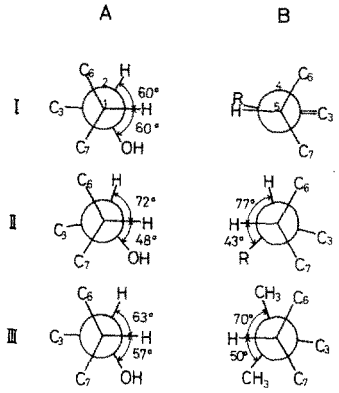


FIG. 4. Newman projections on C_1–C_2 (A) and C_5–C_4 (B) of alcohols and dihedral angles. I, Y-shaped alcohols (*trans*-verbenol and **5a**); II, alcohols with bridged-chair conformation (**6a**, **7a**, and **9a**); alcohol with half bridged-chair conformation (**11a**).

half bridged-chair conformation (III, **11a**) takes a similar position to that of the Y-shaped compounds (57°), but the α-methyl group of **11a** inclines at angle of 50° from the plane (B-III), which is slightly more than in the case of the bridged-chair conformation (43°). These conformational data must be considered in any discussion of the activities of the corresponding acetates.

DISCUSSION

The threshold dosage level of the present active acetates was categorized into two levels, 0.02 mg (**7b**) and 0.5 mg (**5b** and **11b**), as seen in the previous work (Nishino and Takayanagi, 1981). Both threshold response levels are many orders of magnitude higher than those of the natural sex pheromones of the cockroach (10^{-8} mg) (Persoons, 1977). In interpreting this tremendous discrepancy of the activity between the mimics and the natural pheromones, we have already formulated some hypotheses (Nishino and Takayanagi, 1981): (1) male cockroaches possess a special receptor responsible for the mimics, which is different from the sex pheromone receptor; or (2) both mimics and pheromones are perceived by the sex pheromone receptor, but only a few of the receptor sites are able to accommodate the mimics, whereas all of the sites are able to accommodate the natural pheromones.

The elucidation of common structural factors intrinsic to the pheromone response existing between the natural pheromones and their mimics represents an important approach in considering either hypothesis.

The presence of an α-oriented ester group at C-2 was found to be an important factor, especially the carbonyl oxygen atom of the group in the role

of electron donor in the interaction with the receptor. Furthermore, the optimal size [CH_3 (**1**) and CH_2CH_3 (**3**)] of the alkyl group in the ester moiety appeared to fit in the receptor space, since the butyrate (**4**) was inactive owing to its excessive bulkiness for the space. The methyl group at C-4 was also revealed to be a structural requirement which may function in spatial orientations for fitting the molecule into the receptor. The size of this important 4-alkyl group was also expected to determine the bulkiness of the molecule for the receptor space.

As revealed from the bioassay results (Table 1), the methyl analogs were more active than the ethyl ones in the same type of conformation (**1** > **5b** and **6b** ≥ **7b**), but propyl (**9b**) showed no activity. This implies that methyl and ethyl groups were allowed into the receptor space and consequently induced the activity, while the propyl group was too bulky. For the above discussion, the direct comparison among **6b**, **7b** and **9b** was consistent, because the similar bridged-chair conformation was assigned to these acetates (see almost identical coupling constants in the three alcohols, **6a**, **7a**, and **9a**).

The molecular conformation obviously affects the pheromonal activity. The previous work (Nishino and Takayanagi, 1981) indicated that the bridged-chair form was preferred to the Y-shaped form in causing more potent activity (compare **6b** with **1** in Table 1). This was supported in the present work in which **7b** (bridged-chair) was stronger than **5b** (Y-shape). In this case, the conformational change from the bridged-chair to Y-shaped seems to cause an undesirable shift of the carbonyl oxygen of the ester group from the optimal position for binding to an active site in the receptor. Thus, as seen in Figure 4, the original hydroxyl group of the ester moiety apparently shifts [48° (bridged-chair) → 60° (Y-shaped)]. Similarly, this conformational change influences the location of the alkyl group. As seen in B-II and I in Figure 4, the R group shifts 43° (bridged-chair) to 0° (Y-shaped).

The dimethyl acetate **11b**, which corresponds to compound **6b** with a 4β-methyl group, afforded rather weak activity at the 0.5-mg threshold level. The original alcohol (**11a**) of **11b** was found to take a half bridged-chair conformation (Figure 3), which may result from satisfying the spatial congestion between 9- and 4β-methyl groups.

The large difference of the activity between **6b** and **11b** (Table 1) is possibly due to spatial bulkiness of the newly generated 4β-methyl group and/or the conformational change induced. Both the hydroxyl and alkyl groups in **11a** shift slightly from those of the original alcohol of the more active acetate **6b** (II and III in Figure 4), so that the position of the carbonyl oxygen of the acetoxyl group of **11b** is slightly deviated from the position of **6b**, which is the optimal orientation in the receptor space. Nevertheless, the conformational change between **6b** and **11b** is somewhat smaller than that between **6b** and **1** (see Figure 4). Thus, it may be possible to consider that the

obstructive newly generated 4β-methyl group denies a fit of **11b** into the receptor space.

In order to obtain more precise information for the receptor space, future investigations will be directed toward the synthesis and biological evaluations of the corresponding propionates of the active acetates.

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Book Review

Semiochemicals: Their Role in Pest Control. Donald A. Nordlund, Richard L. Jones, and W. Joe Lewis (eds.). New York, John Wiley & Sons, 1981. 306 pp.

Semiochemicals, as this book points out in a very clear and useful chapter on terminology, are "chemicals that mediate interactions between organisms." They include pheromones, allomones, kairomones, synomones, and apneumones.

Technological and scientific advances have allowed the identification and characterization of many semiochemicals. They are now the subject of much fascinating research on insect biology and have begun to take their place as pest-management tools. Of two recent books which include comprehensive discussion of all types of semiochemicals, this provides the more basic biological orientation. Several chapters make major contributions to the literature.

Chapter 3 by L. Schoonhoven on chemical mediators between plants and phytophagous insects is scholarly and comprehensive. It erects five theorems on chemical interaction between plants and insects and examines each critically, exposing anomalies that demand further research. Schoonhoven notes that host recognition does not depend on single compounds and that highly detailed images of a plant's chemical make up are the general rule.

A.P. Arthur (Chapter 6) provides an excellent section on the role of kairomones in host acceptance by parasitoids. Looking to the future, he relates the factors which influence host acceptance to the requirements of artificial diets for parasitoids.

The employment of kairomones in the management of parasitoids is well covered by H.R. Gross in a clear and well written Chapter 8, although it is limited almost solely to a discussion of *Trichogramma*. Gross explores the analysis and interpretation of experimental data and develops progressive seasonal strategies for the release of *Trichogramma*, supplemental hosts, and synthetic kairomones.

Two chapters stand out as exemplary. R.J. Prokopy (Chapter 10) introduces the reader to epideictic pheromones that "elicit dispersal from

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presently or potentially overcrowded food resources, and thereby act to partition intraspecfic foraging activities." He then takes the reader on a truly comprehensive excursion of epideictic pheromones in many insect taxa and indicates preceptively how they may be used in pest management. P.W. Price contributes an original, analytical, and scholarly chapter on semiochemicals in evolutionary time, with particularly good integration of text and figures. He provides an excellent discussion of local and geographic variation in semiochemicals as evidence for natural selection in different parts of a species range, and of the potential for development of resistence to semiochemicals used in pest management. Both Price and S.B. Vinson (Chapter 4) caution plant breeders to consider the parasitoids of the pests against which they are breeding for resistance, as the parasitoids may also rely on the host plant in host habitat finding.

There are other notable contributions. E.F. Knipling presents a well-reasoned plea in the Forward for adjustment of pest control practices to accommodate the use of semiochemicals, particularly on an area basis and at low population levels. The problems of registration of semiochemicals as pesticides are addressed by W.J. Lewis (Chapter 1) and W. Roelofs (Chapter 11). D.A. Nordlund (Chapter 2) suggests that for "multicomponent" pheromones, it would be wise to "think in terms of interactions mediated by several pheromones, each of which elicits a specific . . . .reaction, each part of the total interaction." Adoption of this one compound–one pheromone policy would bring terminology on semiochemicals in line with that in endocrinology, wherein several hormones often mediate a single phenomenon. R.D. Jackson and W.J. Lewis (Chapter 14) clearly summarize the various possible strategies and tactics for using semiochemicals in pest management. Reflecting a recurring theme throughout the book, they are optimistic, but sound a note of caution in reiterating the need for complete information on chemical communication systems before semiochemicals take their place in truly sophisticated pest-management systems.

In its organization the book suffers from its genesis as individual contributions to two separate symposia, rather than as a collection of papers put together for a predesigned book. Therefore, it fails to develop a logical sequence, and practically oriented chapters are interspersed with more basic ones. There is also considerable overlap, particularly in Chapter 12, which shares material with at least six other chapters. The book also overemphasizes parasitoids in relation to their current importance in the study of semiochemicals. Despite the mention of pest control in the title, the role of semiochemicals in pest management is given limited coverage, which is often too brief to be worthwhile to anyone but a novice in the field.

In general, the book is well edited and easy to read. However, some loose editing and reviewing has resulted in flaws such as: grammatical errors, misnomers (e.g., aggregation pheromones referred to as sex pheromones),

omission of recent data, use of major unpublished references, inclusion of statements, and even whole passages, unsupported by reference to the literature, lack of adequate cross-referencing between chapters, and occasional straying away from the subject of semiochemicals.

Researchers and teachers in insect ecology, behavior, or physiology, as well as in pest management, will want this book on their shelves for ready reference. It is unlikely that this book will be used as a text for any course, but it should be required reading for many graduate students. It is an essential item for any serious library that emphasizes basic or applied biology.

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INTRASPECIFIC PAIRING OF PLANARIA, *Dugesia tigrina* AND *Dugesia dorotocephala* (PLATYHELMINTHES: TURBELLARIA), AND OBSERVATIONS ON LIPOPHILIC EXCRETORY-SECRETORY WORM PRODUCTS

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Abstract—Intraspecific pairing of the planaria, *Dugesia tigrina* and *D. dorotocephala*, was studied for 24 hr at 22 ± 1°C in Petri dish cultures containing an agar substrate and a water overlay. Pairing of fed planaria in the light and dark and starved planaria in the light was studied, and worms in contact or within 5 mm of each other were considered paired. Fed planaria of both species paired significantly in the light and dark, whereas starved planaria did not pair. Our results suggest that worm-emitted chemical factors are involved in intraspecific pairing of planaria. Thin-layer chromatographic analysis was used to study the release of lipophilic worm products. Phospholipids, free sterols, and free fatty acids were detected in all trials, and triglycerides in most. Thin-layer densitometric analysis was used to quantitate the weight of free sterols released by planaria.

Key Words—*Dugesia tigrina, Dugesia dorotocephala*, Platyhelminthes, Turbellaria, planaria, intraspecific pairing, chemical attractants, lipids, excretory-secretory products.

INTRODUCTION

Recent studies have demonstrated intraspecific pairing in vitro of trematodes [*Leucochloridiomorpha constantiae* by Fried and Roberts (1972); *Echinostoma revolutum* by Fried et al. (1980); *Amblosoma suwaense* by Fried and Robinson (1981)] and of the nematode, *Nippostrongylus brasiliensis* (Roberts and Thorson, 1977a). In these studies pairing was defined as worms in contact or within 5 mm of each other in Petri dish cultures containing an agar

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substratum and a Locke's solution overlay. Several studies using thin-layer chromatography (TLC) have indicated that sterols released by worms are involved in chemical attraction in parasitic nematodes [*N. brasiliensis* by Roberts and Thorson (1977b)], and trematodes [*L. constantiae* by Fried and Gioscia (1976); *E. revolutum* by Fried et al. (1980); *A. suwaense* by Fried and Robinson (1981)].

Studies are not available on the intraspecific pairing of any planarian species. The present study is concerned with intraspecific pairing of *Dugesia tigrina* and *D. dorotocephala*, and also examines lipophilic excretory–secretory (E-S) products released by planaria into a nonnutrient medium. Pairing studies on planaria were completed using the model described in the aforementioned nematode and trematode studies. To determine the presence of lipophilic products, TLC analysis of worm-free E-S material from planaria was used.

METHODS AND MATERIALS

Dugesia tigrina and *D. dorotocephala* were obtained from commercial suppliers (Carolina Biological Co., Burlington, North Carolina, and Champlain Biological Co., Glen Gardner, New Jersey). Each species was maintained as a stock culture in separate 20-cm-diameter finger bowls (50–150 organisms per bowl), filled with 1000 ml of boiled and then cooled tap water. The tap water contained 0.2 parts per million (ppm) chlorine and 20–25 ppm sodium; the pH was 7.2–7.6. Following boiling and cooling of the water, the chlorine content was nil, and the pH was 6.8–7.0. Stock cultures were maintained at 22 ± 1°C under diffuse, overhead, fluorescent light for approximately 8 hr/day. Planaria were fed once a week on frozen and thawed chicken liver, and the water was changed after 2–4 hr of feeding. Starved planaria were maintained similarly except that they were not fed for 4–12 weeks before use in experiments. The length of fed planaria was 5–15 mm and that of starved planaria 3–7 mm.

Behavior experiments were made at 22 ± 1°C in disposable, sterile, plastic 5-cm-diameter Petri dishes containing 2 ml of an agar substratum, with a 5-ml overlay of boiled and then cooled tap water (Fried and Roberts, 1972). Each dish was placed on a sheet of white paper containing two marks, 2 cm apart, in the center of the dish. Dishes were then placed either under diffuse, overhead, fluorescent light, or in a dark room. Planaria were removed from stock cultures and placed individually head to head, two per dish. Observations were made 0.5, 1, 1.5, 2, 3, 4, and 24 hr later. Unlike trematodes, planaria move very actively when introduced into Petri dish cultures, and usually circle the dish apparently at random for considerable time. Based on previous helminth studies (Fried and Roberts, 1972; Roberts and Thorson, 1977a,b;

Fried et al., 1980), organisms within 5 mm of the lateral body margin of each other were considered paired, even if both organisms were moving. Moreover, a random model was used as the baseline for deciding when pairing was significant. This model assumed that planaria were 10 mm in length and 5 mm in width, were stationary, and within 5 mm of each other at the edge of the Petri dish. The probability of random pairing based on this model was 0.17. Statistical significance of pairing at each time period was determined using the two-sample Z test for proportions.

Intraspecific pairing was studied using *D. tigrina* and *D. dorotocephala.* Fed planaria were studied in light versus dark, whereas starved planaria were studied only in the light. To observe pairing in the dark, the door of the dark room was opened for approximately 15 sec to record data on 24 experimental dishes. All worms were live at the end of each experiment and were returned to stock cultures (Reynierse et al., 1969). No difference in pairing was observed in fresh worms versus those used in previous trials.

To determine if planaria produce lipophilic E-S products, the worm-free incubate of *D. tigrina* and *D. dorotocephala* was tested for neutral lipids. Worm-free incubate was obtained as follows. Within 2 hr or 4 days after feeding, 10 planaria were rinsed in three changes of 0.3% sterile Locke's (Paul, 1975) solution (30 ppm sodium to approximate the salinity of tap water). Ten worms were placed in each culture tube with 1 ml of Locke's in four separate trials for *D. tigrina* and two trials for *D. dorotocephala.* The tubes were then lightly capped, and allowed to stand at 22 ± 1°C for 4 or 24 hr. A Locke's control was maintained similarly for 24 hr. The worm-free incubate and Locke's control were removed and prepared for TLC as described below. All worms were live following incubation and were returned to stock cultures.

The experimental and control incubate fluids were extracted in chloroform–methanol (2:1) (Folch et al., 1957); the hydrophilic layer was discarded and the lipophilic layer dried under nitrogen, and then reconstituted with 100 μl of chloroform. TLC was performed on preadsorbent silica gel glass plates (LK6DF, 20 × 20 cm, Whatman Inc., Clifton, New Jersey). This plate has nineteen 9-mm channels containing a 250-μm layer of silica gel and a 3-cm preadsorbent zone. The detection reagent was impregnated in the plate prior to chromatography by predeveloping the plate in 5% phosphomolybdic acid (Wortmann and Touchstone, 1973) to the sorbent–preadsorbent interface. Each reconstituted sample was applied to a separate lane and, to ensure total application of the sample, each vial was rinsed with 100 μl of chloroform–methanol (2:1) and the rinse was applied to a separate lane. A neutral lipid standard (184A, Nu-Chek-Prep, Inc., Elysian, Minnesota, containing equal amounts of cholesterol, cholesterol oleate, triolein, oleic acid, and methyl oleate) was prepared in chloroform to a concentration of 20 ng/μl, and applications of 10, 20, 40, 60, 80, and 100 μl were made to separate preadsorbent zones using calibrated, disposable micropipets (Microcaps,

Drummond Scientific Co., Broomall, Pennsylvania). Chromatograms were developed 10 or 12 cm from the preadsorbent zone in 100 ml of petroleum ether–diethyl ether–acetic acid (80:20:1) (Mangold, 1969) in a glass rectangular tank (Chromaflex, Kontes Glass Co., Vineland, New Jersey), and the lipids were visualized by heating the chromatogram at 110°C for 5 min.

To quantitate the free sterol fraction, lipid bands were scanned in the lanes along the direction of development using a Kontes fiberoptic densitometer (K-49500) equipped with a Kontes baseline corrector and a strip-chart recorder (Linear Instruments Corp., Irvine, California). The plates were scanned using the visible light source and the reflectance and single-beam (reference head) modes. Chart speed was 6 cm/min, and the plate speed during scanning was 6 cm/min. The attenuation setting was 5. Peak areas were determined by multiplying peak height by peak width at half height. Calibration curves were obtained by plotting peak area (cm^2) for cholesterol standards on the ordinate and the weight on the abscissa. The weight of free sterols in the E-S products was obtained by interpolating the peak area, finding the weight, and dividing by the number of worms used.

RESULTS

In 24 trials which matched a single *D. tigrina* or *D. dorotocephala* 2 cm from an inert object or a frozen or boiled planarian for 2 hr, the percentage of pairing was 15%.

Paired planaria were usually stationary, and members of the pair were about 2 mm apart, and rarely in contact with each other. Each pair was on the surface of the agar, usually within 1 cm of the perimeter of the dish. Members

TABLE 1. INTRASPECIFIC PAIRING OF *Dugesia tigrina* AT 22 ± 1°C

Time (hr)	Fed worms in light $N = 48$[a] No. (%) paired	Starved worms in light $N = 48$ No. (%) paired	Fed worms in dark $N = 44$ No. (%) paired
0.5	20 (42)	5 (10)[b]	4 (9)[b]
1	21 (44)	7 (15)[b]	22 (50)
1.5	30 (63)	6 (13)[b]	25 (57)
2	31 (65)	8 (17)[b]	24 (55)
3	30 (63)	13 (27)	24 (55)
4	30 (63)	11 (23)[b]	31 (70)
24	26 (54)	8 (17)[b]	25 (57)

[a] N = number of trials.
[b] Pairing not significant ($P > 0.05$).

TABLE 2. INTRASPECIFIC PAIRING OF *Dugesia dorotocephala* AT 22 ± 1°C

Time (hr)	Fed worms in light $N = 47$[a] No. (%) paired	Starved worms in light $N = 44$ No. (%) paired	Fed worms in dark $N = 44$ No. (%) paired
0.5	10 (21)[b]	6 (14)[b]	15 (34)
1.0	14 (30)	5 (11)[b]	22 (50)
1.5	16 (34)	9 (20)[b]	26 (59)
2	19 (40)	7 (16)[b]	27 (61)
3	28 (60)	6 (14)[b]	17 (39)
4	26 (55)	9 (20)[b]	17 (39)
24	25 (53)	9 (20)[b]	17 (39)

[a] N = number of trials.
[b] Pairing not significant ($P > 0.05$).

of a pair were usually oriented head-to-head or head-to-tail either in parallel or approximately 60 degrees from each other.

In the light, fed *D. tigrina* paired more frequently ($P < 0.001$) than starved *D. tigrina* at all observations (Table 1).

In the dark, fed *D. tigrina* also paired more frequently ($P < 0.001$) than starved *D. tigrina* at all observations except at 0.5 hr. Fed *D. tigrina* paired more in the light ($P < 0.001$) at 0.5 hr than did fed *D. tigrina* in the dark, although at all other observations there was no significant difference ($P > 0.10$) in pairing of *D. tigrina* in the light versus dark (Table 1).

In the light, fed *D. dorotocephala* paired more frequently ($P < 0.05$) than starved *D. dorotocephala* except at 0.5 hr (Table 2).

Fed *D. dorotocephala* in the dark also paired more frequently ($P < 0.05$) than starved *D. dorotocephala* in the light. Fed *D. dorotocephala* paired more frequently in the dark than in the light during the first 2 hr (at 0.5 hr, $P < 0.10$; at 1 hr, $P < 0.05$; at 1.5 hr, $P < 0.10$; at 2 hr, $P < 0.05$). However, at 3–24 hr, fed *D. dorotocephala* paired more frequently in the light than in the dark (at 3 hr, $P < 0.05$; at 4 hr, $P < 0.10$; at 24 hr, $P < 0.10$) (Table 2).

Beyond 0.5 hr, fed *D. tigrina* and *D. dorotocephala* showed significant ($P < 0.05$) pairing in the light and dark (Tables 1 and 2).

TLC analyses showed that E-S products from *D. tigrina* incubated after feeding for 4 or 24 hr in 0.3% Locke's solution contained phospholipids (at the origin), free sterols ($R_f = 0.14$–0.18), and free fatty acids ($R_f = 0.37$–0.42) in three trials. In two of three trials triglycerides ($R_f = 0.45$–0.53) were also detected. In one trial, two bands that moved near the solvent front were seen. A representative chromatogram is shown in Figure 1. These two bands were also found in control Locke's incubate and were therefore not of worm origin. Worms incubated 4 days after feeding for 4 or 24 hr in 0.3% Locke's also

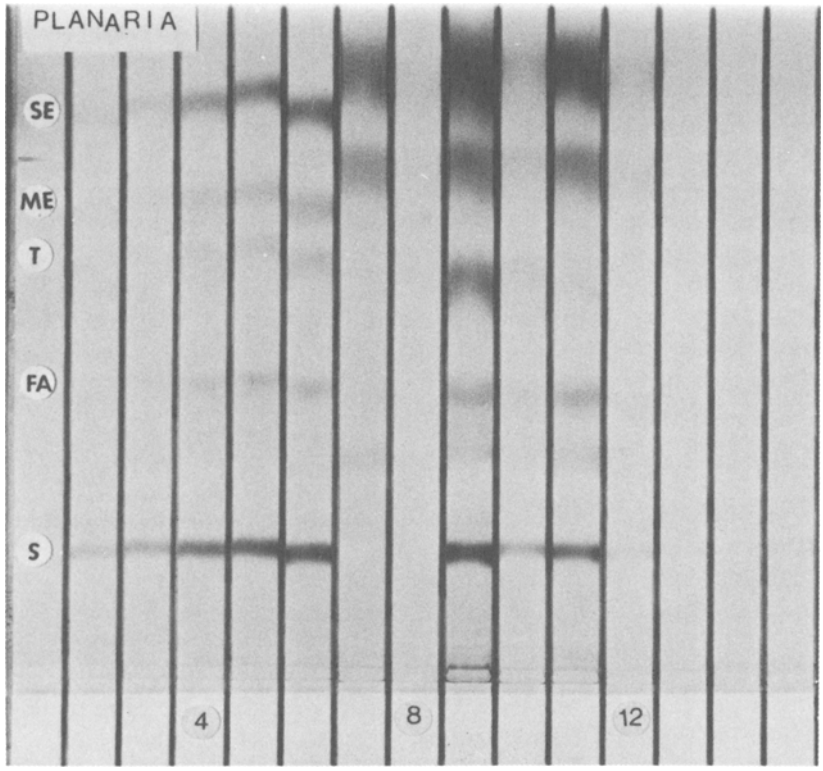

FIG. 1. A photograph of a representative chromatogram showing E-S products of *Dugesia tigrina* incubated 2 hr after feeding. Lanes 2, 3, 4, 5, and 6 contain 0.4, 0.8, 1.2, 1.6 and 2 μg of neutral lipid standard 18-4A, respectively. Lanes 7 and 8 contain an extract of Locke's control solution. Fast-moving bands in lane 7 are unidentified contaminants from the Locke's. Lane 9 contains the total lipid extract from a 24-hr incubate of 10 worms, whereas lane 10 contains a chloroform rinse of that sample. Note phospholipid at the origin of lane 9. Fast-moving bands in lane 9 comigrated with contaminants of lane 7. Lane 11 contains the total lipid sample of a 4-hr incubate from 10 worms, whereas lane 12 contains the chloroform rinse from this sample. Lanes 13–15 were not used. Abbreviations: S = cholesterol; FA = oleic acid; T = triolein; ME = methyl oleate; SE = cholesteryl oleate.

released phospholipids, free sterols, and free fatty acids. Quantitative thin-layer densitometric analyses of E-S products from worms incubated 2 hr after feeding for 4 hr showed a release of 2.7, 20, and 35 ng/worm (average, 19 ng/worm) of free sterols, whereas worms incubated for 24 hr released 20 and 42 ng/worm (average, 31 ng/worm) of free sterols. A representative linear calibration curve is shown in Figure 2. E-S products from worms used 4 days after feeding and then incubated for 4 hr released 1.1 ng/worm of free sterols, whereas worms incubated for 24 hr released 9.9 ng/worm.

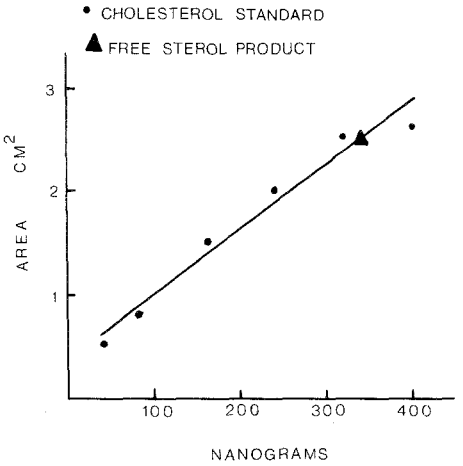

FIG. 2. A representative calibration curve showing the interpolation of *Dugesia tigrina* free sterol worm products from cholesterol standards. In this trial 350 ng of free sterol was released from 10 worms (35 ng/worm) incubated 2 hr after feeding for 4 hr.

TLC analysis showed that E-S products from *D. dorotocephala* incubated 2 hr after feeding in 0.3% Locke's for 4 or 24 hr also contained phospholipids, free sterols, free fatty acids, and triglycerides. Worms incubated 4 days after feeding released phospholipids, free sterols, and free fatty acids. Densitometric analyses of E-S products from worms incubated 2 hr after feeding for 4 hr showed a release of 4.9 ng/worm of free sterols, whereas the release of free sterols from worms incubated for 24 hr was 23 ng/worm. E-S products obtained from worms 4 days after feeding and incubated for 4 hr had 1.6 ng/worm of free sterols.

DISCUSSION

Fed *D. tigrina* and *D. dorotocephala* showed significant intraspecific pairing in the light and dark. Moreover, paired organisms rarely contacted each other, indicating the absence of worm-tactile stimuli in pairing. As shown for trematodes and nematodes (Fried and Roberts, 1972; Fried et al., 1980; Roberts and Thorson, 1977b), our results indicate that worm-emitted chemical factors are involved in pairing of planaria. As mentioned by Roberts and Thorson (1977a), worm pairing in helminths may offer advantages other than reproductive, i.e., feeding and developmental advantages.

Our results show that fed *D. tigrina* and *D. dorotocephala* pair in the absence of light, indicating that visual cues are not essential for intraspecific pairing in these species. In previous studies where an individual planarian was attracted to a planarian aggregation, visual and chemical cues were involved (Reynierse and Gleason, 1975).

When first introduced into a new environment, planaria are very active (Best and Rubinstein, 1967; present study). We observed that it took *D. dorotocephala* about twice as long to cease active movement than it did *D. tigrina*, indicating that the former species is more active than the latter. Additionally, Gunn et al., (1937) showed that light intensity stimulated planaria and increased their klinokinetic activity. This activity declined as the planarian became adapted to light. A planarian in the light takes longer to cease active movement than one in the dark. Fed *D. dorotocephala* in the dark paired more significantly than those in the light within the first 2 hr. The intrinsic activity of this species combined with extrinsic light stimulation may have interfered with planarian pairing behavior during the first 2 hr. Beyond 2 hr visual cues may play an important role in *D. dorotocephala* since these worms paired more significantly in light versus dark from 3 to 24 hr postintroduction. Except at 0.5 hr, pairing of fed *D. tigrina* in the light versus dark did not differ significantly, indicating that this species is more dependent on chemical than visual cues.

Starved planaria paired insignificantly compared with fed worms, suggesting that starvation may interfere with the ability of planaria to produce chemical attractants. Meyer et al. (1970) showed that *D. dorotocephala* could not synthesize sterols and long-chain fatty acids from endogenous reserves. The inability to obtain exogenous lipids could affect the production of chemoattractants in planaria.

Thin-layer chromatographic analysis of lipophilic E-S products of *D. tigrina* and *D. dorotocephala* showed lipid fractions similar to those detected in planarian extracts (Meyer et al., 1970; Fried and Grigo, 1977), except that triglycerides were not as abundant in E-S products versus worm extracts. The presence of free sterols in E-S products in all trials may indicate that these substances are involved in chemical attraction in planaria. Although lipophilic chemoattractants have not been studied in planaria, sterols have been reported to have pheromonal activity in trematodes and nematodes (Roberts and Thorson, 1977b; Fried and Gioscia, 1976; Fried et al., 1980).

Since cholesterol is the only free sterol in *D. dorotocephala* (Meyer et al., 1970), we used this standard in our quantitative densitometric TLC studies. Thin-layer densitometric studies that quantitate free sterol release in flatworms are not available. This study reports for the first time weights of cholesterol released by planaria.

Free fatty acids were also found in *D. tigrina* and *D. dorotocephala* E-S products. These substances may serve as chemical attractants since Mason (1975) showed that free fatty acids are involved in food location in *Dugesia neumani*.

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DEVELOPMENT OF A BIOLUMINESCENCE ASSAY FOR ALDEHYDE PHEROMONES OF INSECTS[1]
I. Sensitivity and Specificity

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Abstract—A rapid, quantitative assay for long-chain aldehydes based on bacterial luminescence was developed. Significant luminescent responses were obtained with both saturated and unsaturated aldehydes of 12–18 carbons. Maximum responses were obtained with the 14- and 16-carbon compounds, including those that are known insect sex pheromones. The bioluminescent response was linearly related to the amount of aldehyde over a 10^4–10^5 concentration range with as little as 0.1 pmol (~20 pg) of aldehyde being detected. The bioluminescent assay represents a new quantitative tool for rapidly measuring aldehyde pheromones of insects.

Key Words—Aldehydes, aldehyde pheromones, bioluminescence, assay for aldehydes, luciferase, insect pheromones.

INTRODUCTION

Bioluminescent marine bacteria utilize long-chain aldehydes as substrates in a bioluminescent reaction catalyzed by luciferase (Hastings, 1978). These luciferases respond in vitro to saturated aldehydes with a straight-chain skeleton of 8–18 carbons (Hastings et al., 1963, 1969; Watanabe and Nakamura, 1972; Meighen and Bartlet, 1980). The aldehyde is oxidized along

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with reduced flavin mononucleotide ($FMNH_2$) producing blue-green light according to the following scheme

$$FMNH_2 + O_2 + CH_3(CH_2)_n CHO \xrightarrow{\text{luciferase}} FMN + H_2O + CH_3(CH_2)_n COOH + h\nu_{490}$$

The reaction is initiated when $FMNH_2$ is rapidly mixed with 0_2 and aldehyde in the presence of the enzyme. $FMNH_2$ and 0_2 must be kept separate prior to the analysis since they combine readily in a nonenzymic reaction as follows:

$$FMNH_2 \xrightarrow{O_2} FMN + H_2O_2$$

Two types of assays are available, depending on the order in which the components are mixed together. In the standard assay, $FMNH_2$ is injected into a solution of aldehyde and luciferase (Hastings et al., 1978). In the second assay, the aldehyde is injected into a solution of luciferase and $FMNH_2$. In this case, the $FMNH_2$ is maintained in a reduced state by the addition of a small excess of sodium dithionite, and hence this system is generally referred to as the dithionite assay (Meighen and Hastings, 1971; Meighen and MacKenzie, 1973).

The initial maximum light intensity in both assays is dependent on the concentration of the reaction components ($FMNH_2$, O_2, aldehyde, luciferase), the species of bacterial luciferase, and the structure of the aldehyde. By using a fixed amount of bacterial luciferase and a high concentration of $FMNH_2$ (and O_2) for initiation of the reaction, the maximum light intensity will depend only on the amount and structure of the aldehyde. In addition, the rate of decay of luminescence under the above conditions also depends on the species of luciferase and the chemical structure of the aldehyde (Meighen and Bartlet, 1980).

Studies to determine the effects of substitution or desaturation of the aldehydes on the relative bioluminescence responses are limited. Spudich and Hastings (1963) have shown that the bioluminescent response of *Photobacterium fischeri* luciferase to high concentrations of 9-undecenal is similar to that of undecanal, whereas little or no response is obtained with 2-decenal compared to decanal. We have recently shown (Meighen et al., 1981) that some of these bacterial luciferases can respond as strongly to unsaturated aldehydes at very low concentrations as to the putative in vivo substrate, tetradecanal (Shimomura et al., 1974; Ulitzur and Hastings, 1979; Riendeau and Meighen, 1979).

The purpose of this paper is to compare the two bioluminescent assays and to describe some of the factors which affect their sensitivity and optimum responsiveness. In particular, using selected aldehydes, the effect of unsaturation, its position, and the length of the carbon chain on relative

bioluminescent responses are investigated. Many unsaturated aldehydes are known sex pheromones of moths and beetles, and we show that the very high sensitivity and quantitative nature of the bioluminescence assay is applicable to insect pheromone detection and quantification.

METHODS AND MATERIALS

Luciferases were purified from two bioluminescent species of bacteria, *Beneckea harveyi* and *Photobacterium phosphoreum* (NCMB 844), according to procedures similar to those described by Gunsalus-Miguel et al. (1972). The enzymes were stored as stock solutions (~10 mg/ml) in 30–40% glycerol, 0.01 M dithiothreitol, 0.05 M phosphate, pH 7.0, at -20° C and were stable for at least six months without any loss of activity. Secondary stocks were prepared by diluting the enzyme 1:20 into 0.001 M NH_2OH, 0.05 M mercaptoethanol, 0.05 phosphate, pH 7.0, at 4° and were used within two weeks. A typical enzyme preparation from 10 liters of bacterial culture will yield enough luciferase to perform at least 20,000 assays.

Aldehydes were obtained from Aldrich, Chemsamp Co., Zoecon Corp., or were synthesized. Isomeric and molecular purities of the unsaturated aldehydes were checked by gas chromatography and found to be >95%. Stock solutions of aldehydes were prepared in dimethylformamide and stored at 4°. Aqueous aldehyde solutions were prepared by dilution of the dimethylformamide stocks in water (>0.1% v/v) and were used within 20 min. All other chemicals were reagent grade. Phosphate buffers were made by mixing appropriate amounts of 1.0 M NaH_2PO_4 and 1.0 M K_2HPO_4.

Dithionite Assay. One ml of an aqueous solution of the test aldehyde was injected into 1.0 ml of 0.001 M NH_2OH, 0.05 M mercaptoethanol, 0.05 M phosphate, pH 7.0, containing 10 μl of a 1 : 20 dilution of luciferase (~5 μg) and 50 μM $FMNH_2$ reduced just before analyses by addition of a small amount (~0.5 mg) of solid sodium dithionite (Meighen and MacKenzie, 1973; Meighen and Hastings, 1971). Light emission was detected by a photomultiplier tube and recorded graphically with a pen recorder. Each sample was analyzed two to three times, and the average of the maximum light intensities, recorded in light units (LU), was used as a measure of the amount of aldehyde in the sample. One light unit corresponds to 6×10^9 quanta/sec based on the light standard of Hastings and Weber (1963).

Standard Assay. One ml of 50 μM $FMNH_2$ (catalytically reduced over platinized asbestos) was injected into 1.0 ml of 0.05 M phosphate, 0.001 M mercaptoethanol, pH 7.0, containing luciferase (~5 μg) and the test aldehyde. Light intensity was measured and recorded as described for the dithionite assay.

Evaluation of Bioluminescent Assays. The sensitivities of the two assay

types were compared by determining their background (endogenous) responses and the responses obtained with 100 pmol of either dodecanal or tetradecanal. Background responses were measured in the standard assay by injection of $FMNH_2$ into an assay medium containing no exogenously added aldehyde and in the dithionite assay by injection of 1.0 ml of water containing no added aldehyde. The effect of hydroxylamine in the two assays was determined by adding 0.001 M NH_2OH to the assay medium before injection of the final component.

The dependence of the bioluminescence response on enzyme concentration was determined using the dithionite assay and measuring the maximum light intensity on injection of either 1.0 ml of water or 10 pmol of (*E*)-11-tetradecenal in 1.0 ml of water into the assay medium containing various amounts of *B. harveyi* luciferase (5–50 μl of a 0.5 mg/ml solution). The dependence of the maximum light intensity on the amount of aldehyde was investigated by injection of different amounts of (*E*)-11-tetradecenal in 1.0 ml of water into a dithionite assay medium containing *B. harveyi* luciferase.

The effect of aldehyde chain length was determined with the dithionite assay by the response of *B. harveyi* or *P. phosphoreum* luciferase to 100 pmol of aldehyde. Similarly, the effect of unsaturation was investigated using 100 pmol of selected unsaturated 12-, 14-, and 16-carbon aldehydes and comparing them with tetradecanal.

RESULTS AND DISCUSSION

Comparision of Assay Sensitivities. The response of the two assay systems to background and to the same quantities of dodecanal and tetradecanal are shown for *B. harveyi* luciferase in Figure 1. In both assay systems, the responses to the aldehydes are similar. Much higher (10 to 40-fold) maximum light intensities were obtained for tetradecanal (Figure 1C) than for dodecanal (Figure 1B) and differences in the rates of decay of luminescence are evident for the two aldehydes. Essentially the same effects were observed with the *P. phosphoreum* luciferase.

However, the major differences between the assays is in their background responses. In the absence of exogenous aldehyde (Figure 1A), the dithionite assay produces significantly lower background responses than the standard assay, enabling detection of lower concentrations of long-chain aldehydes. The decreased background response in the dithionite assay was partially achieved by the addition of hydroxylamine (NH_2OH) to the assay medium (Table 1). This is presumably due to the formation of an oxime with endogenous aldehyde. In the dithionite assay, however, little effect on injected aldehyde is observed (Table 1) since oxidation and concurrent bioluminescence are more rapid than oxime formation. The standard assay is not

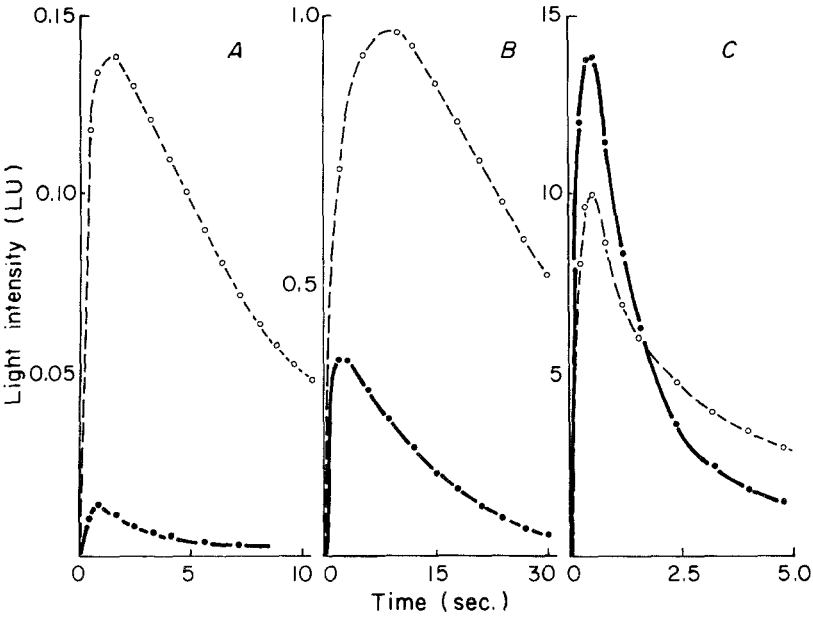

FIG 1. Comparison of the bioluminescent response of *B. harveyi* luciferase in the standard (--O--) and dithionite (—●—) assays: (A) background response; (B) 100-pmol of dodecanal (18 ng); (C) 100 pmol of tetradecanal (21 ng).

amenable to hydroxylamine treatment (Table 1) since the added aldehyde and hydroxylamine are in contact sufficiently long for complete oxime formation to occur. Since the lower limit of sensitivity of the bioluminescence assay is dictated by the endogenous response of luciferase, the hydroxylamine–dithionite method clearly provides the assay of choice for quantitative analysis of low levels of long-chain aldehydes.

TABLE 1. EFFECT OF HYDROXYLAMINE ON BIOLUMINESCENCE ASSAY SYSTEMS

Assay system (± NH_2OH)[a]	Maximum light intensities (LU)		
	No aldehyde	Dodecanal	Tetradecanal
Dithionite (–)	0.028	0.30	18
Dithionite (+)	0.013	0.33	14
Standard (–)	0.14	0.90	10
Standard (+)	0.021	0.017	0.022

[a](+) indicates presence of 0.001 M NH_2OH added to assay prior to the additional of the final component. The dithionite (+) and the standard (–) assays are described in Methods and Materials.

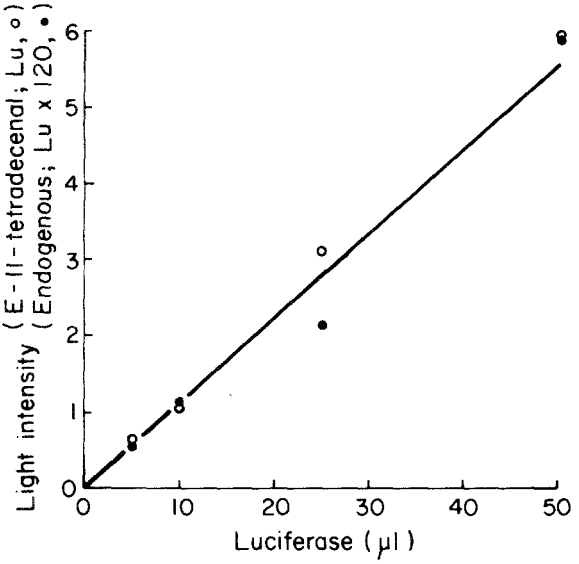

FIG 2. Dependence of the maximum light intensity response on the amount of added *B. harveyi* luciferase with either 10 pmol of (*E*)-11-tetradecenal (O) or no added exogeneous aldehyde (●) in the dithionite assay.

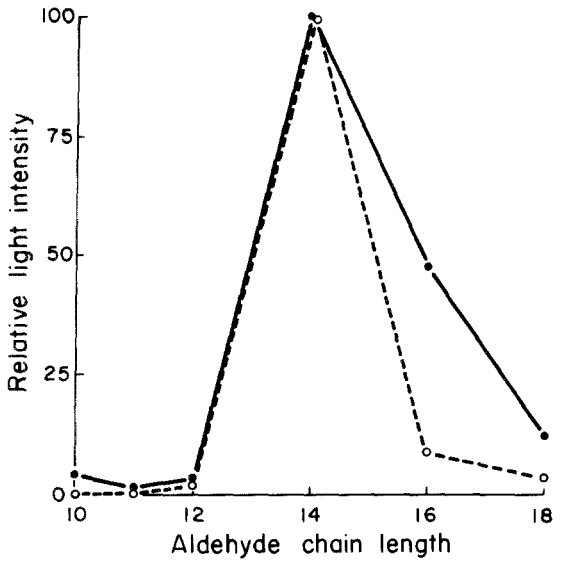

FIG 3. Relative maximum light intensities in the dithionite assay as a function of aldehyde chain length for 100 pmol of the saturated aldehyde with *B. harveyi* (●) and *P. phosphoreum* luciferases (O). The responses are reported as a percentage of that observed with tetradecanal for each of the luciferases.

Effect of Enzyme Concentration. As Figure 2 shows, a roughly linear increase in light intensity is obtained with increasing enzyme concentration, but no gain in sensitivity is achieved because the background emission rises in an approximately linear fashion. Although the relationship shown in Figure 2 was obtained with (*E*)-11-tetradecenal, it occurs with all aldehydes we have examined to date. Thus for quantitative measurement of aldehydes in this study, a standard enzyme concentration was chosen so that the light emission was high enough to be easily detected by our instrumentation for bioluminescence measurements.

Chain-Length Dependence. The chain-length dependences of the luciferases of *B. harveyi* and *P. phosphoreum* in the dithionite assay are depicted in Figure 3. Both luciferases showed the strongest preference for tetradecanal and more modest responses to hexadecanal and octadecanal. The response to dodecanal, although low, is significantly above background, as are the responses to undecanal and decanal. The response of *P. phosphoreum* luciferase to the substrates was slightly more restrictive than that of *B. harveyi* luciferase.

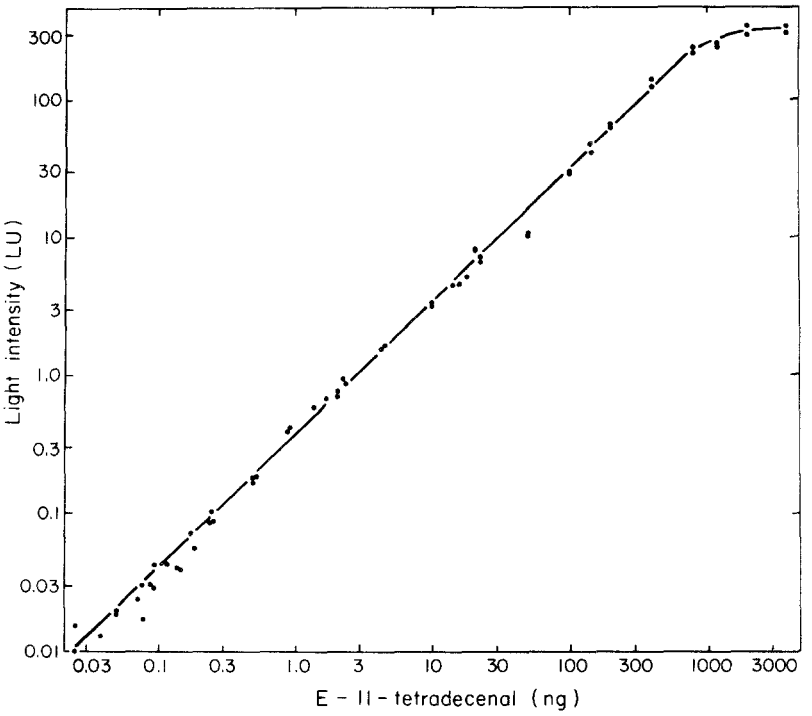

FIG 4. Maximum light intensity response of *B. harveyi* luciferase to different amounts of (*E*)-11-tetradecenal plotted on logarithmic scales. Each point represents the average of two independent dithionite assays after background correction.

Although previous studies (Hastings et al., 1963; Watanabe and Nakamura, 1972) have shown that bioluminescent responses vary with aldehyde chain length with a high specificity for saturated aldehydes of 8–18 carbons, the present study has described this specificity at low, nonsaturating concentrations of aldehyde under conditions where the bioluminescent response should be proportional to aldehyde concentration.[5] It is at these concentrations that we have demonstrated that the assay responds quantitatively to the aldehyde concentration (see next section).

Determination of Aldehyde Concentration. The bioluminescent response of *B. harveyi* luciferase to increasing concentrations of (*E*)-11-tetradecenal (Figure 4) is linear over nearly five orders of magnitude of concentration, from 20 pg to 600 ng ($0.1–3 \times 10^3$ pmol). At higher aldehyde concentrations, the bioluminescent response remained relatively constant, presumably reflecting saturation of the binding sites of the enzyme at these concentrations.[5]

Similar linear plots were obtained with other aldehydes and with both species of luciferase. These linear relationships form the basis of the bioluminescent assay as a tool for quantitatively measuring long-chain aldehydes, including those that are insect sex pheromones.

Response of Selected Unsaturated Aldehydes. The presence, position, and isomerism of double bonds in straight-chain aldehydes affected the magnitude (Table 2) and decay characteristics (Figure 5) of the bioluminescent response elicited from the two species of luciferases tested. For the 12-and 14-carbon aldehydes, the unsaturated compounds elicited responses slightly less than the saturated congener, for the 16-carbon aldehydes with the double bond more distal than the 8 position, the responses were much greater than hexadecanal (Table 2). The luciferases themselves respond quite differently to the various aldehydes. With one clear exception [(*Z*)-9-tetradecenal], greater responses were usually obtained with *B. harveyi* luciferase.

A small but significant difference (~20%) in the rates of decay of

[5] On the basis that luciferase obeys Michaelis-Menten kinetics in the dithionite assay (see Meighen and Hastings, 1971; Meighen and MacKenzie, 1973) and using saturating concentrations of $FMNH_2$ and 0_2, the relative bioluminescence response or activity of luciferase at different aldehyde concentrations (v) is given by $v = V_m A/(K_m + A)$. In this equation, V_m is the bioluminescence response of luciferase to high and saturating aldehyde concentrations and is only dependent on the total amount of luciferase (E) and the rate of turnover (k) of luciferase in the bioluminescent reaction ($V_m = k\,E$); A is the aldehyde concentration; K_m is the aldehyde concentration required for luciferase to give a bioluminescence response 50% of that observed at saturating aldehyde concentrations and is the parameter that reflects the apparent affinity of binding of the aldehyde to the enzyme. Although the bioluminescence response will be constant at high aldehyde concentration ($v = V_m$ if $A >> K_m$), at low aldehyde concentrations ($A < K_m$), the bioluminescence response will be directly proportional to the aldehyde concentration ($v = V_m A/K_m$).

TABLE 2. BIOLUMINESCENT RESPONSES TO 100 PMOL OF SATURATED AND UNSATURATED ALDEHYDES RELATIVE TO 100 PMOL OF TETRADECANAL[a]

Aldehyde pheromone	Luciferase	
	B. harveyi	P. phosphoreum
None (background)	<0.1	<0.1
(*E*)-7-Dodecenal	1.1	0.3
(*Z*)-9-Dodecenal	0.6	0.6
(*Z*,*E*)-5,7-Dodecadienal	1.2	0.2
Dodecanal	2.4	2.2
(*Z*)-9-Tetradecenal	30	60
(*Z*)-11-Tetradecenal	80	30
(*E*)-11-Tetradecenal	100	75
Tetradecanal	100	100
(*Z*)-7-Hexadecenal	25	3
(*Z*)-9-Hexadecenal	100	40
(*Z*)-11-Hexadecenal	120	70
(*Z*,*Z*)-11,13-Hexadecadienal	160	20
Hexadecanal	40	10

[a]Maximum light intensities in the dithionite assay.

luminescence for the *Z* and *E* isomers of 11-tetradecenal were observed in these experiments (Figure 5). An even larger difference (~ 3-fold) occurs between the rates of decay of luminescence of (*E*)-7-dodecenal and (*Z*)-9-dodecenal (Figure 5), and the decay rates of both these aldehydes, as well as dodecanal, are considerably slower than the 14-carbon aldehydes (see also Figure 1). The differences in the rate of decay of luminescence and, in particular, the differences in relative bioluminescent responses of the two luciferases to different aldehydes with respect to maximum light intensity (Table 2) may provide criteria for distinguishing qualitatively between the unsaturated aldehydes.

Summary. To summarize, the results of the above experiments show that the dithionite assay with luciferase from either *B. harveyi* or *P. phosphoreum* can quantitatively measure low levels of saturated and unsaturated aldehyde pheromones of 14–16 carbons and possibly those outside those chain lengths. The range of concentrations over which the response is linear coincides with the quantities of aldehyde pheromones often found in insects. For example, in the spruce budworm, individual females contain an average of 1–2 ng/gland (Silk et al., 1980). The following papers (Grant et al., 1982; Szittner et al., 1982) demonstrate the application of the bioluminescence assay to the

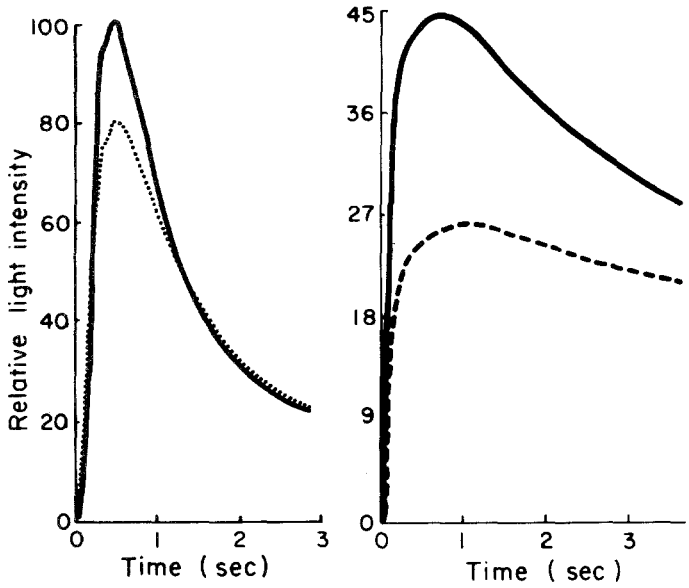

FIG 5. Bioluminescent response of *B. harveyi* luciferase to selected unsaturated aldehydes (100 pmol) in the dithionite assay. Light intensity is reported as a percentage of the maximum light intensity obtained with an identical amount of saturated aldehyde of the same chain length. Left: (*E*)-11-tetradecenal (solid line) and (*Z*)-11-tetradecenal (dotted line). Right (*E*)-7-dodecenal (solid line) and (*Z*)-9-dodecenal (dotted line).

measurement of the aldehyde content of single gland extracts of aldehyde-producing moths and the analysis of the levels of aldehyde pheromone in air.

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DEVELOPMENT OF A BIOLUMINESCENCE ASSAY FOR ALDEHYDE PHEROMONES OF INSECTS[1]
II. Analysis of Pheromone Glands

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Abstract—Pheromone levels in the glands of individual female moths of the spruce budworm (*Choristoneura fumiferana*), the western spruce budworm (*C. occidentalis*), the navel orangeworm (*Amyelois transitella*), and the corn earworm (*Heliothis zea*) were quantitively measured by means of a new bacterial bioluminescence assay specific for aldehydes. The sensitivity and rapidity of the bioluminescent assay enabled studies to be conducted on the dependence of the pheromone levels in the spruce budworm on age and the effect of photoperiod on the pheromone levels in the corn earworm. The bioluminescence assay provides a rapid and sensitive approach for studying aldehyde pheromone levels and their regulation in insects.

Key Words—Aldehyde pheromone, bioluminescence, corn earworm, *Heliothis zea*, insect pheromone, navel orangeworm, *Amyelois transitella*, western spruce budworm, *Choristoneura occidentalis*, spruce budworm, *Choristoneura fumiferana*, Lepidoptera, assay for aldehydes, Pyralidae, Noctuidae, Tortricidae.

INTRODUCTION

In recent years, a rapidly increasing number of insects have been shown to use aldehydes as part of their sex pheromone blend. Over 20 species of moths in 10

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families [5] and four species of dermestid beetles have at least one pheromone component that is a long-chain aliphatic aldehyde. Table 1 lists examples of major forest, agricultural, and stored-products pests and their aldehyde pheromones. In addition, many other moth species are known to be attracted to aliphatic aldehydes (Ando et al., 1977; Underhill et al., 1977; Weatherston et al., 1978), suggesting that they also utilize aldehydes as sex pheromones. Often, for species in which aldehydes have been identified as the major sex pheromone component, the minor components are also aldehydes as, for example, in the spruce budworm and the corn earworm (Table 1).

The previous paper (Meighen et al., 1982) described a bioluminescent assay which utilizes bacterial luciferases to quantitatively measure saturated and unsaturated aliphatic aldehydes with chain lengths of 12–18 carbons. The optimum responses for the luciferases studied were obtained with aldehydes having a backbone of 14 or 16 carbons which fortuitously encompasses most of the aldehyde sex pheromones identified to date. As little as 0.1 pmol of these unsaturated aldehydes can be detected. This paper reports the application of the bioluminescence assay for rapid quantitative analysis of sex pheromone extracts of lepidopteran species possessing aldehyde pheromones.

METHODS AND MATERIALS

Analysis of Aldehyde Pheromones. Synthetic aldehyde pheromones were analyzed using the dithionite bioluminescence assay described in the preceding paper (Meighen et al., 1982). One ml of an aqueous solution of the pheromone was injected into the assay solution and the maximum light intensity recorded as a measure of the amount of aldehyde present. Aldehyde stock solutions (Meighen et al., 1982) were prepared in either dimethylformamide or heptane.

Analysis of Insect Extracts. Laboratory-reared (Grisdale, 1970, 1973), 2-and 3-day-old spruce budworm moths, *Choristoneura fumiferana,* and western spruce budworm moths, *C. occidentalis*, were maintained on continuous light. Glands were excised between 1500 and 1700 hr and extracted for 10 min with 10 μl of heptane in a small tube as described by Klun et al. (1979). The extract was transferred in a Hamilton syringe to a glass vial, the heptane removed by evaporation for 2 min under low vacuum, and then redissolved in 10 ml of water and vortexed for 10 sec. One-ml aliquots were assayed 10 min later, and the average of the initial maximum light intensities for two to three assays of each sample was recorded.

Laboratory-reared navel orangeworm moths, *Amyelois transitella* (supplied as pupae by USDA, Fresno, California) were maintained on a 12:12

[5] The families are Noctuidae, Tortricidae, Lasiocampidae, Yponomentidae, Pyralidae, Sphingidae, Bombycidae, Saturnidae, Arctiidae, and Pterophoridae.

TABLE 1. REPRESENTATIVE PEST SPECIES WITH SEX PHEROMONES CONTAINING AT LEAST ONE ALIPHATIC ALDEHYDE COMPONENT

Order	Species (common name)	Family	Aldehydes Present	Reference
Lepidoptera				
	Choristoneura fumiferana (spruce budworm)	Tortricidae	(*E*)-11-Tetradecenal (*Z*)-11-Tetradecenal Tetradecanal	Silk et al. (1980) Sanders and Weatherston (1976)
	Argyrotaenia citrana (orange tortrix)	Tortricidae	(*Z*)-11-Tetradecenal	Hill et al. (1975)
	Heliothis zea (corn earworm)	Noctuidae	(*Z*)-11-Hexadecenal (*Z*)-9-Hexadecenal (*Z*)-7-Hexadecenal Hexadecanal	Klun et al. (1979)
	H. virescens (tobacco budworm)	Noctuidae	(*Z*)-11-Hexadecenal (*Z*)-9-Hexadecenal (*Z*)-7-Hexadecenal Hexadecanal (*Z*)-9-Tetradecenal Tetradecanal	Klun et al. (1979)
	Earias insulana (spiny bollworm)	Noctuidae	(*E*,*E*)-10,12-Hexadecadienal	Hall et al. (1980)
	Amyelois transitella (naval orangeworm)	Pyralidae	(*Z*,*Z*)-11,13-Hexadecadienal	Coffelt et al. (1979)
	Manduca sexta (tobacco hornworm)	Sphingidae	(*E*,*Z*)-10,12-Hexadecadienal	Starratt et al. (1979)
	Plutella xylostella (diamondback moth)	Yponomeutidae	(*Z*)-11-Hexadecenal	Tamaki et al. (1977)
	Malacosoma disstria (forest tent caterpillar)	Lasiocampidae	(*Z*,*E*)-5,7-Dodecadienal	Chisholm et al. (1980)
Coleoptera				
	Trogoderma granarium (khapra beetle)	Dermestidae	(*E*)-14-Methyl-8-hexadecenal (*Z*)-14-Methyl-8-hexadecenal	Cross et al. (1976)

light–dark photoperiod with photophase commencing at 0700 hr. Glands from 1- to 3-day-old females were excised between 1200 and 1600 hr and analyzed in a manner similar to the budworm glands, except the sample was dissolved in 3.5 ml rather than 10 ml of water.

Corn earworms were reared on the same diet as the budworm. Adults were maintained on two light cycles. Some females were maintained on the same photoperiod as the navel orangeworm and their glands were removed during the photophase, between 1200 and 1600 hr, and analyzed as described for the budworm glands. The remaining females were maintained on a reversed light cycle (16:8 light–dark) with the photophase commencing at 1700 hr. Glands were cut and analyzed between 1100 and 1500 hr during the insect's scotophase.

As a check for possible extraneous nonpheromone aldehydes associated with body tissue, male and female heads with attached antennae and segments of female abdominal tissue immediately adjacent to the pheromone gland were also analyzed for each species (Table 2). The amount of abdominal tissue analyzed was approximately twice that contained in an excised gland.

A final experiment was undertaken to assess the effect of age on pheromone levels in individual female spruce budworm moths. Emerging adults were collected, dated, and maintained in continuous light at ambient

TABLE 2. QUANTITATIVE ANALYSIS OF ALDEHYDE PHEROMONES IN INSECT EXTRACTS MEASURED BY BIOLUMINESCENT ASSAY

Insect[a]	Sample	Number analyzed	Average (ng) ± SD	Range (ng)
Spruce budworm (male)	Head/antennae	6	0.0 ± 0.1	0.0 - 0.1
Spruce budworm	Head/antennae	6	0.1 ± 0.1	0.0 - 0.2
Spruce budworm	Abdomen	6	0.0 ± 0.1	0.0 - 0.1
Spruce budworm	Gland	40	2.2 ± 1.6	0.0 - 5.8
Western spruce budworm	Gland	10	2.8 ± 1.5	0.0 - 5.2
Navel orangeworm	Head/antennae	9	0.03 ± 0.03	0.0 - 0.07
Navel orangeworm	Abdomen	20	0.00 ± 0.03	0.0 - 0.05
Navel orangeworm	Gland	45	0.56 ± 1.4	0.0 - 7.0[b]
Corn earworm	Abdomen	4	0.0 ± 0.1	0.0 - 0.1
Corn earworm	Gland[c]	27	0.9 ± 2.0	0.0 - 8.0
Corn earworm	Gland[d]	33	4.7 ± 5.6	0.0 - 19.0

[a] Extracts were from female insects unless indicated otherwise.

[b] Two glands, analyzed independently on different days, gave the maximum response (7.0 ng), whereas all other gland extracts had contents less than 1.5 ng.

[c] Insects on 12: 12 light - dark light cycle. The glands were excised and analyzed between 1200 and 1700 hr during insect's photophase.

[d] Insects on reversed 16: 8 light - dark photoperiod with dark cycle at 900–1700 hr. The glands were excised and analyzed between 1200 and 1700 hr during insect's scotophase.

laboratory temperature and humidity, although the insects had access to water. Glands from females 0 (<4 hr) to 7 days old were analyzed at 1500–1700 hr.

For all experiments, the bioluminescent readings were corrected for the average response of a blank which consisted of 10 μl of heptane carried through the same process as a pheromone extract. The responses were converted into nanograms based on the response elicited by a known amount of the appropriate aldehyde standard in 10 μl of heptane and treated as an extract. The aldehyde standards were (*E*)-11-tetradecenal for the two budworm species, (*Z,Z*)-11,13-hexadecadienal for the navel orangeworm, and (*Z*)-11-hexadecenal for the corn earworm. In each case, the standard chosen represents the major identified pheromone component (Table I).

RESULTS AND DISCUSSION

Analyses of extracts from individual female spruce budworm moths produced bioluminescent responses equivalent to an average of 2.2 ng of aldehyde pheromone per gland (Table 2). Glands of the western budworm contained a similar quantity of aldehyde. These values for both species are in close agreement with those obtained independently by other workers using similar extraction techniques and gas–liquid chromatography analysis (Silk et al., 1980, personal communication; Slessor, unpublished data). Figure 1 directly compares the bioluminescent response of a typical gland extract from a single female budworm moth to the response obtained with 2.1 ng (10 pmol) of the (*E*)-11-tetradecenal standard and to a blank control with no exogenous aldehyde (i.e., background response).

The levels of aldehyde pheromone measured for individual glands were variable, even if moths came from the same batch of pupae. For example, the following quantities (ng) of aldehyde were measured sequentially in 7 glands from 3-day-old virgin female budworm moths maintained in continuous light: 1.2, 2.9, 2.3, 1.5, 0.6, 4.2, 1.8 ($x \pm$ SD = 2.1 ng $\pm$ 1.2). Although 5.8 ng of aldehyde is the maximum amount measured in the data presented here (Table 2), quantities up to 14 ng have been measured on occasion and, as Table 2 shows, some glands had no detectable aldehyde. Such variability may be a reflection of the use of a continuous light regime for maintaining the females, but this variability was also observed in the other species analyzed by the bioluminescent assay and has been observed when the analysis was done with capillary chromatography (Slessor, unpublished data) of single female insects.

Glands of individual female navel orangeworm moths had relatively low levels of aldehyde pheromone. The average quantity detected was only 0.6 ng/female, although quantities as great as 7.0 ng were measured. However,

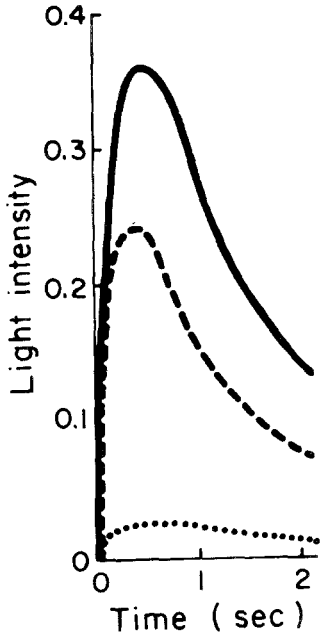

FIG. 1. Bioluminescent responses to an extract of the gland of one spruce budworm moth, *Choristoneura fumiferana* (solid line), a standard containing 10 pmol of (*E*)-11-tetradecenal (dashed line), and a blank containing no exogenous aldehyde (dotted line). The gland was extracted as described in Methods and Materials, except the sample was redissolved in 3.5 ml rather than 10 ml of water. Bioluminescence responses are for 1.0-ml aliquots. The gland response (about 1.5 times the standard after correction for the background response) indicates that the extract of this female contains 3 ng of (*E*)-11-tetradecenal.

these glands were analyzed during the insect's photophase (noncalling period) and are probably lower than would be expected if the glands were excised during calling. Nonetheless, the results are in reasonable agreement with those of Coffelt et al. (1979), who reported an average of 1.0–1.5 ng of (*Z,Z*)-11,13-hexadecadienal for calling female navel orangeworm moths analyzed by gas chromatography.

The effect of photoperiod on the level of pheromone in the female moth was demonstrated for the corn earworm (Table 2). The average quantity of aldehyde pheromone in females analyzed during their scotophase was 4.7 ng, more than 5-fold higher than levels measured in the photophase. Similarly the upper value of measured pheromone in the scotophase (19.0 ng) was over twice the upper value (8.0 ng) for corn earworm moths in their photophase. Klun et al. (1979) reported an average of 10–20 ng of (*Z*)-11-hexadecenal per female, and this component represented about 90% of the pheromone.

In contrast to the glands of the various insects analyzed, extracts of their abdomens or the head and antennae of individual male or female moths did not contain measurable amounts (<0.1 ng) of aldehyde pheromone (Table 2). This indicates that the aldehyde measured in the glands with the bioluminescent assay is due to the pheromone and not to nonpheromone aldehyde associated with insect tissue. In the case of budworms, internal controls, in which a fixed amount of (*E*)-11-tetradecenal (10 ng) was added to the excised tissue (abdomen, glands, or head and antennae) in heptane, demonstrated that some pheromone was lost during the extraction procedure (Table 3). This loss of pheromone is primarily due to the absorption of aldehyde on the tissue since internal controls in which the pheromone was added to the extract after removal of the tissue (Table 3) gave values close to that observed for the standard in heptane. These latter controls indicate that components inhibitory to the bioluminescence assay were not present in significant amounts in the extracts. Since very little pheromone, if any (<0.1 ng), was detected in the extracts of the head and antennae or abdomen, correction for the relative losses measured in the respective internal controls will not significantly affect these results. Although correction for the pheromone loss, measured in the internal control for the gland extract, would lead to pheromone levels approximately two times higher in the budworm gland extracts, it should be

TABLE 3. EXTRACTS OF SPRUCE BUDWORM: STANDARD, INTERNAL CONTROLS, BLANK

Sample[a]	Average light intensity [LU/ml (No.) ± SD]	Percentage of standard
Blank		
H, heptane, 10 μl	0.018 (12) ± 0.008	
Standard		
A	0.54 (20) ± 0.09	100
Internal controls		
A + head/antennae	0.40 (10) ± 0.09	74
A + abdomen	0.24 (10) ± 0.05	44
A + gland	0.31 (10) ± 0.06	57 (37)[c]
H + head/antennae (+ B)[b]	0.58 (7) ± 0.09	107
H + abdomen (+ B)[b]	0.38 (6) ± 0.08	70
H + gland (+ B)[b]	0.49 (6) ± 0.11	91 (70)[c]

[a] A and B are standards of 50 pmol of (*E*)-11-tetradecenal in 10 μl of heptane and dimethylformamide, respectively. Unless indicated otherwise, samples were treated in a process identical to that described in Methods and Materials for extraction of the budworm tissue and the maximum luminescence for 1.0 ml of the aqueous sample (LU/ml) recorded.

[b] B was added to the other components after they had been dissolved in H_2O (see Methods and Materials).

[c] Percentage of standard after subtraction of the average luminescene response (0.11 LU/ml) expected for the extract of the budworm gland.

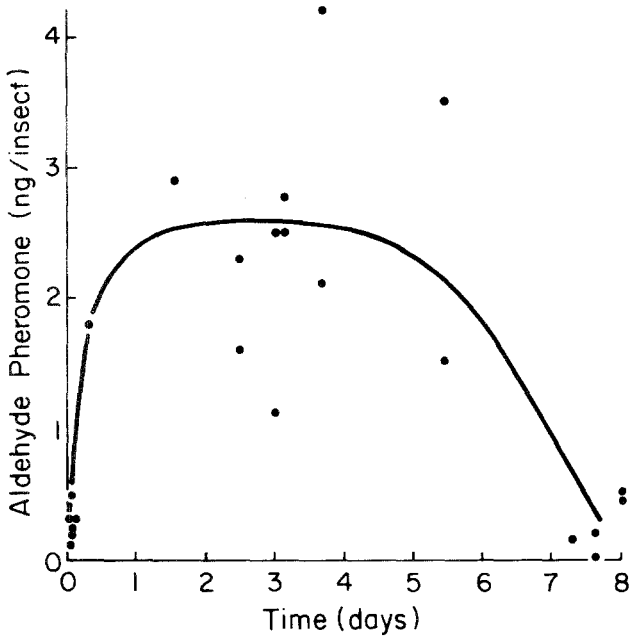

FIG. 2. Dependence of aldehyde pheromone levels in extracts of the glands of female budworms on time after emergence from pupae. Insects were maintained on continuous light, and the gland was excised between 1200 and 1700 hr. Each point represents the average value for analysis of 4–7 individual female moths. One batch of insects analyzed after 2–3 days had an average value of 7 ng (data not plotted) for the pheromone level in the gland extracts.

recognized that these measured levels are only relative values since repeated extraction of the same gland leads to additional recovery of pheromone. Consequently, only the amount of aldehyde pheromone obtained in the extract immediately after removal of the gland is reported.

Figure 2 shows the effect of insect maturity on pheromone present in glands of virgin female spruce budworms. Almost all glands analyzed shortly after emergence (<4 hr) had very low levels of aldehyde pheromone ($\bar{X} = 0.3$ ng). However, by day one the amount of pheromone rose to an average value of 2–3 ng/insect and remained relatively constant at that level through the 5th day, after which the pheromone level declined. By the 7th day, the glands contained an average of only 0.3 ng/gland. Sanders and Lucuik (1972), using male responses to extracts from rinses of containers holding virgin females of known ages, obtained results very similar to those in Figure 2. Males responses were low to extracts from 1-day-old females, peaked with extracts from 3-day-old females, and declined significantly with extracts from females

older than 5 days. These results are not directly comparable to ours because their method was indirect and may include the effect of age on behavior (i.e., calling). Nonetheless, the agreement of the two sets of results provides strong support for the validity of these measurements.

It was recognized in developing the bioluminescent assay for quantitative analysis of aldehyde pheromone extracts that the level of pheromone detected is dependent on a number of physiological and experimental factors. The assay reliably detected both the effects of age (spruce budworm) and photoperiod (corn earworm) on the pheromone levels of these insects. Such studies often rely on the indirect measurement of pheromone levels such as that used by Sanders and Lucuik (1972) because of the greater effort involved in direct methods such as gas chromatography. With respect to the assay itself, the method of pheromone extraction and sample processing, as well as the presence of factors inhibitory to the luciferase enzyme, can also affect the measured levels. However, any loss of pheromone ($\sim$10–20%) during sample processing was corrected for by using synthetic standards taken through an identical process. Relatively little inhibition of the bioluminescence assay was caused by material extracted from the gland. Although extraction of the aldehyde pheromone from the gland was certainly not complete and, indeed, pheromone could be demonstrated to be lost on mixing with the gland, changing the extraction conditions by decreasing the extraction time to less than a minute did not significantly affect the level of extracted pheromone detected in different sets of insects. Moreover, the results obtained for the four species studied here agree very closely with those obtained using similar solvent extraction (i.e., gland rinses) and analysis by gas chromatography.

The present paper has demonstrated that the levels of aldehyde pheromone in organic solvent extracts of glands of the spruce budworm, the corn earworm, and the navel orangeworm can be measured using the bacterial bioluminescence assay. The conversion of the bioluminescent response into an absolute amount of aldehyde is dependent on prior knowledge of the aldehyde composition of the pheromone and the specific response of luciferase to that aldehyde (Meighen et al., 1982). The work-up procedure requires only a small amount of manipulation, so that it takes less than 30 min from excision of the gland to the extraction and quantitation of the pheromone present in the extract. The assay itself is extremely rapid, requiring only a few seconds, and the level of aldehyde pheromone in each sample can be measured in duplicate or triplicate in about one minute. Consequently, excision of ten tissue samples and analysis by the assay was completed within 40 min. Application of the bioluminescence assay to the analysis of the level and regulation of aldehyde pheromones in the glands of other insects containing long-chain aldehydes (see references in Table I; McDonough and Kamm, 1979; Nesbitt et al., 1977, 1979a,b; Cross et al., 1977) appears readily feasible.

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DEVELOPMENT OF A BIOLUMINESCENCE ASSAY FOR ALDEHYDE PHEROMONES OF INSECTS[1]
III. Analysis of Airborne Pheromone

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Abstract—A newly developed bioluminescent assay was used to measure quantitatively the amount of (*E*)-11-tetradecenal, the major component of the sex pheromone of the spruce budworm, trapped on Porapak Q®. The bioluminescent response was linearly related to the amount of aldehyde either deposited on the absorbent or trapped from an airstream. However, the recovery of pheromone from Porapak was dependent on whether the air was prefiltered (through Porapak) or taken directly from the atmosphere. Furthermore, pheromone on Porapak was lost with time during the flow of air through the absorbent, indicating that trapping of aldehyde pheromone should be conducted for short periods of time for optimal recoveries. The applicability of the assay system for the rapid and direct measurement of the release rates of aldehyde pheromone lures was demonstrated for pheromone lures used for baiting spruce budworm traps.

Key Words—Aldehydes, bioluminescence, insect pheromones, Porapak Q, spruce budworm, *Choristoneura fumiferana*, Lepidoptera, (*E*)-11-tetradecenal, trapping, bioassay for aldehydes.

INTRODUCTION

In order to correlate levels of airborne pheromone emitted by insects, trap lures, and controlled-release formulations with the behavioral effects they manifest in the target species, it is necessary to measure the atmospheric

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concentration. Direct methods of achieving this have combined gas chromatographic analysis with various methods of trapping the airborne pheromone, including cold traps (Browne et al., 1974), glass surface absorption (Baker et al., 1980), and adsorption on solid porous absorbents (Byrne et al., 1975; Caro et al., 1980; Cross et al., 1980). In general, systems which seem best suited to measuring atmospheric levels have made use of some sort of porous absorbent such as Porapak Q, and these have been shown to give high recoveries of pheromonal compounds (Cross et al., 1980; Wiesner et al., 1980).

Since an increasing number of important pest insects are known to release long-chain aldehydes as sex pheromones (Grant et al., 1982), an efficient trapping and analysis system for these airborne aldehyde pheromones would be useful. We have recently developed a bioluminescent assay (Meighen et al., 1982) which specifically measures aldehyde pheromones, particularly those with 14 to 16-carbon chains. Since the assay is a rapid and sensitive procedure, the present studies were directed toward determining its applicability for measuring small quantities of airborne aldehyde trapped on Porapak Q. In particular, we wanted to measure trapped pheromone under laboratory conditions that could be used for measuring release rates of lures or controlled-release formulations and that could potentially be extended to atmospheric studies.

METHODS AND MATERIALS

Materials. Bacterial luciferase was purified to homogeneity from the bioluminescent bacteria, *Beneckea harveyi* (Gunsalus-Miguel et al., 1972) and stored as a stock solution (~10 mg/ml) in 30–40% glycerol, 0.01 M dithiothreitol, 0.05 M phosphate, pH 7.0, at −20° C (Meighen et al., 1982).

(*E*)-11-Tetradecenal (Chem. Samp Co.) was used throughout the study as a model aldehyde pheromone because it is the major pheromonal component of the spruce budworm, *Choristoneura fumiferana* (Sanders and Weatherston, 1976; Silk et al., 1980). Stock solutions (0.01 M) of (*E*)-11-tetradecenal were prepared by dilution of the neat liquid into hexane and then stored at −20° C. The purity was checked by gas chromatography and found to be >95%; the *E*-to-*Z* ratio was 99:1. Hexane, certified grade (H-301), was obtained from Fisher. All other chemicals were reagent grade. Phosphate buffers were made by mixing the appropriate amounts of 1.0 M NaH_2PO_4 and 1.0 M K_2HOP_4.

Porapak Q, 50–80 mesh (Waters Assoc.), was extensively washed with hexane (~5–10 ml/g per wash equilibrated for >1 hr) before use to reduce the levels of endogenous aldehyde and substances inhibitory to the bioluminescence assay. After 20–25 hexane washes, most of the inhibitory material

was removed, as shown by standards containing a fixed amount of aldehyde added to a hexane extract of Porapak which gave about 80% of the luminescence response of the same amount of aldehyde added to pure hexane. A check was also made for a low endogenous (background) response since this affects the sensitivity of the assay (Meighen et al., 1982). The washed Porapak was dried under vacuum and stored in a sealed flask. Controls measuring the bioluminescence responses of a hexane extract of Porapak before (background) and after (standard) addition of (*E*)-11-tetradecenal were routinely run with every set of analyses. In all experiments, 200-mg quantities of Porapak were used for trapping the aldehyde or as a prefilter.

Bioluminescence Analysis of Porapak Q Extracts. Porpak Q (200 mg) containing trapped or deposited (*E*)-11-tetradecenal was extracted for 30 min by incubation in 1.0 ml of hexane unless otherwise noted. Because hexane inhibits the bacterial bioluminescent reaction, it is necessary to evaporate the hexane and redissolve the aldehyde pheromone in water before analysis. An aliquot (0.2 ml) of the extract was transferred to a 6 ml glass vial prerinsed with water, and the hexane was removed under a controlled flow of N_2 filtered through Porapak (2 liters/min, 7 min). Shorter periods of time (<5 min) did not remove all the hexane, whereas longer periods of time (>15 min) resulted in a lower luminescence response for standards, indicating that the aldehyde pheromone was being lost. Distilled water (3.5 ml) was added to the glass vial, vortexed for 5–10 sec, and then allowed to stand for 10 min before analysis with the bioluminescence assay. Standards of (*E*)-11-tetradecenal prepared in dimethylformamide and added directly to water gave the same luminescence response as those evaporated from hexane for 5–15 min. The luminescence response is reported in light units (LU) (Meighen et al., 1982) for analyses of 1.0-ml aliquots of the sample or alternatively as a percentage of controls in which a known amount of (*E*)-11-tetradecenal was deposited directly on Porapak Q and extracted immediately. The responses of the controls were typically 60–100% of the value obtained if the aldehyde is added to a hexane extract of Porapak Q. For each set of analyses, these controls as well as the background response were measured. Experiments were conducted at room temperature (21 ± 1°C).

Analysis of Aldehyde Pheromone Deposited on Porapak. To establish the effectiveness of the bioluminescent assay in analyzing extracts of Porapak containing aldehyde pheromone, known quantities (2 ng to 2 μg) of (*E*)-11-tetradecenal in 10 μl of hexane were deposited on 200 mg of Porapak in a constricted glass tube and extracted immediately after solvent evaporation (~5 min). The effects of time and airflow on the recovery of the pheromone from the absorbent were determined by depositing 50 ng or 2 μg of the aldehyde on Porapak and leaving it for periods of up to 17 hr in an airflow of 2 liters/min before extraction and analysis.

Analysis of Airborne Aldehyde Pheromone. Either a fixed amount of (*E*)-11-tetradecenal in 10 μl hexane deposited on aluminum foil ($\sim$2 cm^2) immediately before an experiment or a 1-cm polyvinyl lure (r = 2 mm) containing 0.03% by weight of 96% (*E*)-11-tetradecenal and 4% (*Z*)-11-tetradecenal (Sanders, 1981) were used as sources of airborne pheromone. Four PVC lures (courtesy of Dr. C. Sanders, Great Lakes Forest Research Centre), stored at $-20°$, were equilibrated at room temperature (2–24 hr) before analyses of their individual release rates. At the time of analysis, the lures were 0–1 day old (cf. Figure 2, Sanders, 1981). Either of the above pheromone emitters were placed in the center of a glass tube (r = 1.5 cm, length = 8 cm) with an inlet and outlet of 4 mm radius. Porapak Q (200 mg) was placed directly in the outlet and maintained by glass wool in a restriction in the glass tube. In some cases, a second Porapak Q trap (200 mg) was placed either upstream in the inlet to prefilter the air or immediately downstream from the first Porapak Q trap to check for possible aldehyde breakthrough. The rate of airflow was determined by a calibrated flowmeter placed between the last Porapak Q trap and the source of vacuum.

RESULTS

Analysis of Deposited Pheromone. The bioluminescence response to extracts of Porapak was directly proportional to the amount of aldehyde deposited on the Porapak Q (Figure 1). This response is linear up to 2 μg. The lower limit of sensitivity, determined by the background response for the Porapak extracts, was approximately 2 ng.[4] The recovery of pheromone is relatively constant over the range investigated for any particular experiment, as shown by the close fit of the points to the line in Figure 1, with a correlation coefficient of 0.995 for the regression equation. Depending on the particular batch and/or treatment of the Porapak, the recovery was found to vary between 60 and 100%, with an average recovery of 90% in this experiment. Since the absolute luminescence response for Porapak extracts in LU/μg also varied to some degree with the preparation of Porapak and luciferase over the course (1 year) of these experiments (i.e., compare slope in Figure 1 with control given in Figure 2 legend), appropriate controls were run with each set of analyses (see Methods and Materials).

When air at 2 liters/min passed through the absorbent, the recovery of deposited pheromone decreased with time with the relative loss being

[4] The lower limits for detection of aldehyde are determined by the relative background responses and the proportion of the sample that was analyzed. In these experiments, only 6% of the Porapak extract was analyzed in each assay with the background response being fivefold higher than that observed for the assay medium itself, thus accounting for the differences in sensitivity from that reported earlier (Meighen et al., 1982).

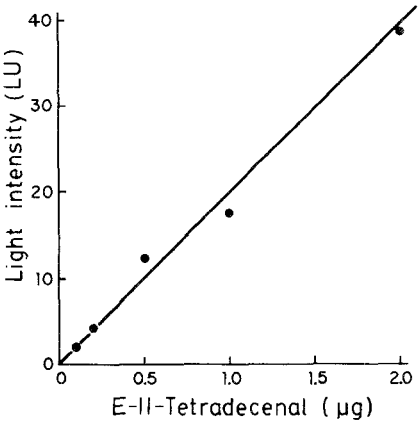

FIG. 1. Analysis of known amounts of (*E*)-11-tetradecenal deposited on Porapak Q and extracted within 5 min. Each point is the average of two independent experiments. Linear regression analysis gave a correlation coefficient of 0.995.

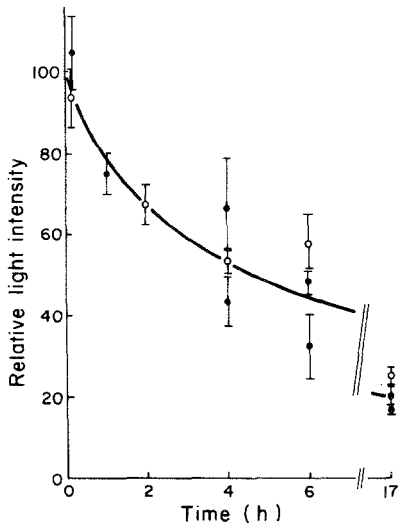

FIG. 2. Effect of time and airflow (2 liters/min) on recovery of two quantities (50 ng, ○; and 2 μg, ●) of (*E*)-11-tetradecenal deposited on Porapak Q. Each point represents the average of 4–6 independent analyses with the standard error of the mean given by the vertical bars. The data is given as the percentage of the average responses (1.6 LU and 62 LU) elicited by the same quantity of pheromone deposited on Porapak Q and extracted immediately.

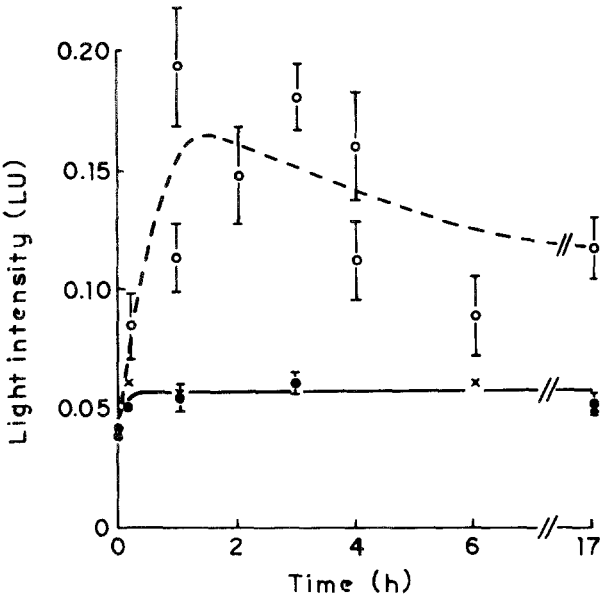

FIG. 3. Effect of prefiltering air (2 liters/min) through Porapak Q on the endogenous (background) response to extracts of a downstream Porapak Q trap with no exogenous aldehyde: with prefilter (●), without prefilter (○). In some cases, (*E*)-11-tetradecenal (2 μg) was deposited on the prefilter (x). Each point is the average of 4–6 analyses with the standard error of the mean given by the vertical bars.

independent of the amount of aldehyde (50 ng or 2 μg) initially deposited (Figure 2). This loss was somewhat greater at higher flow rates and less if the rate of airflow was decreased.

The chemical fate of the lost aldehyde was not determined. However, the decrease of bioluminescent response (i.e., lower aldehyde levels) was not due to the trapping of components inhibitory to the assay since addition of (*E*)-11-tetradecenal to extracts of untreated Porapak with airflow produced the same response as the addition of the same quantity of this aldehyde to extracts of untreated Porapak not previously exposed to air. Furthermore, analysis of extracts of a second Porapak Q trap placed downstream (Figure 3) showed that the decrease in the aldehyde levels was not due to "breakthrough," since the bioluminescent responses to extracts of the downstream trap remained constant.

This "breakthrough" test led to the discovery that the background responses of Porapak extracts of the downstream absorbent (0.05 LU) was about threefold less than the background response (0.1–0.2 LU) of the upstream absorbent (Figure 3). This means that analysis by bioluminescent assay of pheromone trapped on Porapak exposed to prefiltered air is about

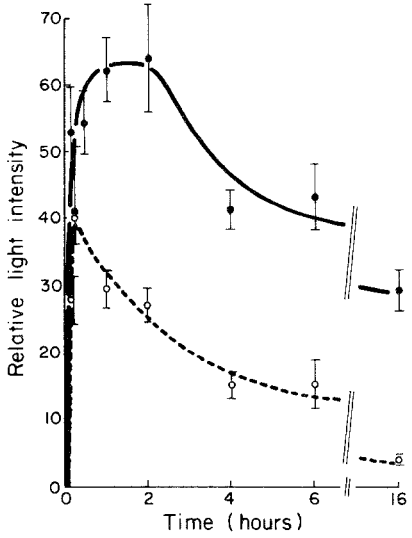

FIG. 4. Effect of time and prefiltration of air on recovery of airborne (*E*)-11-tetradecenal emitted from a tin foil surface (~2 cm^2) containing 2 μg of the pheromone. Air (2 liters/min) was either prefiltered through Porapak Q (●) or not filtered (○). Bioluminescent responses, with the standard error of the mean given by the vertical bars, are expressed as a percentage of the response elicited by 2 μg of the pheromone deposited on Porapak Q and extracted immediately.

threefold more sensitive than the analysis of pheromone trapped on Porapak exposed to laboratory air.

Analysis of Airborne Pheromone. When a fixed amount of (*E*)-11-tetradecenal was placed on foil in an airstream (2 liters/min) rather than deposited directly on the Porapak Q trap, most of the pheromone was trapped within the first hour (Figure 4), with the bioluminescence responses being up to 40% of that observed with control extracts of Porapak Q containing the same amount deposited on the absorbent and extracted immediately. Periods of airflow longer than 1 hr showed that there was a progressive loss in the amount of trapped aldehyde extractable from Porapak similar to that observed when the aldehyde was deposited directly on the Porapak. Higher recoveries of pheromone (>60%) were obtained if the air, also maintained at a flow rate of 2 liters/min, was prefiltered through a Porapak Q trap placed upstream to the aldehyde (Figure 4). Loss of pheromone at longer periods of time also was reduced when prefiltered air was used. Extracts of the glass tube and foil (after 1 hr) contained less than 5% of the pheromone.

The amount of (*E*)-11-tetradecenal trapped (for 1 hr) from a stream of prefiltered air was linearly dependent on the amount of pheromone placed in the airstream over the range investigated (50 ng to 2 μg) (Figure 5). The

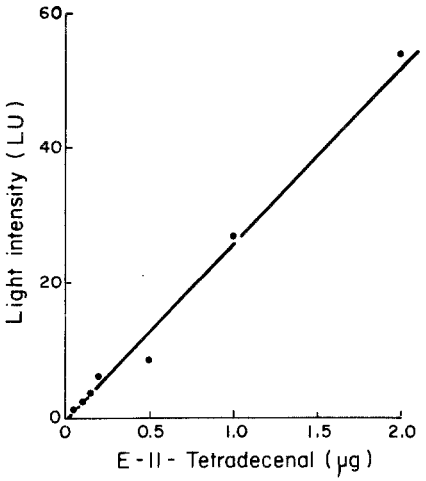

FIG. 5. Dependence of bioluminescence response on the amount of aldehyde trapped on Porapak Q. Known amounts of (*E*)-11-tetradecenal varying from 50 ng to 2 μg were placed for 1 hr in an airstream (2 liters/min) prefiltered through Porapak Q, and the extracts of the Porapak Q trap then analyzed. The average response for the aldehyde trapped on Porapak Q in four independent experiments is given in light units (LU). Linear regression analysis gave a correlation coefficient of 0.995.

bioluminescence response of the trapped pheromone in this particular experiment was 70–80% of the response of controls (2 μg) in which the (*E*)-11-tetradecenal had been deposited on Porapak Q and immediately extracted. This high recovery, combined with the quantitative relationship between the amount of pheromone trapped on Porapak and the amount released into the airstream, can be used to rapidly measure low rates of pheromone release from lures.

Analysis of Release Rates of Pheromone Lures. Figure 6 shows the bioluminescence responses with time to trapped aldehyde released from polyvinyl chloride (PVC) lures containing 0.03% by weight of spruce budworm pheromone (Sanders, 1981). Relatively low recoveries of pheromone were obtained if nonfiltered air was used (lower curves) and curvature can be seen in the plots at longer times. Much higher responses were obtained if prefiltered air was used with an essentially linear release of pheromone over a period of 8 hr, although curvature could be observed after 16 hr (data not shown). These results are consistent with the data shown in Figure 4, where lower recoveries of pheromone were obtained with nonfiltered air and there was a decrease in the trapped aldehyde with time.

The high recovery of pheromone using prefiltered air and the linear rate of release allows calculation of the release rate of the lures. From the slope of the plot in Figure 6 (2.2 LU/hr) and the standard curve in Figure 5 (24

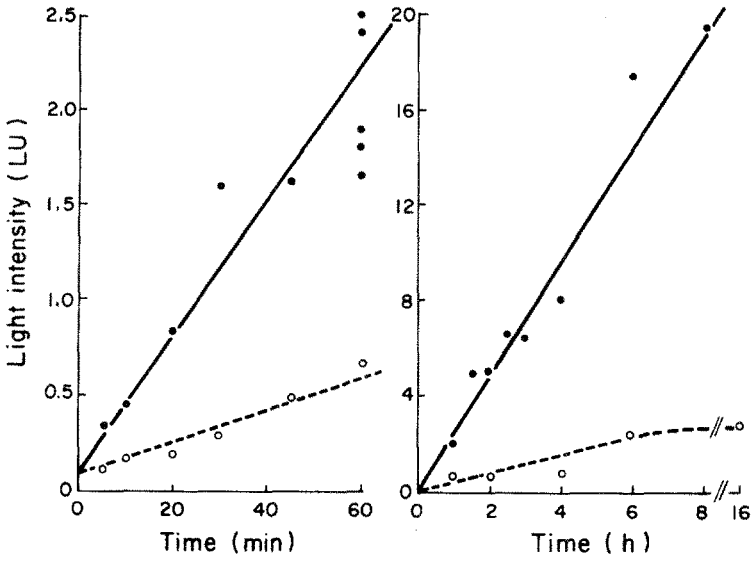

FIG. 6. Recovery on Porapak Q of spruce budworm pheromone released from PVC lures containing 0.03% pheromone. Air (2 liters/min) was either prefiltered through Porapak (●) or not filtered (○). Each point is the average of the bioluminescent responses for 4 lures equilibrated at room temperature (<24 hr) before analysis. Linear regression analysis of the upper lines (●) gave correlation coefficients of 0.92 (left) and 0.96 (right), respectively.

LU/μg), the release rate of 0.03% lures (0–1 day old) can be calculated to be 0.09 μg/hr or 2.2 μg/day. Sanders (1981, in Figure 2) reported a release rate of ~20 μg/day, measured by weight loss, for similar 1-day-old PVC lures containing 0.03% by weight of spruce budworm pheromone (*E*)-(11)-tetradecenal. Experiments by Weatherston et al. (1981) on the release rate of gossyplure from hollow fibers also showed that lower rates were obtained on quantifying trapped volatiles in comparison to methods involving residue analysis (e.g., weight loss).

DISCUSSION

The absorption of volatile material on Porapak Q and the subsequent analysis for trapped long-chain aldehydes by the bacterial bioluminescence assay provides a very rapid method for measurements of airborne aldehyde phermones. Using absolute amounts of aldehyde pheromone placed in an airstream, over 60% of the aldehyde could be trapped and then detected in the bioluminescence assay under optimal conditions (prefiltered air; <2 hr

trapping). Pheromone lures with rates of release of 0.1 μg/day or greater of aldehyde would easily be measured using this assay system.

Application of the bioluminescence system to measure airborne pheromone released into the atmosphere is more difficult and less sensitive (about sixfold), since the recovery of pheromone is lower and the endogenous background is higher when the air is not prefiltered. Longer periods of trapping (>1–2 hr) are not advantageous since the recoveries of aldehyde progressively decreased after about 1 hr of trapping. This loss in aldehyde pheromone is not due to the trapping of a component inhibitory to the bioluminescence assay, since aldehyde added to extracts of air-treated Porapak Q gave the same bioluminescence response as that added to untreated Porapak independent of the source of air (laboratory, greenhouse, or field air in contact with a variety of trees and plants). These studies suggest that trapping of atmospheric aldehydes should be conducted for only short periods of time (1–2 hr) for optimum results. Low recoveries (23%) of airborne pheromone have also been reported by Shapas and Burkholder (1978) for the aldehyde pheromone of *Trogoderma glabrum*. In contrast, high recoveries of nonaldehyde volatile compounds have been obtained on Porapak Q (Byrne et al., 1975), although relatively high quantities (in the mg range) were trapped in comparison to the present studies (<2 μg).

The bioluminescent assay system could be readily applied to measure other airborne aldehyde pheromones (Meighen et al., 1982; Grant et al., 1982), particularly for the direct analysis of the release rates of pheromone from lures or insects. Recent experiments have shown that both long-chain alcohols and acetate esters at low concentration can be converted enzymically with a high efficiency into long-chain aldehydes. Consequently, the use of the bioluminescence assay to measure long-chain alcohols and acetate esters should result in this system having a potentially wide range of applications for analysis of airborne pheromone.

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HAIRPENCIL PHEROMONE COMPONENTS OF MALE ORIENTAL FRUIT MOTHS, *Grapholitha molesta*[1,2]

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Abstract—Male *Grapholitha molesta* hairpencil components are ethyl *trans*-cinnamate, mellein, methyl jasmonate, and methyl 2-epijasmonate. The natural behavioral effect elicited by hairpencil-displaying males during courtship in attracting sex-pheromone-releasing females from several centimeters away can be duplicated by mixtures of ethyl *trans*-cinnamate in various combinations with the other components.

Key Words—Hairpencil, pheromone, *Grapholitha molesta*, Lipidoptera, Tortricidae, Oriental fruit moth, ethyl *trans*-cinnamate, mellein, methyl jasmonate, methyl 2-epijasmonate.

INTRODUCTION

Male Lepidoptera possess an unusually varied array of accessory scent-producing organs, usually groups of elongated, hairlike scales (hairpencils) that are bundled into special pouches, then everted and splayed in the vicinity of a female during courtship (Birch, 1974; Weatherston and Percy, 1977). Studies of most species have revealed, however, that the volatile chemicals from these scales exert a minimal observable effect on female behavior, and the lack of an overt female response has hindered the identification of behaviorally active constituents. In those cases where both the behavior and

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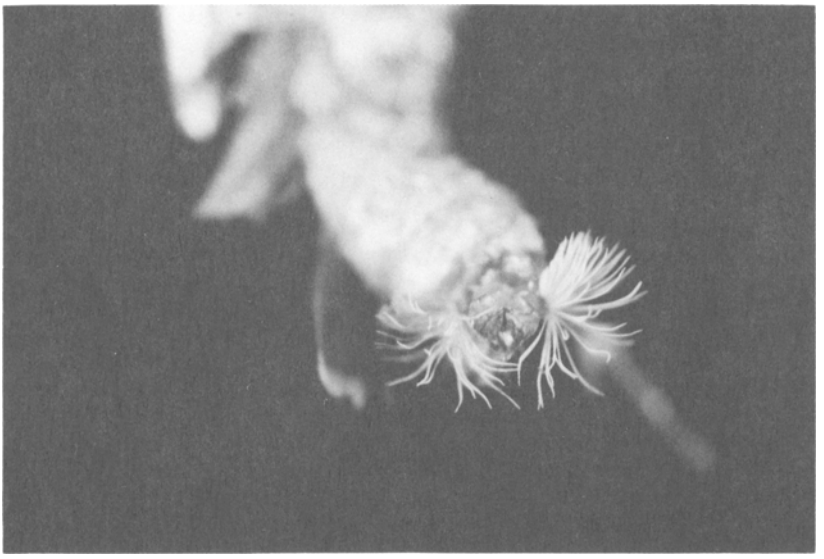

FIG. 1. Male *G. molesta* with extruded abdominal hairpencils.

chemistry have been worked out, the compounds have elicited female "acceptance" through inferred quiescence (Pliske and Eisner, 1969) or abdominal extension (Grula et al., 1980).

Courtship in *Grapholitha molesta* (Busck), the Oriental fruit moth, is thus far unique in the Lepidoptera, in that males attract females after they themselves have been attracted to the female's vicinity by her sex pheromone (Baker and Cardé, 1979). A few centimeters from the female, males turn away and repeatedly extrude and retract their abdominal hairpencils (Figure 1), propelling volatile chemicals over the female with wind generated from wing vibration. The female immediately walks toward the source of the odor and with her head touches the tip of the male's abdomen, evoking from him a copulatory attempt (Baker and Cardé, 1979). The overt movement of females toward displaying males provided a rare opportunity to define a lepidopteran courtship pheromone that attracts females (Baker et al., 1981).

METHODS AND MATERIALS

Crude Pheromone Extract. G. molesta males were reared on small green thinning apples on a 16:8 light–dark photoperiod regime at 25° C and variable humidity. Newly emerged males were held 5 days at 25° C (16:8 light–dark), and then each male's paired hairpencils plus claspers were excised and

immersed for several minutes in a vial containing Skellysolve B (Skelly B). The extract was removed from the residue and stored at −20° until use.

Behavioral Assays. Female *G. molesta* moths were held in isolation in 33 × 27 × 31-cm cages on a 16 : 8 light–dark photoperiod regime and transferred onto 3.8 × 3.8-cm sheet-metal platforms during their mating activity period beginning at 3 hr before darkness. The platforms were placed in a 25 × 30 × 5-cm cage in the wind flow beside a 25 × 25-cm sheet-metal observation arena for acclimation to the 20° C, 60–80% relative humidity, 700 lux light intensity, and 71 cm/sec wind. Individual 4 to 5-day-old females on their platforms then were placed onto the observation arena. A laminar wind field was created by cheesecloth baffles placed between the fan and arena.

Chemical treatments were prepared by placing 1 ng of authentic compound or 1 male equivalent (ME) of natural extract in 1 μl Skelly B followed by 5 μl of Skelly B onto a 5 × 7-mm filter paper (Whatman No. 1) skewered to a metal thumbtack. After solvent evaporation, the thumbtack was placed with forceps in the arena so that the treated filter paper hung just above the surface 2 cm upwind of a female. Females were scored as to whether they walked upwind and touched the paper; walked upwind to, but did not touch, the paper; began walking upwind; or did not move at all during a 10-sec exposure to the treatment. One filter paper preparation was used for two females and then discarded. Each day a treatment was tested against 1 ME crude extract and a 5-μl Skelly B blank. Females emitting sex pheromone ("calling") sometimes respond by walking to changes in wind flow (Baker and Cardé, 1979), such as produced by inserting a treatment, and so if crude extract responses were not significantly higher than the solvent blank on a particular day, the whole replicate was repeated. The arena surface and metal plates were washed with acetone between uses.

Electroantennogram Assays. EAG responses were obtained using the techniques previously described (Roelofs, 1977). Test samples (ca. 3 ME) were deposited in glass capillary tubes by evaporating 3 μl of a Skelly B solution in the tube. Samples were assayed by puffing 1 ml of air through the tube and into an airstream flowing over an antenna taken from a 3 to 5-day-old female *G. molesta* moth. A mean response amplitude to each fraction was obtained from nine replications of three repeated puffs each. Amplitudes were read from an oscilloscope or directly from digital display (Bjostad and Roelofs, 1980).

Chemical Analyses. Extracts of 1000 male equivalents were processed by removing the solvent under a stream of nitrogen and analyzing the residue by GLC. GLC was carried out using a 3% OV-101 glass column (methyl silicone on 100–120 mesh Gas-Chrom Q, 2 m × 4 mm ID) and a 3% XF-1150 glass column (cyanoethyl methyl silicone on 100–120 mesh Chromosorb W-AW-BWCS, 2 m × 2 mm ID). Proton magnetic resonance spectra were obtained with a Varian XL 100A (100 MHz) instrument using $CDCl_3$ as solvent and

tetramethylsilane as an internal standard. Mass spectra were recorded with an HP5985A GC-MS using a 30-m OV-101 capillary column.

Microhydrogenations were carried out by bubbling hydrogen vigorously through a solution of the sample (ca. 0.5 μg) in Skelly B(100 μl) containing a catalytic amount of platinum oxide at 0° C for 2 min. Ozonolyses were conducted by bubbling ozone through a syringe needle into a solution of sample (ca. 1 μg) in CS_2 (100 μl) for 30 sec. The reaction vial was held in a dry ice-acetone bath throughout the reaction. Excess triphenylphosphine was added and the reaction mixture concentrated under nitrogen for GC analysis. Epimerization of methyl 2-epijasmonate (IV) to methyl jasmonate (III) was effected by adding 2 μl of 1% *p*-toluenesulfonic acid in Skelly B to a solution of 20 ng of IV in Skelly B. The mixture was held at room temperature overnight, and then it was concentrated under nitrogen for GLC analysis.

Chemicals. Ethyl *trans*-cinnamate was obtained from Aldrich Chemical Co., and ethyl *cis*-cinnamate was prepared according to the procedure by Wittig and Haag (1955) to yield a 1:4.5 ratio of *cis–trans*. The mixture was separated by column chromotography using a 15% $AgNO_3$-Florisil column eluted with 10% ether in Skelly B. Ethyl 3-phenylpropanoate was obtained by Jones oxidation of 3-phenyl-1-propanal (Aldrich Chem. Co.) followed by ethylation with ethanol in the presence of a catalytic amount of sulfuric acid.

Samples of (+)- and (−)-mellein were obtained from Drs. D. Aldridge (ICI), N. Davis (Auburn U.), and J. Moore (U. North Alabama). (±)-Methyl jasmonate was provided by International Flavor and Fragrances, and methyl 2-epijasmonate was isolated from lemon peels (Nishida, Acree, and Roelofs, unpublished).

RESULTS

Isolation, Bioassay, and Identification of Hairpencil Components. Crude hairpencil extract from 5000 male *G. molesta* was processed and fractionated on the OV-101 GLC column (150° C) into 12 fractions (Figure 2). EAG assay (Figure 3) of these fractions showed female antennal response (0.6 mV ± 0.2 SD; $N = 9$) only for fraction 3, although the crude extract elicited a much higher response (1.25 mV ± 0.31 SD; $N = 9$).

Behavioral assays were used to test the fractions for their attractiveness to calling females in an arena in moving air. Only fraction 3 produced a significant amount (29%: $N = 35$) of upwind walking by females compared to the solvent blank (3%). After identification of the only component (compound I) found in fraction 3, synthetic I was added to the GLC fractions and various combinations thereof.

A blend of I (10 ng) with fractions 6 and 7 (3 ME each) gave significantly greater attraction of females (80% response; $N = 40$) than I (10 ng) alone

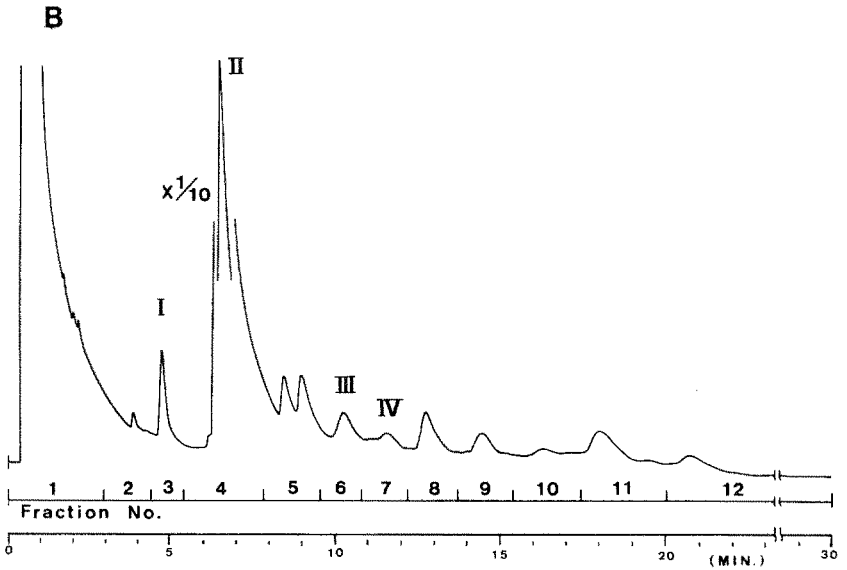

FIG. 2. GLC (OV-101) tracing of male *G. molesta* hairpencil extract.

(45%) (significantly different at the 5% level according to a χ^2 2 × 2 test of independence with Yates' correction). The response to a Skelly B blank ($N = 40$) was 13%. The components of fractions 6 and 7 were labeled compounds III and IV, respectively.

The predominant compound was found in fraction 4. This fraction was inactive by itself in bioassays, but this major hairpencil compound was labeled compound II and identified for testing in combination with the other active components.

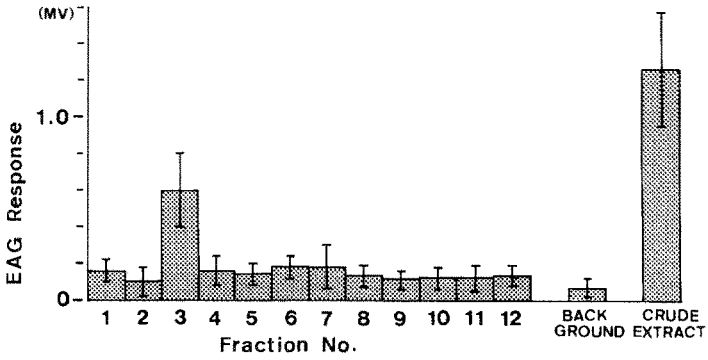

FIG. 3. Mean EAG response amplitudes to GLC fractions (Figure 2). $N = 27$; nine replicates of three responses to each fraciton in a randomized, complete-block design.

Compound I. The only observable compound in fraction 3 was present at a rate of 0.5 ng/male. The retention times on OV-101 and XF-1150 (4.8 and 3.9 min, respectively) were identical to those of ethyl *trans*-cinnamate, with no evidence of the *cis* isomer (3.3 and 2.9 min, respectively). The mass spectrum of compound I (Figure 5A) was identical to that of synthetic ethyl *trans*-cinnamate, as was the UV spectrum [λ_{max} 270 nm (ϵ 15,000) in Skelly B]. Additional evidence in identifying compound I to be ethyl *trans*-cinnamate (Figure 4) was that catalytic hydrogenation of compound I produced a product with GLC retention times identical to ethyl 3-phenylpropanoate on OV-101 and XF-1150.

Compound II. Compound II was isolated from fraction 4 at a yield of 20 ng/male. Removal of the solvent left a fine crystalline residue that was shown to be *R*-(−)-mellen (II, Figure 4) by instrumental analyses. A mass spectrum (Figure 5B) of compound II exhibited a molecular ion peak at 178, which was similar to that of authentic mellein (Brand et al., 1973). A UV spectrum of compound II in ethanol exhibited absorption bands at λ_{max} 210 nm (ϵ 20,000), 247 nm (ϵ 4000), and 313 nm (ϵ 5000), all of which showed a bathochromic shift in the presence of sodium hydroxide to λ_{max} 228 nm (ϵ 16,500), 252 nm (ϵ 4500), and 350 nm (ϵ 7000). The NMR spectrum was identical to that of an authentic sample. One proton singlet at δ 11.03 suggested the presence of a phenolic proton hydrogen-bonding with a carbonyl oxygen at an *ortho* position. Three aromatic protons were indicated in adjacent positions, since a proton at δ 7.41 (1H, double doublet, J = 7.3 and 8.4) was coupled with a proton at δ 6.90 (1H, doublet, J = 8.4) and at δ 6.70 (1H, doublet, J = 7.3). One methine proton at δ 4.73 (1H, sextet, J = 7) was indicated to be adjacent

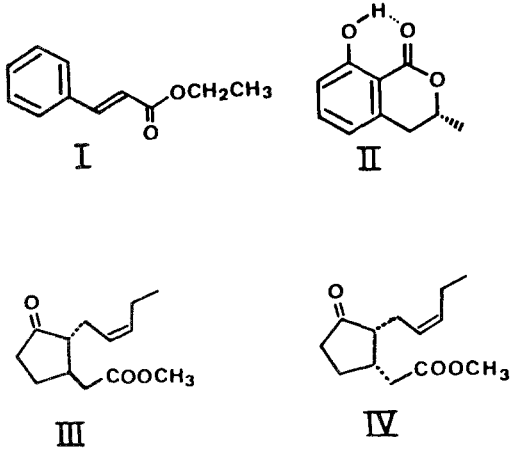

FIG. 4. Structures of the four components isolated from *G. molesta* hairpencils (Figure 2).

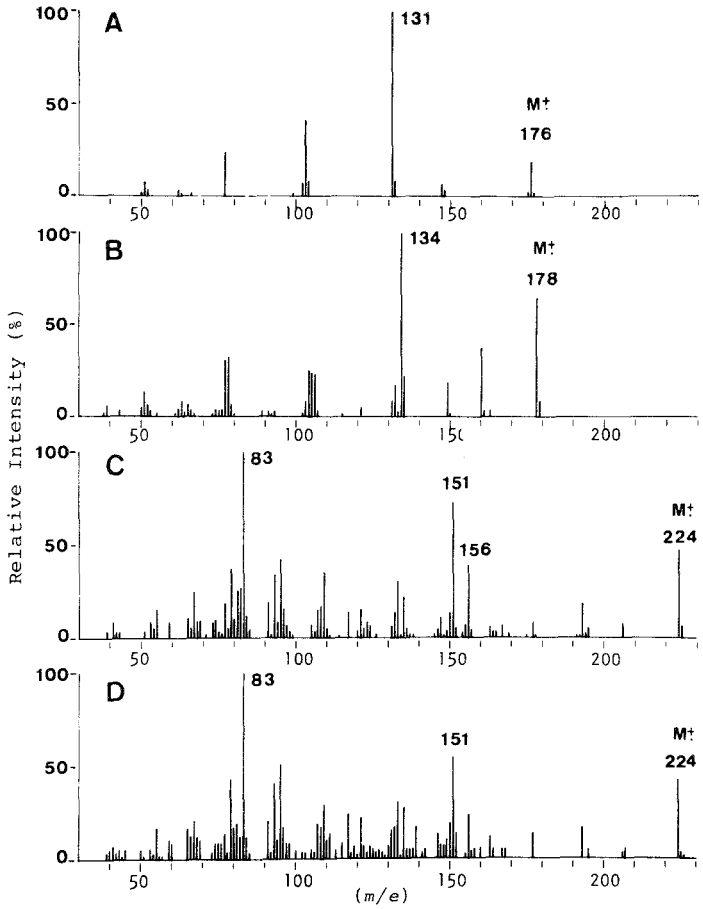

FIG. 5. Mass spectra of components isolated from *G. molesta* hairpencils.

to an oxygen atom and was coupled with secondary methyl protons at δ 1.53 (3H, doublet, $J = 6.3$) and benzylic protons at δ 2.93 (2H, doublet, $J = 7.0$). The optical rotation of II gave a negative sign $[\alpha] = -133°, c = 0.01$ in $CHCl_3$. This confirmed II to be *R*-(−)-mellein, since Arakawa et al. (1969) had determined that the absolute configuration of (−)-mellein was *R*.

Compounds III and IV. Fractions 6 and 7 (Figure 2) were characterized by a distinct herbal odor, similar to that obtained when the male *G. molesta* hairpencils are extruded. Compound III was isolated from fraction 6 (0.3 ng/male) by collection from the XF-1150 column and found to be methyl jasmonate (Figure 4, III) by the physical data and comparison to an authentic sample. The natural component was identical to that of an authentic sample of methyl jasmonate possessing a Z double bond (Table 1). The mass spectrum

TABLE 1. GLC (OV-101) RETENTION TIMES (MIN) OF HAIRPENCIL COMPONENTS III AND IV (FIGURE 2), THEIR DERIVATIVES, AND SOME SYNTHETICS

	III	Me jasmonate	*E* isomer	IV	Me 2-epijasmonate
Untreated	10.2	10.2	9.9	11.6	11.6
Hydrogenated	10.5	10.5		12.0	12.0
Ozonolysis-aldehyde	6.0	6.0			6.2
Epimerized				10.2	10.2

(Fig. 5C) exhibited a molecular ion at *m/e* 224 and was identical to that of the authentic sample. Ozonolysis of component III produced an aldehyde that was identical by GLC (Table 1) and mass spectrometry (M^+ 198) to that obtained by ozonolysis of the synthetic sample. Hydrogenation of component III produced a product whose mass spectrum (M^+ 226) and GLC retention time on OV-101 (Table 1) was identical to that of methyl dihydrojasmonate.

Compound IV was isolated from fraction 7 (0.01 ng/male) by collection from the XF-1150 column and identified to be methyl 2-epijasmonate. GLC retention times (Table 1) of the natural compound and its hydrogenated product were identical to those of authentic sample. Mass spectra of the natural sample and of its ozonolysis product were similar to those of the authentic samples, and compound IV was readily epimerized to III in the

TABLE 2. BEHAVIORAL RESPONSE OF *G. Molesta* FEMALES TO COMPOUNDS ISOLATED FROM MALE HAIRPENCILS[a]

	No. females walking upwind			No. females walking to source			No. females touching source		
Treatment	Treatment	Crude	Blank	Treatment	Crude	Blank	Treatment	Crude	Blank
I	11*	11*	4	7	10*	3	3	8	3
II	3	11	4	1	9	3	1	8	2
III	4	11*	2	1	10*	0	1	10*	0
IV	4	14*	3	4	10*	3	1	9	3
I+II	6	13*	3	4	12*	1	2	12*	1
I+III	5	8	2	3	6*	0	1	5	0
I+IV	16*	13*	2	12*	11*	2	10**	11*	2
I+II+III	12*	11*	0	7*	6*	0	6	3	0

[a]Treatments were 1 ng of each authentic compound and 1 ME for crude. $N = 20$ for each treatment. Responses are significantly different from the blank under the same behavior if followed by $^*(P < 0.05)$ according to a χ^2 2×2 test to independence with Yates' correction. Response under same behavior is significantly greater ($P < 0.05$) than to I alone if followed by **

presence of *p*-toluenesulfonic acid (Table 1) (Demole and Stall, 1962; Tanaka and Torii, 1975; Fukui et al., 1977). Mixtures of authentic III and IV had the characteristic odor of extruded hairpencils.

Biological Activity of Hairpencil Compounds. Authentic samples of compounds I–IV were tested alone and in combination for behavioral response activity with female *G. molesta* (Table 2). Compound I was the only synthetic to elicit significant female attraction, although it was less active than crude extract in behavioral responses of walking to the source and touching the source. Combinations of I + IV and of I + II + III were as active as crude extract in eliciting all behavioral responses.

DISCUSSION

This is the first report of characterized male moth hairpencil compounds that are behaviorally active in attracting females. Previous studies have identified the volatile chemicals extracted from hairpencils (Meinwald et al., 1966, 1969, 1971, 1974; Aplin and Birch, 1970; Culvenor and Edgard, 1972; Edgar et al., 1973, 1979; Grant et al, 1972; Jacobson et al., 1976; Bestmann et al., 1977; Petty et al., 1977) or other specialized scales (Lundgren and Bergstrom, 1975; Edgar et al., 1976; Honda, 1980) without knowledge of the behavior these compounds elicited. Other studies attempted to define the behavioral roles played by scent through ablation (Myers and Brower, 1969; Birch, 1970; Grant, 1974, 1976; Gothilf and Shorey, 1976; Hirai, 1977; Thibout, 1978; Ono, 1979; Rutowski, 1977, 1980), trapping experiments (Dahm et al., 1971; Finn and Payne, 1977), or observational inference (Clearwater, 1972; Pliske, 1975; Barrer and Hill, 1980).

Female EAG responses were helpful in determining the GLC area of activity later found to be a behaviorally active compound I, ethyl *trans*-cinnamate. Fraction 3 (I) was the only significantly EAG-active area from extracts (Figure 2). Female EAGs have been used in previous studies to measure responses to potentially behaviorally active male-produced compounds (Schneider and Seibt, 1969; Grant, 1970, 1971; Birch, 1971; Payne and Finn, 1977; Chow et al., 1980).

Although the behavioral activity of compounds I and IV is clear from the data, additional studies are needed to determine whether II and III further increase activity when added to the other two components. Both II and III were inactive alone or individually in combination with I, but in a trinary blend with I, elicited significant upwind movement from females.

The percentages of attraction of females might at first appear unduly low. However, even during normal courtship, many females do not respond to displaying males (Baker and Cardé, 1979). Also, a pulsed wind of 45–90 cm/sec did not accompany the chemical treatments as it does during a display; this additional wind significantly increases female locomotion (Baker and

Cardé, 1979). We do not know whether a pulsed pheromone emission pattern, most likely the case during display, would further increase the percentage of responding females.

The four identified compounds have been reported previously in the literature, but have heretofore not been found in the Lepidoptera. Ethyl *trans*-cinnamate is similar to 2-phenylethanol, which has been found in the hairpencils of a number of lepidopteran species (Aplin and Birch, 1970; Jacobson et al., 1976; Bestman et al., 1977; Edgar et al., 1979). Mellein is a known fungal metabolite (Nishikawa, 1933; Yabuta and Sumiki, 1933; Patterson et al., 1966; Aldridge et al., 1971; Cole et al., 1971) and has been found in a number of ant species (Brand et al., 1973). Methyl jasmonate is a constituent of jasmine oil and known in the perfume industry as the "queen of aroma." It closely resembles *cis*-jasmone, which was identified from male *Amauris ochlea* butterfly hairpencils (Petty et al., 1977).

Although methyl 2-epijasmonate can be isolated from lemon (Nishida, Acree, and Roelofs, unpublished) and methyl jasmonate from jasmine, and could possibly be present in fruits fed upon by *G. molesta* larvae, it is not known whether *G. molesta* males are dependent on their various fruit and nut tree host species for immediate precursors to the hairpencil compounds, which produce an herbal-like odor. Preliminary studies indicate *G. molesta* hairpencils from males reared on an artificial diet do not possess the characteristic herbal odor of hairpencils from males reared on the usual small green apples. Elimination of hairpencil extrusion prevents attraction of females (Baker and Cardé, 1979), and it is possible that a reduction of these compounds due to larval feeding on deficient hosts might affect male mating success. Danaidone, known to increase mating success in *Danaus gillippus* males (Pliske and Eisner, 1969) and apparently also a constituent of the hairpencil secretion of *D. chrysippus* (Meinwald et al., 1971), likely is metabolized from dihydropyrrolidizine alkaloids, which the latter adult males ingest when visiting dried *Heliotropium* plants (Edgar and Culvenor, 1975). Rigorous work would be needed to determine first whether the *G. molesta* courtship pheromone components are present in their fruit hosts and then whether they are ingested and sequestered through to adulthood and used as the odorous components that attract females.

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ALLELOPATHIC PROPERTIES OF α-TERTHIENYL AND PHENYLHEPTATRIYNE, NATURALLY OCCURRING COMPOUNDS FROM SPECIES OF ASTERACEAE

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Abstract—Alpha-terthienyl (α-T), a naturally occurring polyacetylene derivative from roots of *Tagetes erecta* L., and phenylheptatriyne (PHT), from leaves of *Bidens pilosa* L., were tested as possible allelopathic agents against four seedling species (*Asclepias syriaca* L., *Chenopodium album* L., *Phleum pratense* L., *Trifolium pratense* L.). *Asclepias* was the most sensitive of the species. Allelopathic activity was enhanced in the presence of sunlight or sources of near-UV, with LC_{50}s for *A. syriaca* of 0.15 ppm and 0.66 ppm with α-T and PHT, respectively; 0.27 and 0.85 for *C. album*; 0.79 and 1.43 for *P. pratense*, and 1.93 and 1.82 for *T. pratense*. Near-UV exposure was saturating but never more than found in summer sunlight at Ottawa, Canada. Growth inhibition was observed with seedlings treated with α-T and PHT but without near-UV irradiation. Germination of seedlings was also sensitive to α-T and PHT with or without near-UV treatment. α-T was extracted from soil surrounding the roots of *Tagetes*. Concentrations calculated for the soil (0.4 ppm) indicate that seedling growth could be significantly hindered. The activity and specificity of α-T was sufficiently high to warrant future field trials to assess its potential as a natural weed-control agent.

Key Words—Allelopathy, α-terthienyl, phenylheptatriyne, *Asclepias syriaca* L., *Chenopodium album* L., *Phleum pratense* L., *Trifolium pratense* L., *Tagetes erecta* L., *Bidens pilosa* L., Asteraceae, soil compounds.

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INTRODUCTION

Species of the Asteraceae are well known for their aggressive character and broad ecological amplitude. The family contains many species that are prone to become weedy, given a disturbed habitat. While the family is not noted for its importance as a food source, it is well known for its ability to compete successfully with agricultural crops (Frankton, 1971). Many species contain physiologically active compounds that have been utilized in folk medicine for their curative properties (Wat et al., 1980), while many of the substances synthesized by the family are toxic or show other significant physiological activity (Heywood et al., 1977).

During the past two decades many new compounds have been identified in the Asteraceae (Heywood et al., 1977), including, for example, polyacetylenes and their thiophene derivatives which find their greatest diversity in the family (Bohlmann et al., 1973). While a number of reports indicate that polyacetylenes are physiologically very active against other organisms, the function of such compounds in the plant has not been seriously questioned until very recently (Towers and Wat, 1978).

Recently, we have begun a systematic study of the biological activity of polyacetylenes which has revealed for the first time that many polyacetylenes show high cytotoxic activity under natural sunlight or near-UV light (Towers et al., 1977). The photosensitizing effect of the pure polyacetylenes has been demonstrated with nematodes (Gommers and Geerligs, 1973), bacteria and fungi (Towers et al., 1977), mosquito and black fly larvae (Arnason et al., 1981b), and selected marine and freshwater algae (Arnason et al., 1981a).

Only two reports in the literature were found that describe the effects of polyacetylenes on higher plants (Ichihara et al., 1976; Kobayashi et al., 1980). These workers found that C-10 polyacetylenes of composites, especially *cis*-dehydromatricariaester and *cis*-lachnophylumester were inhibitory to plant growth. The role of near-UV in this process was not investigated by these workers.

Many secondary metabolites are thought to have been selected for in plant evolution because they provide a protective advantage against pathogens, insects, and competing plants, a fact that seems to have been ignored in this technical age of synthetic biocides. By contrast, in many parts of the Third World the toxic effect of certain plants is used in folk medicine to control infections and in agriculture to control insect pests (Towers and Wat, 1978). Because synthetic biocides are coming under increasing scrutiny regarding their possible long-term effect in both natural and agroecosystems, we extended our study of the allelopathic properties of polyacetylenes and derivatives to their herbicidal effects on two selected forage plants and two weeds.

The compounds used in the study (Figure 1) were α-terthienyl (α-T), a

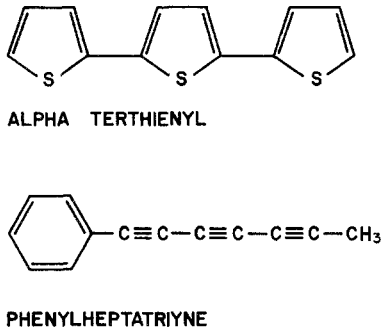

FIG. 1. The structure of alpha-terthienyl (α-T) and phenylheptatriyne (PHT).

thiophene derivative naturally occurring in roots of the common marigold (*Tagetes* spp.), and phenylheptatriyne (PHT), from the leaves of *Bidens pilosa* L. (Bohlmann et al., 1973). *B. pilosa* is a pantropical weed that is a serious problem in agriculture in the humid tropics in the Central American lowlands. The compounds were tested against seedlings of *Asclepias syriaca* L. and *Chenopodium album* L., two common weeds, and *Phleum pratense* L. and *Trifolium pratense* L., two common forage species of eastern North America.

METHODS AND MATERIALS

Plant Sources. Dehiscing pods of *Asclepias syriaca* were collected in Ottawa, Ontario, Canada. The seeds were stratified in distilled water at 3° C for three weeks to induce a high level of germination. *Chenopodium album* seeds were supplied by Agriculture Canada from sources in southern Ontario. *Phleum pratense*, *Trifolium pratense*, and *Tagetes erecta* seeds were obtained from a local farm seed supplier. *Tagetes* was grown in a greenhouse from seed, while *Bidens pilosa* was harvested from wild populations growing in vacant lots in Miami, Florida.

Chemicals. The α-terthienyl was isolated and purified from *Tagetes erecta* root tissue (Chan et al., 1975), and phenylheptatriyne was obtained from leaf tissue of *Bidens pilosa* (Towers et al., 1977).

Survival Curves. To determine the toxic effects of α-T and PHT on seedlings of the four plant species, germinated seedling of identical length (10 cm) were placed in Petri dishes lined with Whatman No. 1 filter paper. Since the activity of α-T and PHT is enhanced by near-UV, one trial consisted of polyacetylene and near-UV exposure. The UV exposure was omitted in one trial to observe dark effects of the pure compounds. The toxic effect of near-UV exposure was monitored by running trials without α-T or PHT treatment. Controls were run without UV exposure and without polyacetylene

treatment. EtOH was added to the controls to monitor its possible toxic effects.

Solutions of the polyacetylenes were made from stock diluted in Hoagland's solution to achieve the following concentrations: 10, 5, 1, 0.1, 0.8, 0.5, and 0.01 ppm. Five milliliters were sprayed directly on the seedlings in each plate. There were 100 seedlings per trial.

The near-UV exposure was made using four Westinghouse blacklight blue tubes (Westinghouse F20T12/BLB). After a 16-hr exposure of the uncovered seedlings, all plates were then placed in a growth chamber with a temperature regime of 22° C–30° C and a 16-hr photoperiod (fluorescent and incandescent). Seedling death was assayed each day for five days. The experiments were carried out in quintuplicate. From the data collected, probit plots were obtained from which LC_{50} and LC_{90} values were determined for all plant species.

Using a YSI 65-A radiometer and a Kodak Wratten filter #2A, incident UV radiation of less than 400 nm was estimated to be $3 W/m^2$. Similar measurement on solar radiation indicated that the UV component of summer sunlight at Ottawa (45° N latitude) was the same order of magnitude ($8 W/m^2$, measured at midday on a clear day in June).

Germination Studies. The effect of α-T and PHT on germination were tested as follows. Seeds for each plant were placed in Petri dishes lined with Whatman No. 1 filter paper. A total of 800 seeds were separated into four sets of plates. The plates were moistened with 5 ml of the appropriate polyacetylene solutions. Three concentrations were used: 10, 1, and 0.1 ppm, with or without near-UV. The near-UV exposure was 8 hr, after which the seeds were covered in foil and placed in the growth chamber under conditions identical to the seedling trial. Germination was assayed over a 2-week period. The experiment was carried out in triplicate.

Light Saturation curve. To test the effect of varying near-UV exposure on the toxicity of the polyacetylenes, *Asclepias syriaca* was chosen since it was the most sensitive of all seedlings tested. One hundred seedlings of identical length were chosen and 0.3 ppm α-T was applied as stated previously for the survival curves. Plates were exposed to near-UV light for periods varying from 2 to 24 hr. Seedling death was assayed each day for five days. The toxicity levels of α-T were considered to have reached saturation when little increase in toxicity could be observed with increased near-UV exposure. The experiment was carried out in triplicate.

Growth Curves. Seedlings of the four species were placed in Petri plates containing Hoagland's solution with α-T and PHT concentrations of 100, 30, 10, 3, 1, and 0.3 ppm. Treatments and controls were established as previously described for the survival curve. The near-UV treatment was given for 6 hr each day for 14 days. The fresh weight was taken at the end of the fourteenth

day. Graphed results represent percent mean growth of the test seedlings versus the controls. Ten seedlings were measured for each data point.

Extraction of Active Compounds from Soil. One hundred grams of soil were obtained from soil surrounding the roots of *Tagetes* and run through a 2-mm sieve to remove root particles. The resulting soil was suspended in 200 ml of ethanol and mixed in a shaker for 24 hr. After filtration the ethanolic solution was extracted with 500 ml of petroleum ether which was subsequently brought down in vacuo to a 5-ml-thick residue. Phototoxic activity was assayed by a standard assay method using yeast (Chan et al., 1975).

The α-T was separated by thin-layer chromatography of 100 μl of soil extract. A reference α-T solution was developed with the extract chromatograms. All plates were developed in 1:9 anhydrous ethyl ether and petroleum ether on silica gel G-25 UV254. A second solvent system (1:1 hexane and chloroform) was used to confirm the presence of α-T in the extract. Plates were analyzed under UV light to detect the blue fluorescing compound α-T. To quantitate the relative amounts of α-T in the soil, the TLC fluorescent spots were scraped from plates and eluted with ethanol and determined spectrophotometrically. The complete procedure was repeated three times.

RESULTS

The probit plots (Figure 2) represent data points that lie between 10 and 90% seedling survival for seedlings treated with α-T or PHT in the presence of near-UV radiation. At least three points of toxicity were found for α-T and PHT for the four species tested. The symptoms of α-T and PHT damage were remarkably similar in all four species. *Asclepias syriaca* showed the most acute signs of damage, characterized by necrosis of the roots and chlorosis of the leaves. The leaf response of *Trifolium pratense*, *Chenopodium album*, and *Phleum pratense* was similar. However, no root damage as seen in *Asclepias syriaca* was observed. The final criterion for seedling death was a complete chlorosis and wilting of leaf and stem tissue. No "dark effect" was observed at the concentrations used and no toxicity was observed with the near-UV controls. In all cases, it was difficult to obtain data points in the intermediate toxicity range and many replicates were needed.

LC_{50} and LC_{90} values (Table 1) show that *A. syriaca* was the most sensitive species followed by *C. album*, *T. pratense*, and the monocot *P. pratense*.

Figure 3 represents the effects of varying the exposure time to near-UV of *Asclepias syriaca* seedlings treated with 0.3 ppm α-T. Minimal increases in seedling death were observed after 16 hr of near-UV exposure (2.5 W/m^2). Saturation was assumed to occur at this point, and all experiments involving seedling mortality had this minimum level of UV exposure.

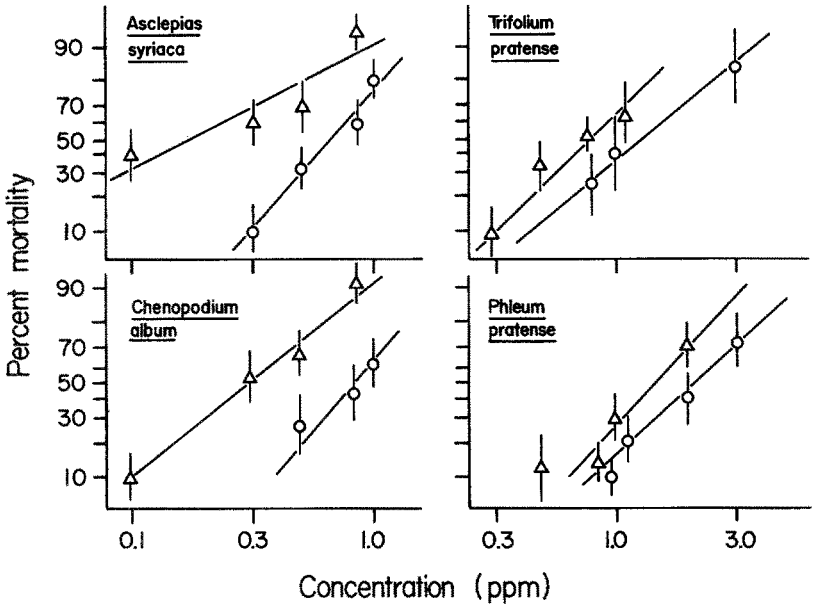

FIG. 2. The log of seedling mortality over seedling survival (probit plot) for four plant species exposed to varying concentrations of α-T (triangles) and PHT (circles). Near-UV exposure was 16 hr at 3 W/m^2. Data points represent intermediate toxicity between 100% seedling survival and 100% seedling mortality. Standard error is included.

TABLE 1. LETHAL CONCENTRATION LEVELS FOR SEEDLINGS TREATED WITH α-T AND PHT EXPOSED TO NEAR-UV.

Test species	α-T	PHT	Lethal conc.
Asclepias	0.15	0.66	LC^{50}
syriaca	0.79	1.51	LC^{90}
Chenopodium	0.27	0.83	LC^{50}
album	0.83	1.57	LC^{90}
Phleum	1.82	2.88	LC^{50}
pratense	3.63	5.08	LC^{90}
Trifolium	0.79	1.43	LC^{50}
pratense	1.99	3.63	LC^{90}

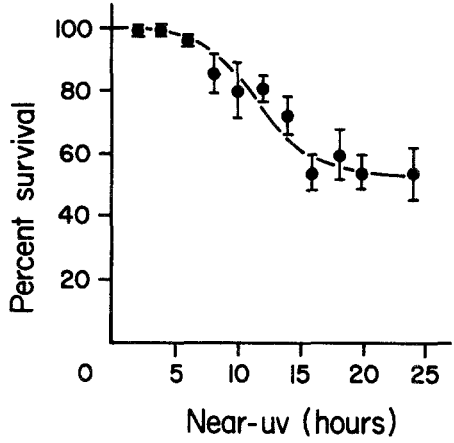

FIG. 3. The effect of near-UV exposure on seedling survival of *Asclepias syriaca* treated with 0.3 ppm α-T. Intensity was 3 W/m^2.

Even under conditions which are nonlethal, α-T and PHT possess demonstrable allelopathic potential (Figures 4 and 5). Growth inhibition is shown for α-T and PHT treatment without near-UV irradiation or with daily near-UV fluences that are approximately one-half saturation (8 hr) for the phototoxic effect (Figure 3). Without near-UV treatment, α-T and PHT inhibit the growth of all species at concentrations of approximately 10 ppm and higher, with α-T showing somewhat greater activity. With near-UV treatment, a similar pattern of growth inhibition was observed at lower concentrations.

Germination of the four test species was also sensitive to α-T or PHT treatment (Table 2). As with the growth experiments, significant inhibition of germination was observed even without near-UV treatment. Surprisingly, *C. album*, the smallest seed, was the least sensitive.

The extracts of soil taken from pure stands of *Tagetes* were found to have phototoxic activity in assays using yeast. Thin-layer chromatography of the crude extract revealed the presence of two blue fluorescent spots. Cochromatography with authentic α-T revealed that the lower spot was α-T ($R_f = 0.66$ with ethyl ether–petroleum ether, 1:9; and $R_f = 0.80$ for chloroform–hexane, 1:1; UV absorption bands at 253 and 350 nm). The upper spot ($R_f = 0.81$ with ethyl ether–petroleum ether, 1:9; and $R_f = 0.89$ with chloroform–hexane 1:1; UV absorption bands at 253 and 346 nm) was tentatively identified as the bithienyl. By using published values for the extinction coefficient (Bohlmann et al., 1973), the concentration of α-T in the soil was estimated to be 0.48 ± 0.21 ppm. A recovery estimate of α-T placed in soil and extracted by an identical procedure gave a value of 81%.

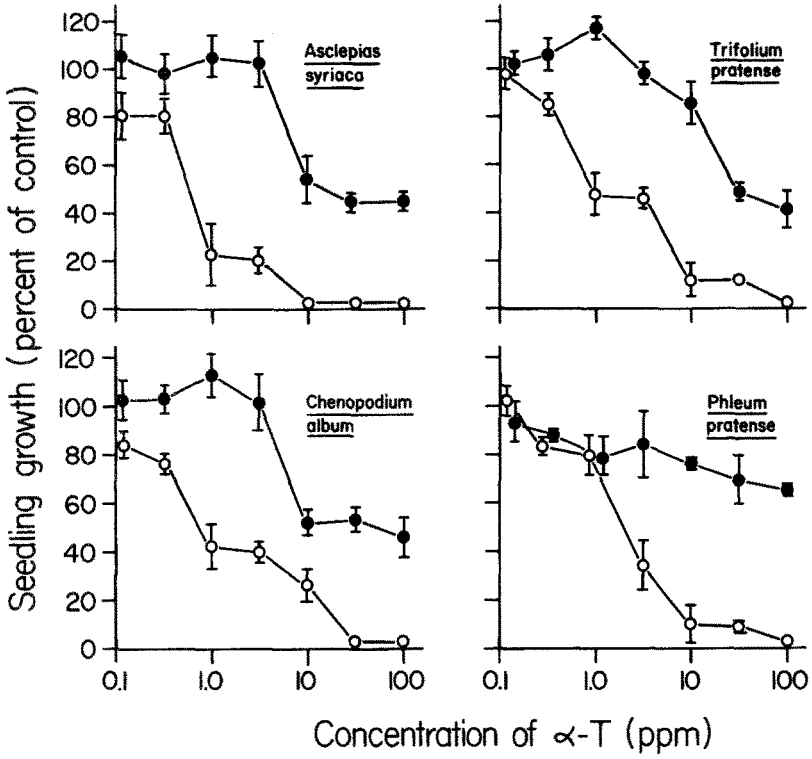

FIG. 4. The growth of four seedling species (fresh weight) as a percent of control growth is plotted against the concentration of α-T in the growth medium. Near-UV exposure (open symbols) was for 8 hr/day for 14 days. Plants given no near-UV treatment are represented by closed circles. Growth was monitored after 14 days. Each data point represents combined results of ten seedlings.

DISCUSSION

To demonstrate that a naturally occurring plant compound is allelopathic, it must first be shown that it is toxic towards competing plants. The probit plots indicate that both α-T and PHT are toxic towards four species of seedlings when irradiated with near-UV. Although probably not the natural competitors of *Bidens* or *Tagetes*, the species are in widely separated families and demonstrate the broad spectrum of activity of these compounds. In addition, the growth curves and germination study indicate that these compounds are inhibitory with or without near-UV exposure.

The role of near-UV in the action of allelopathic substances requires clarification. These compounds could be absorbed from the soil by newly emergent competing seedlings to a degree where the sun's near-UV rays could

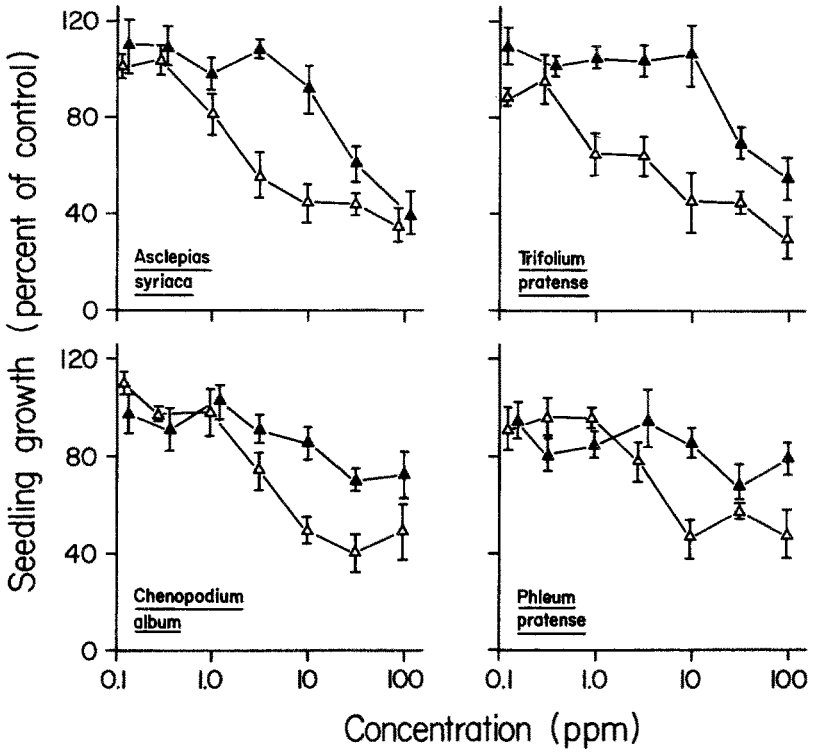

FIG. 5. As in Figure 4 using PHT.

TABLE 2. EFFECT OF α-T AND PHT ON SEED GERMINATION[a]

Species	Concentration (ppm)	α-T		PHT	
		+UV	−UV	+UV	−UV
Ascelepias syriaca	10	19.4 ± 4.9	46.8 ± 6.3	42.1 ± 3.8	54.4 ± 8.4
	1	48.7 ± 8.1	63.2 ± 9.4	83.1 ± 3.4	79.6 ± 4.5
	0.1	96.2 ± 1.8	101.1 ± 2.0	98.2 ± 7.3	104.7 ± 0.9
Chenopodium album	10	49.2 ± 3.4	100.1 ± 8.8	106.0 ± 1.7	101.1 ± 0.6
	1	81.6 ± 8.1	97.6 ± 1.3	99.1 ± 2.9	87.8 ± 1.1
	0.1	100.4 ± 7.4	102.6 ± 2.1	100.1 ± 0.9	97.4 ± 4.8
Phleum pratense	10	27.4 ± 4.6	48.8 ± 6.1	61.4 ± 6.4	69.1 ± 8.8
	1	81.3 ± 6.2	100.1 ± 1.1	100.0 ± 0.7	97.9 ± 2.2
	0.1	100.9 ± 8.4	99.7 ± 9.1	102.0 ± 1.2	96.1 ± 0.9
Trifolium pratense	10	22.1 ± 4.4	59.1 ± 7.7	59.0 ± 8.8	71.0 ± 10.1
	1	76.4 ± 8.3	92.9 ± 3.4	84.0 ± 10.1	97 ± 4.4
	0.1	97.4 ± 5.1	103.8 ± 1.7	99.0 ± 1.2	101.0 ± 0.3

[a]Germination expressed as percent of control ± SE.

invoke photosensitized damage. Growth inhibition shown by the dark activity of α-T and PHT also indicates that significant effects on competing seedlings could occur even without the presence of UV light. However, the near-UV radiation levels from the lamps are not as high as the near-UV irradiation from the sun incident at the earth surface, an important consideration when one looks at the natural functions of these compounds. Saturation occurred at much higher doses with plants than previously found for other organisms such as mosquito larvae (1 hr) (Arnason et al., 1981b). This effect may be due to the presence of UV screening compounds such as flavonoids in the plants.

The second stage in identifying an allelopathic compound is to demonstrate that the chemical is present in the soil in sufficient quantitities to inhibit growth after being released from the plant body. There are four known methods by which plants can release these compounds into the soil (Tukey, 1969). They are: (1) the decomposition of leaf litter through mechanical or biological means; (2) highly volatile compounds could be vaporized and exert their effects on other plants; (3) the roots could release the compounds by direct exudation or by mechanical or biological decomposition; and (4) leaching of the leaves by rain to the soil. The most likely possibility for *Tagetes* spp. is (3), since α-T is found in such high concentrations in the root tissue. For *Bidens pilosa*, (1) and (4) are the two most likely since PHT is found at high concentrations in the leaf tissue. PHT is also found in the roots of other Asteraceae (Bohlmann et al., 1973).

In the case of *Tagetes*, α-T was found in the soil of pure stands in concentrations as high as 0.4 ppm, which would be more than enough to inhibit competing seedlings if one considers the role of near-UV. One could also consider the possibility that competing seedlings could absorb these lipophilic compounds and concentrate them in membranes to highly inhibitory levels. No pure stands of *Bidens* were available, so soil assays of PHT were not attempted.

The next step in assessing the allelopathy of α-T is to show that it is active not only in controlled in vitro experiments but also under natural conditions. We are currently investigating this problem and attempting to determine how the host plant protects itself from autotoxic effects.

We are also investigating the toxic mechanism for both α-T and PHT. Alpha-T was found to be more phytotoxic in near-UV to the plant seedling than PHT, which agrees with previously published data on polyacetylene phototoxicology (Arnason et al., 1980). Also, work on the mechanism of action of α-T and related polyacetylenes has revealed that the action spectrum for photosensitization resembles the absorption spectrum with the photoreceptor molecule being the polyacetylene or thiophene compound. The research to date has related known photosensitizers to the activity of these compounds. There are two known groups of photosensitizers, the largest being the photodynamic compounds which produce toxic species of oxygen

like OH· (hydroxyl radical) and $^1O_2$ (singlet oxygen). The second group does not require O_2 to exert damage, like the furanocoumarins whose toxicity is caused by a UV-induced cross-linking of DNA. Alpha-T has been shown to be photodynamic, as anaerobic conditions inhibit its activity and quenchers of activated species of O_2 such as SOD and NaN_3 lower toxicity (Arnason et al., 1980). PHT photosensitization studies indicated survival of *E. coli* was enhanced in aerobic conditions. In addition, SOD and NaN_3 did not effect the survival curves. Thus activated species of oxygen do not appear to be involved in the toxic process, and PHT is nonphotodynamic in vivo. Studies indicate, however, that there is no cross-linking of DNA as in photosensitization with the furanocoumarins (Towers et al., 1977). It is thus believed that PHT represents a new class of photosensitizer, possibly producing toxic free radicals.

Asclepias syriaca was the most sensitive of the seedlings tested, while the monocot *P. pratense* was the least sensitive of all seedlings exposed to α-T and PHT in the presence of near UV. The ratio in LC_{50}'s for these two species is 12:1 for α-T. This selectivity is potentially useful since milkweed is a common problem of overgrown pastures in eastern North America. Herbicides such as 2,4-D are not very effective against this species and cutting leads to regeneration of plants from the adventitious root systems.

Alpha-terthienyl was found to have such high toxicity that additional experiments are now being conducted to ascertain its potential as a natural weed control agent through potted plant and weed plot field trials. The use of allelopathic agents poses an alternative in weed control where the potential for biodegradation and reduced environmental impact are attractive aspects of using natural products in pest control.

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SEX PHEROMONE OF EUROPEAN GRAPEVINE MOTH (*Lobesia botrana*) Its Chemical Transformations In Sunlight and Heat

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Abstract—Photo- and radical isomerization of (*E*, *Z*)-7,9-dodecadien-1-yl acetate (DDA) leads to an equilibrium mixture of all four possible geometric isomers of 7,9-DDA in the ratio of *E*,*E*, 69–76%; *Z*,*E*, 11–13%; *E*,*Z*, 12–15%; and *Z*,*Z*, 1–3%. Iodine catalysis of the isomerization takes place even in dark at room temperature and is probably a radical reaction.

Key Words—(*E*, *Z*)-7,9-Dodecadien-1-yl acetate, European grapevine moth, *Lobesia botrana*, Lepidoptera, Tortricidae, sex pheromone, photoisomerization, conjugated diene.

INTRODUCTION

Pheromones are probably biodegradable and in many cases are also chemodegradable via oxidation, isomerization, hydrolysis, etc. These chemical transformations can reduce the biological activity of the pheromones to a low level, thus causing them to be ineffective in pest control. The typical conditions in the field are strong and direct sunlight, high temperature (from 25–30°C up to 45–50°C), presence of oxygen, and in some cases "aqueous atmosphere" because of irrigation or rainfall. Thus, all possible pathways of pheromone loss on field application (besides volatility) should be taken into consideration. A study of the chemical transformation of (*Z*,*E*)-9,11-tetradecadien-1-yl acetate (9,11-TDDA), the main component of the sex pheromone of the Egyptian cotton leafworm (*Spodoptera littoralis*), under field conditions, has been published recently (Shani and Klug, 1980a,b). In this paper we describe the photo- and radical isomerization of synthetic (*E*,*Z*)-7,9-dodecadien-1-yl acetate (I), the sex pheromone of European grape-

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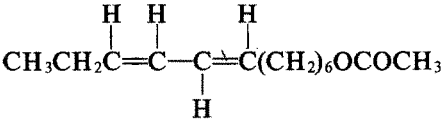
vine moth (EGVM) (*Lobesia botrana*), first identified by Roelofs and Arn (Roelofs et al., 1973; Buser et al., 1974) and shown to be very active in the field in Israel (Ideses et al., 1982).



I

METHODS AND MATERIALS

UV spectra were determined with a Cary 17 in cyclohexane solution. GLC analyses were performed with a Packard model 417 on a stainless-steel capillary column of DEGS SCOT 15 m × 0.5 mm, flow rate (He) of 2.7 ml/min at 130°C, and on 5% FFAP on Chromosorb W, stainless-steel 2-m × 3-mm column, flow rate (Ar) of 30 ml/min at 150°C. Retention time of the four 7,9-isomers on the capillary column were as follows: *Z,E*, 19.1 min; *E,Z*, 20.0 min; *Z,Z*, 20.8 min; and *E,E*, 21.2 min. Aliquots of 1 μl were injected from a diluted sample. Volume of solvent in tested samples was adjusted daily to the original volume (because of slow evaporation). Preparative TLC was used to purify different samples by loading 30–80 mg of crude material on a 20 × 20-cm glass plate with a thickness of 1 mm. The eluting system was 10% ether in petroleum ether (60–80°C) with recovery of ca. 80%. Petroleum ether (60–80°C) was dried over $CaCl_2$ and distilled. Ether was dried over $CaCl_2$, then on Na, and distilled. Cyclohexane (AR grade) and toluene (CP grade) were used without purification.

Illumination in Sunlight and Heating at 50° C. Samples of 100 mg of DDA (I) were dissolved in 5 ml cyclohexane in a Pyrex flask and illuminated in sunlight or submitted to continuous heating in an oil bath at 50°C. The changes were followed by GLC.

Illumination and Heating with Antioxidant. The same solutions as above were prepared, and 100 mg of BHT (2,6-di-*tert*-butyl-4-methylphenol) and 100 mg of BHA[3(2)-*tert*-butyl-4-hydroxyanisole] were added, each to a different sample. Illumination, heating, and monitoring were conducted as above.

Heating with Thiophenol. A solution of 50 mg of DDA and 1 drop of thiophenol in 5 ml toluene was heated at 100°C under N_2 atmosphere. Samples were withdrawn and quenched by solid K_2CO_3 and then injected to the GLC.

Isomerization of DDA with I_2 in the Dark. A solution of 5 mg of I_2 in 2.5 ml cyclohexane was added rapidly (in the dark) to a solution of 100 mg of

DDA in 2.5 ml cyclohexane. Samples were withdrawn immediately, quenched by $NaHSO_3$ solution, and then injected to the GLC.

RESULTS AND DISCUSSION

Based on our experience with 9,11-TDDA, we studied the effect, on I, of light and heat, in the presence of air (oxygen) and radical sources under inert atmosphere: (1) illumination in direct sunlight; (2) illumination in direct sunlight with antioxidant; (3) heating at 50°C; (4) heating at 50°C with antioxidant; (5) heating at 100°C with thiophenol; (6) mixing with iodine in the dark at room temperature; and (7) illumination in direct sunlight with rose bengal (Ideses et al., 1982a). All reactions, except (3) and (4), showed geometrical isomerization as summarized in Tables 1 and 2 and illustrated in Figures 1–5. The main features of these results are as follows. Heating alone did not cause any change in isomer composition (Table 1 entries 3, 4) but caused fast decomposition of the pheromone (Table 2, entry 4) which could be reduced by addition of BHA as antioxidant (Table 2, entry 5). Illumination caused rapid decomposition (Table 1, entry 1; Table 2, entry 1) which was reduced by addition of antioxidant, BHA being more effective than BHT

TABLE 1. ISOMERIZATION OF (*E*,*Z*)-7,9-DDA BY VARIOUS METHODS

		Percentage of components[a] by GLC[b]			
Method	Period	*E*,*Z*	*E*,*E*	*Z*,*E*	*Z*,*Z*
1. Direct sunlight	3 days[c]	87	13		
2. Direct sunlight with BHA	45 days	18	64	14	4
3. Heating at 50°C	10 days	90	10		
4. Heating at 50°C with BHA	30 days	90	10		
5. Heating at 100°C with thiophenol	30 min	15	69	13	3
6. Mixing with I_2 at room temperature					
In sunlight	4 hr	12	75	11	2
In dark	26 hr	12	75	11	2
7. Direct sunlight with rose bengal	2 hr	12	76	12	<1

[a]Composition of starting mixture of *E*,*Z*:*E*,*E* isomers is 90:10.
[b]Composition at equilibrium.
[c]Decomposition is much faster than isomerization, as no DDA was detected in GLC or in TLC after 3 days.

TABLE 2. EFFECT OF HEAT, SUNLIGHT, AND OXYGEN ON STABILITY OF (*E*,*Z*)-7,9-DDA

Tested mixture[a]	Conditions	Period (days)	Total 7,9-DDA (% by TLC)
1. (*E*,*Z*)-7,9-DDA	Sunlight	6	<1
2. (*E*,*Z*)-7,9-DDA + BHT	Sunlight	17	<1
3. (*E*,*Z*)-7,9-DDA + BHA	Sunlight	40	20
4. (*E*,*Z*)-7,9-DDA	50°C	10	6
5. (*E*,*Z*)-7,9-DDA + BHA	50°C	30	50

[a]Each sample contained 100 mg of DDA and 100 mg of antioxidant (when mentioned) in 10 ml cyclohexane.

(Table 2, entries 2 and 3). Extended illumination in the presence of antioxidant caused isomerization towards the equilibrium mixture (Table 1, entry 2). Isomerization was also caused by heating with thiophenol (Hall et al., 1980), illumination in the presence of rose bengal, and by adding iodine, even in the dark (Table 1, entries 5–7). The equilibrium mixture of geometric isomers contains 69–76% of the *E, E,* 11–13% of the *Z, E,* 12–15% of the *E, Z,* and 1–3% of the *Z, Z* isomer.

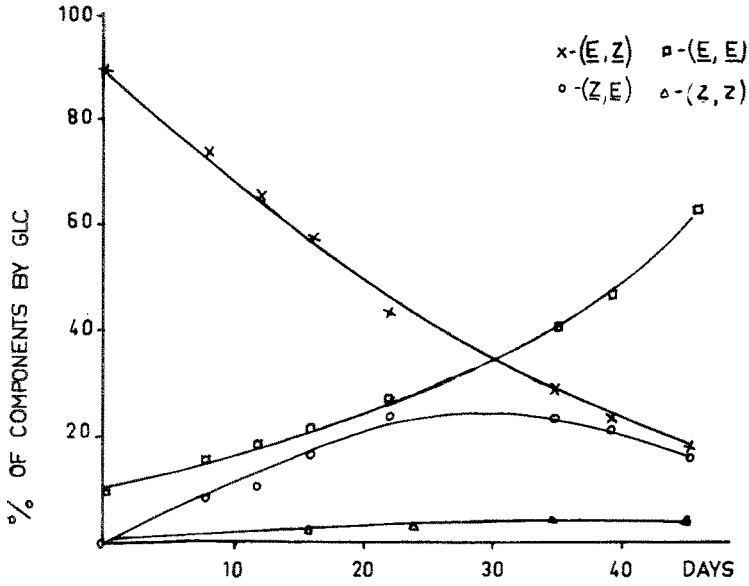


FIG. 1. Photoisomerization of (*E*, *Z*)-7,9-DDA in sunlight in the presence of BHA as an antioxidant.

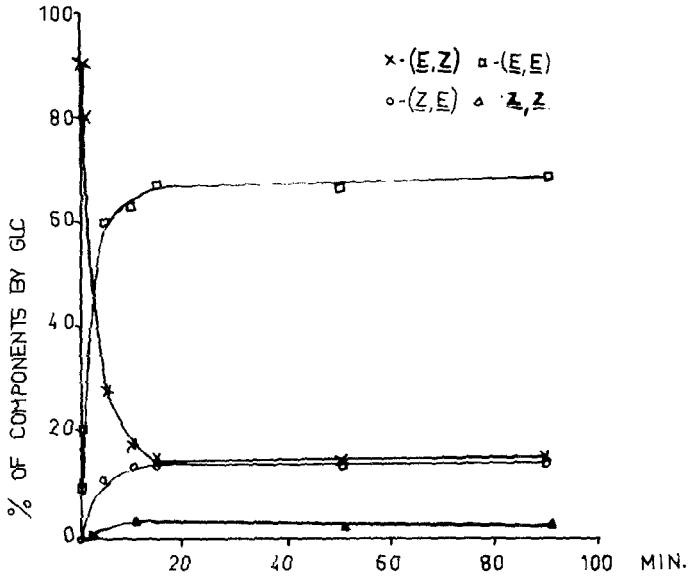


FIG. 2. Isomerization of (*E*, *Z*)-7,9-DDA with thiophenol.

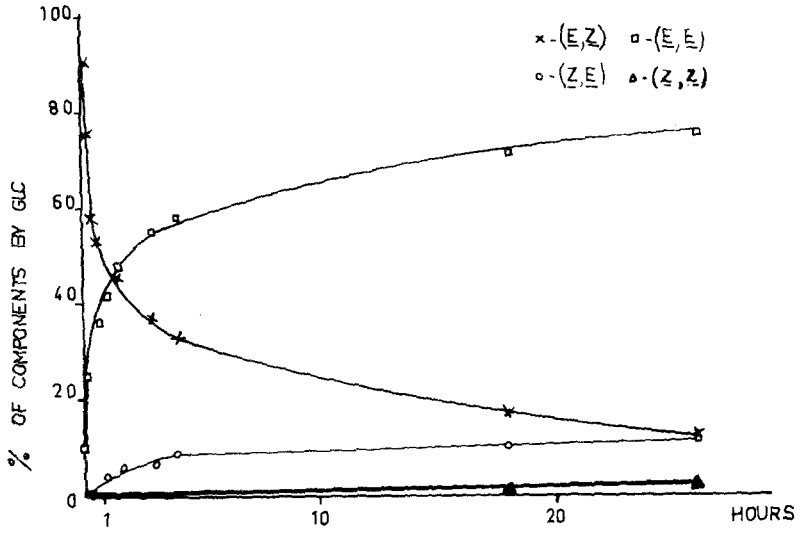


FIG. 3. Isomerization of (*E*, *Z*)-7,9-DDA with I_2 in dark.

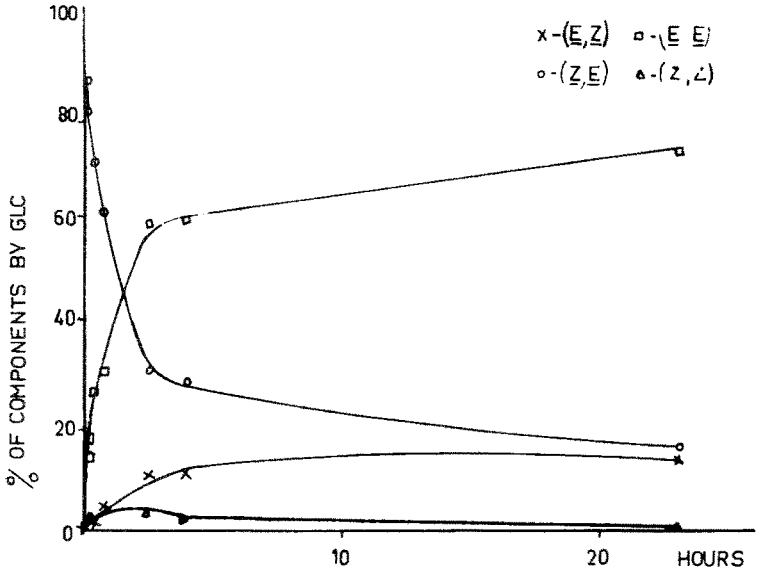


FIG. 4. Isomerization of (*Z*, *E*)-9,11-TDDA with I_2 in dark (sex pheromone of Egyptian cotton leafworm).

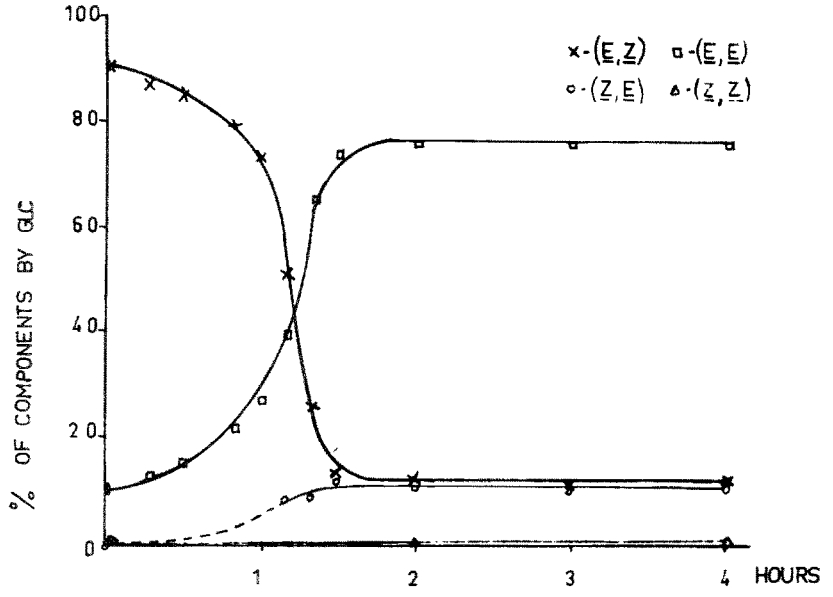


FIG. 5. Photoisomerization of (*E*, *Z*)-7,9-DDA with rose bengal in sunlight.

Iodine is considered to be an isomerization catalyst in light. However, we have found that the process proceeded in the dark and at room temperature. The possibility of π-complex or charge transfer (CT) (Benesi and Hildebrand, 1949) of iodine and π electrons of the diene unit was ruled out as no change was detected in the UV spectrum of I in the presence of iodine. These two chromophores absorbed at $\lambda_{max}^{C_6H_{12}}$ 232.5 (30,700) (DDA) and 521.8 (1016) (I_2).

This result implies a radical mechanism for isomerization by iodine. We might rationalize this by the weak I–I bond (37 kcal/mol) (March, 1977) as compared to S–H (82 kcal/mol) (March, 1977). Thiophenol is a source of radicals at 100°C, and it could be assumed that some fragmentation such as $I_2 \rightleftharpoons 2I\bullet$ occurs at room temperature even in the dark.

We thus reconfirmed that isomerization of conjugated dienes to an equilibrium mixture is catalyzed either by a radical mechanism (Table 1, entries 5 and 6) or by electronic excitation, either by direct irradiation (Table 1, entries 1 and 2) (Shani and Klug, 1980b), or by energy transfer (Table 1, entry 7).

We assumed a radical mechanism for the oxidation of DDA under heating without an antioxidant (Ideses et al., 1982a). The importance of the antioxidant is in preventing this oxidation and deterioration of the pheromone, but it does not stop or prevent photoisomerization (Shani and Klug, 1980b) (Table 1, entry 2; Figure 1). As in the case of *Spodoptera littoralis*, the *E,E* isomer does not interfere with the biological activity (Shani and Klug, 1980b; Ideses et al., 1982). It also appears that about 3–4% (18% × 20%) (Table 1, entry 2; Table 2, entry 3) of the starting DDA is still present in the solution after 40–45 days. This coincides with the drastic drop of the pheromone activity in the field, when the pheromone carriers of EGVM are replaced in the field traps. Similar amounts of active 9,11-TDDA were found in solution and on carriers after 1 month (Shani and Klug, 1980b).

The agreement of the semiquantitative results from the field and the laboratory might be explained by the assumption that two factors (1) and (2) compensate for each other: (1) the carriers are usually not under direct sunlight so less isomerization and deterioration of the pheromone is expected; and (2) the pheromone is being volatilized from the carriers, a process which does not occur in solution.

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PHEROMONE ATTRACTION IN THE SOYBEAN CYST NEMATODE *Heterodera glycines* RACE 3

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Abstract—Male *Heterodera glycines* responded to female nematodes during in vitro bioassay. The male's response was dosage-dependent and significant with a pheromone source of more than five females. Male responsiveness was influenced by the pheromone diffusion and response times. Males were most responsive at three days after emergence from the host plant, while females were also most attractive at the same age. Light intensities that ranged from dark to bright had no effect on female location by the male, although bioassay in a nitrogen atmosphere eliminated sexual communication. Mate location was not significant below 25° C and declined slightly at 30 or 33° C. Bioassay at pHs from 5 to 8.5 showed a bimodal effect, with maximal attraction around pH 6.

Key Words—Nematode pheromones, nematode, mate location, *Heterodera glycines,* soybean cyst nematode.

INTRODUCTION

No current investigations of *Heterodera* pheromones are evident despite the broad economic significance of these nematodes and the initiation of study over a decade ago. Since insect management through pheromone-based confusion of the male appears practical, a similar biorational approach to control of injurious nematodes would seem feasible after sufficient study.

Green (1966) initially reported the attraction of male *Heterodera rostochiensis* and *H. schachtii* to their females by in vitro bioassay. The pheromone of female *H. schachtii* was less labile or more concentrated than that of *H. rostochiensis.* The pheromone of *H. schachtii* appeared nonvolatile in a second bioassay system and thus moved through an aqueous agar medium (Green, 1967). In contrast, Greet et al. (1968) reported that the attractants had

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a volatile component in *Heterodera*. Regardless of this conflict, the authors knowledgeably standardized their procedures by dose–response analysis of female pheromone activity from *H. rostochiensis* and *H. schachtii*.

H. rostochiensis males were also slightly attracted to each other in bioassay but were usually more attracted to one of a group of females. Grouped females exhibited increased attractiveness for responding males. However, multiple males that were responding to female pheromone obstructed each other during copulatory attempts (Green et al., 1970).

Green and Greet (1972) reported that female *H. schachtii* and *H. rostochiensis* produced pheromone throughout their body, and thus concluded the chemical component(s) originated in the hypodermis or pseudocoelom. However, the tail of *H. schachtii* was more attractive, which suggested that the egg sac may act as a pheromone carrier.

Perhaps the most thorough study of nematode pheromone diversity to date was performed by Green and Plumb (1970). Cross-specific pairings of ten *Heterodera* species were conducted by in vitro bioassay. Based on the male responses, at least six attractants were postulated with the likelihood of one additional substance.

The above investigations suggest that a well-defined system of pheromone communication is present and probably necessary in various species of *Heterodera*, as one might suspect from the sedentary behavior of the female helminths. Accordingly, we have recently initiated preliminary research on pheromone-mediated reproduction in the soybean cyst nematode *Heterodera glycines* Race 3. A pheromone-based scheme of biological control of this worm should prove environmentally appealing and would complement the current control practices.

METHODS AND MATERIALS

Soybean plants (Union cultivar) were raised in 2 to 3-in. clay pots with a sterile sand–vermiculite mixture in growth chambers at 28°C and a 18:6 light–dark cycle. Hoagland's solution was given about every four days by foliage feeding, and plants were watered daily. When the first pair of trifolate leaves expanded, the soil around the host plant was inoculated with 50–100 manually crushed cysts of *Heterodera glycines* Race 3.

After 13–15 days for nematode development, infected plants were removed and their roots were rinsed. Then the roots were suspended in 250–450 ml of aerated water. Other inoculated plants were left in the original container for maintenance of the life cycle.

Initially, males were recovered from the aerated solutions at 1–3 days postemergence for usage as pheromone responders. Females were taken from the roots at 1–5 days postemergence for bioassay as a pheromone source. Females were removed by clipping a 3-mm segment of root.

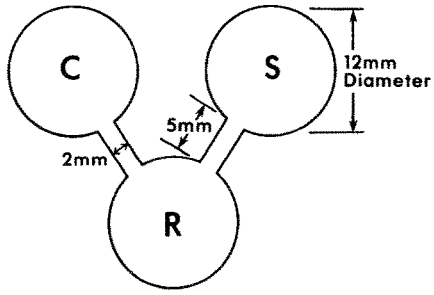

FIG. 1. Bioassay chamber for pheromone attraction of *H. glycines*. C = control chamber for roots only, S = pheromone source chamber for females and roots, R = response chamber for males.

Bioassay devices were modified from Samoiloff et al. (1973) and were lab-fabricated from Plexiglas. The chambers consisted of three 12-mm circles that were connected at a 45° angle by 2-mm-wide and 5-mm-long channels (Figure 1). The chambers were filled with 1.2 ml of 0.2% sterile nutrient agar (Fisher). Determination of initial dose–response conditions were obtained by placing 1, 3, 5, 10, 12, 15, or 20 females in the left or right chamber and equivalent amounts of roots in the opposite side as a control for any host response. After 8 hr for pheromone production and diffusion, single male *H. glycines* were placed in the middle chamber. Their bioassay responses were determined after 14, 24, and 36 hr. All bioassays were conducted under uniform light at ambient temperatures (24–25° C) unless otherwise stated. The responses for each dosage of females were obtained by subtracting the males that responded to the root controls from the males that responded to females and dividing by the total trials. Thus, percentages in this study represent net positive responses. Forty replicates were performed for each dosage.

Based on initial results, subsequent investigations employed the 24-hr male response to an established 8-hr pheromone gradient from females. The influences of nematode age were determined by collecting males on the initial day of emergence with maintenance in tap water for 1, 2, 3, 4, or 5 days prior to bioassay of their responses to a dosage of 10 females at one day of age. At least 20 replicates were done for each male age.

Pheromone production by females was studied by the recovery of females at 1, 3, 6, 12, or 17 days postemergence. Males were removed daily to reduce mating. Dosages of 10 females were used to obtain the responses of 3-day-old males. Twenty replicates or more were performed for each female age.

Other conditions were tested by a standard bioassay with 10 females at one day postemergence as a pheromone source for the response of 3-day-old males. Twenty replicates were done for each of the following treatments.

Temperature effects on the pheromone system were investigated by bioassay at 16, 20, 25, 30, and 33° C. Incandescent light intensities of 7.5, 4.7, 1.9, 0.5, and 0 Joules/m^2 sec were used to examine any influence on pheromone communication. Illumination of the bioassay chambers was determined with a YSI-Kettering radiometer (model 65A) in an enclosed room. No temperature differences were noted. Bioassay of the males' response in a nitrogen atmosphere was conducted in a vacuum vessel after purging with nitrogen.

The pH of the bioassay medium was altered to examine any changes in mate location. Agar at several pHs from 5 to 8.5, after adjustment with 0.1 N hydrochloric acid or 0.1 N sodium hydroxide, was used to examine any effect of pH on mate location. Some bioassays were conducted in media which were buffered at pH 6 and 6.5 with sodium phosphate (dibasic) and potassium hydrogen phosphate (monobasic) to prevent changes of the pH by the females.

Data were evaluated by linear regression or analysis of variance. The 0.05 probability level was considered significant.

RESULTS

Initial response of male *H. glycines* after 24 hr to various dosages of female worms is given in Figure 2 for an 8-hr diffusion period. The male's response to increased number of females was linear ($r = 0.94$). One or three females of *H. glycines* elicited only a slight response which was not different from zero (MSE = 10.5). However, the use of over five females caused a significant male response. Maximal male responsiveness (67%) was observed with a 12-female dosage.

Bioassay of high number of females revealed reduced attractiveness to the male. Female dosages of 15 and 20 caused 15 and 33% male responses, respectively. This may represent a bioassay artifact that is caused by male habituation or sensory adaptation with extreme pheromone dosages.

The influences of time on the male responses were determined after an 8-hr period for pheromone diffusion for selected female dosages (Figure 3). No significant male responses were found to three females until 36 hr of bioassay time (MSE = 3.5). Male responsiveness to a pheromone source of five or more females significantly increased at all examined times (MSE = 3.4). However, no significant difference was found between 24 and 36 hr of response when 12 females were used as a pheromone source. Thus, the male's response in bioassay is dependent on the female dosage and an adequate period for response. Subsequent experimentation was standardized at a 24-hr period for male responses.

The bioassay responses to a dosage of 10 females differed with the postemergence age of the tested males (Figure 4). The responses of 1- or

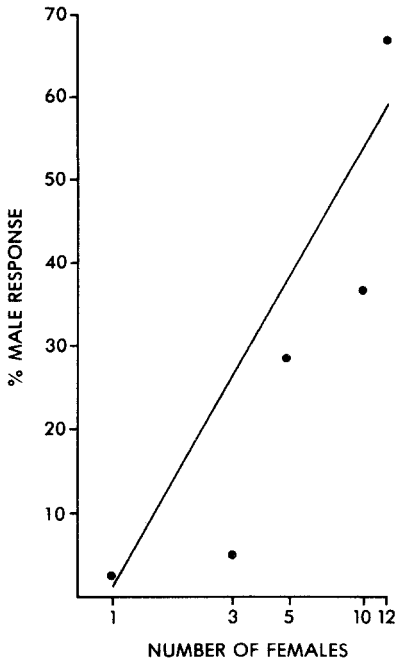

FIG. 2. Bioassay responses of male *H. glycines* to various dosages of female nematodes (MSE = 10.53).

2-day-old males did not differ from each other, but were significantly less than older ages. Maximal responses were obtained from males at three days postemergence. Four- or 5-day-old males were identical in their responses, but less responsive than 3-day-old males.

Males were most responsive to female *H. glycines* that were one or 3 days of age (Figure 5). No difference was found between these ages. However, the male's attraction to 6-day-old females was reduced by 71% while that to older females was not significant (MSE = 8.76). Based on these results, pheromone production by females diminishes with age or as the cyst becomes tanned.

Figure 6 shows the response of males to females at various light intensities. No differences were observed, and all male responses are within the range that was expected from dosage–response analysis (MSE = 2.47). Although *H. glycines* occupies a subterranean habitat, the pheromone system is functional with bright to moderate illumination as well as dark. Thus, light has no effect on bioassay at the tested levels.

However, environmental temperature has a pronounced influence on chemocommunication (Figure 7). Our results indicated that there was no significant male responsiveness to females or no female production of

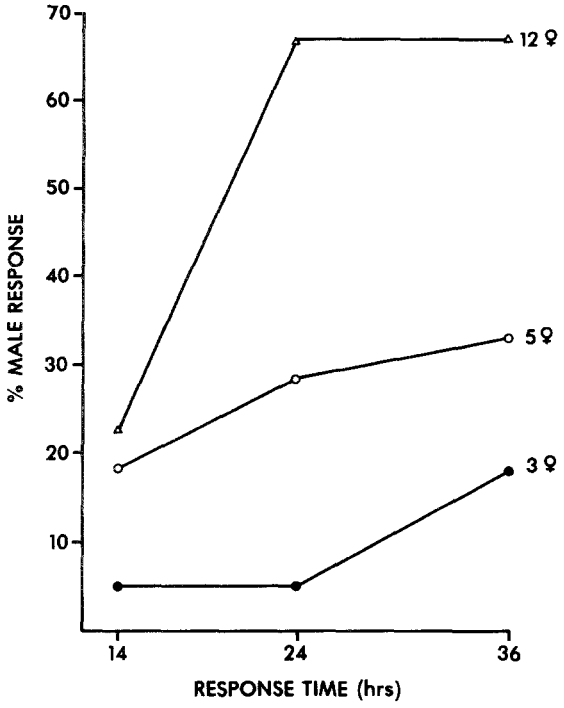

FIG. 3. Bioassay responses of male *H. glycines* to selected dosages of female nematodes after 14, 24, and 36 hr (MSE = 2.01, 3.49, and 11.7, respectively).

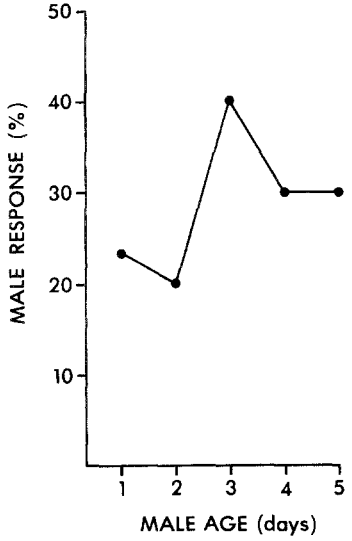

FIG. 4. Bioassay responses of males of various postemergence ages to a pheromone source of *H. glycines* females at three days postemergence (MSE = 3.03).

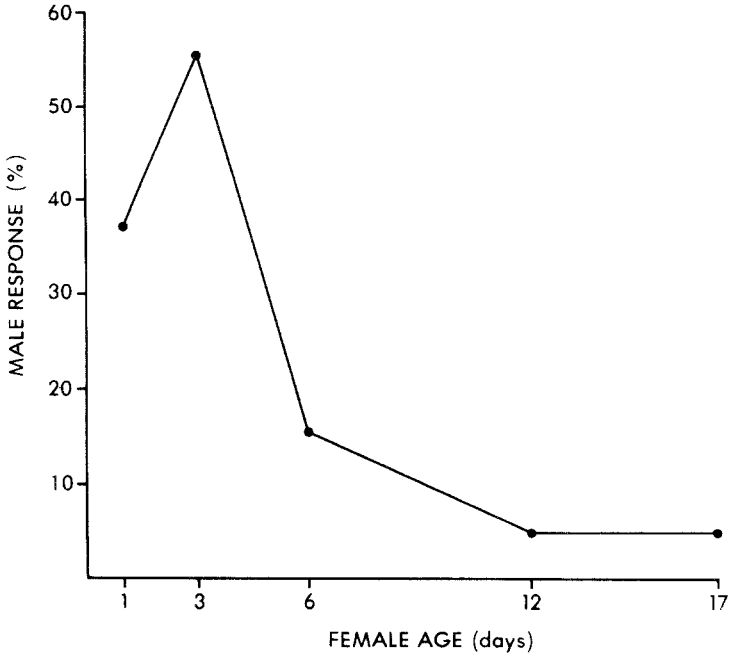

FIG. 5. Bioassay responses of 3-day-old males to *H. glycines* females of various postemergence ages (MSE = 8.76).

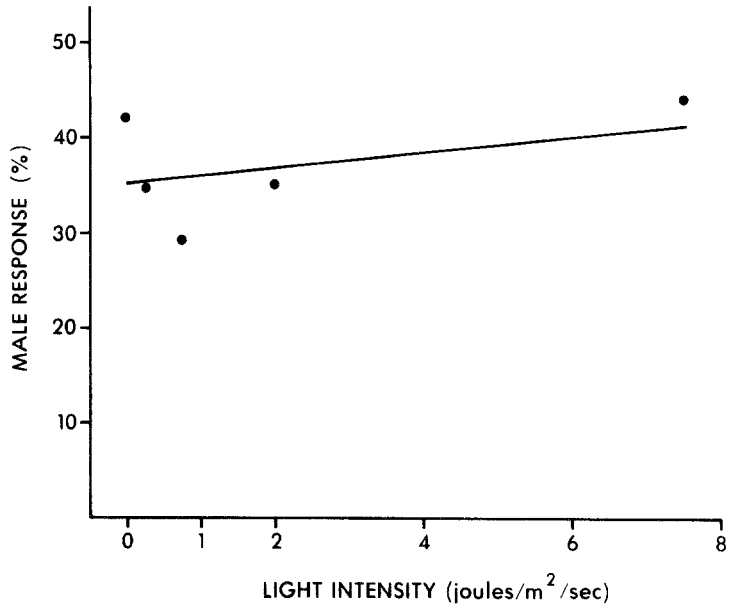

FIG. 6. Bioassay responses of male *H. glycines* to females at different light intensities of bioassay (MSE = 2.47).

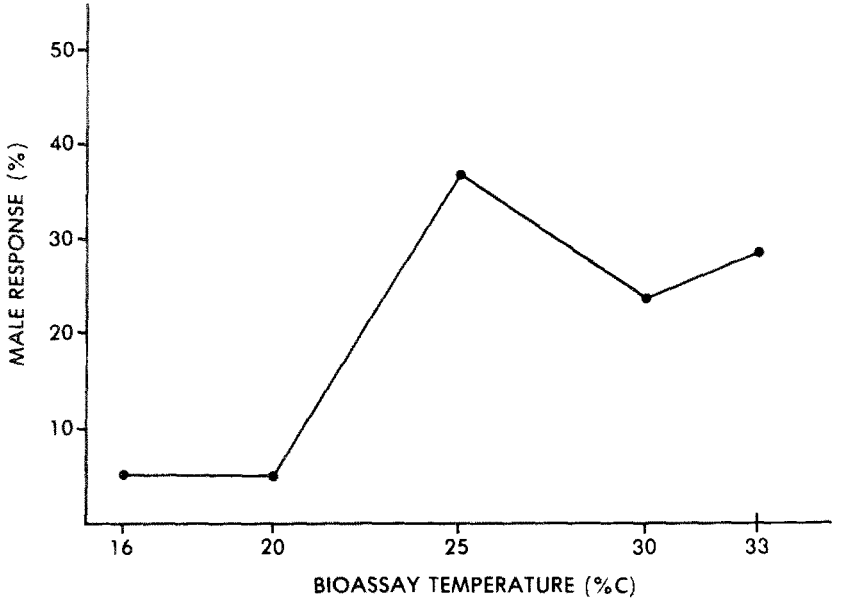

FIG. 7. Bioassay responses of male *H. glycines* to females at different temperatures of bioassay (MSE = 5.67).

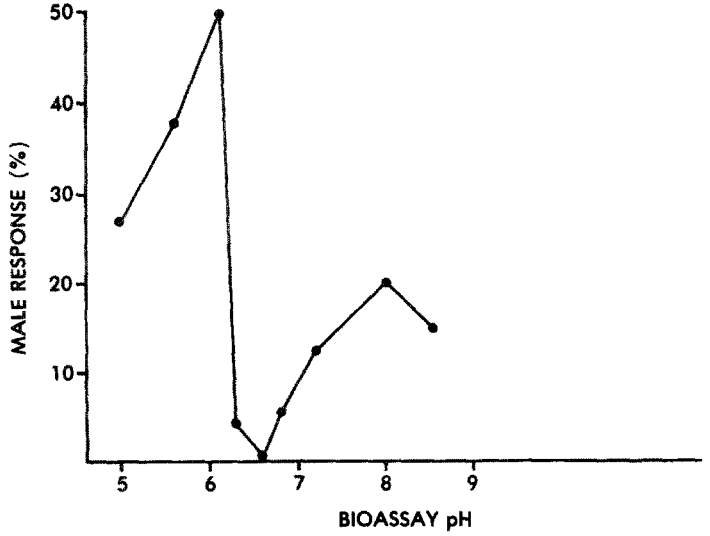

FIG. 8. Bioassay responses of male *H. glycines* to females at the indicated pH values (MSE = 5.18).

pheromone (or both) below 25°C (MSE = 5.67). Higher temperatures of bioassay revealed a slight, but insignificant, decline in pheromone communication.

A nitrogen atmosphere eradicated attraction between the sexes during bioassay. The male's response to females was zero, although some male movement occurred at random. Thus, a level of aerobism may be necessary for mate location in *H. glycines*.

The pH of the in vitro bioassay revealed a bimodal influence on orientation of males to a female source (Figure 8). The male's responsiveness increased linearly from pH 5 to a maximum at 6.1, but was insignificant from 6.3 to 6.8 (MSE = 5.18). A linear increase in response occurred at pH above 6.3 to a greatly diminished peak response at pH 8. Bioassay in buffered agar at pH 6 and 6.5 gave male responses of 18.2 and 4.3, respectively.

DISCUSSION

Heterodera glycines apparently utilizes pheromone communication for mate location, as expected from the previous reports concerning *H. rostochiensis* and *H. schachtii*. However, the species specificity of pheromone-mediated orientation is uncertain, based on the cross-attraction between species of *Heterodera* (Green and Plumb, 1970) and the hybridization between *H. schachtii* and *H. glycines* (Potter and Fox, 1965).

The greater responsiveness of males to a few females indicates that the in vitro bioassay design may be more appropriate when compared to that used for the zooparasite *Nippostrongylus brasiliensis* (Bone et al., 1977). We suspect that the agar gel serves to stabilize pheromone gradients and other parameters which, when coupled to longer diffusion and response periods, enables a more precise orientation by males. Interestingly, the reduction in male responsiveness with higher dosages of females is found also in *N. brasiliensis, H. rostochiensis,* and *H. schachtii* (Bone et al., 1978; Greet et al., 1968). This suggests that the effect may be a universal artifact of bioassay design, although a natural biological occurrence and purpose may exist also. Regardless, examination of a range of dosages is an experimental necessity but regrettably has not been followed in most studies of helminth chemocommunication.

Male *H. glycines* emerge from the host plant prior to the females, but emergence continues for several days (Skotland, 1957). Our age studies seem to indicate that some males are responsive to pheromone soon after emergence, although responsiveness increases over the next few days. Therefore, the delayed emergence of females ensures that adequate numbers of receptive males are present. However, continued maturation of the female may enhance mate location.

A number of factors that potentially influence mate location by *Heterodera* have been examined in this or previous studies. Green (1966) found that fumigation with methyl bromide, heating, and ultraviolet irradiation decreased the number of attracted males during bioassay. Sublethal dosages of the nematocide aldicarb prevented the movement of *H. schachtii* males toward females (Hough and Thomason, 1975). Based on this study, incandescent light has no effect on mate location, while reduced oxygen tension may disrupt communication between the sexes, probably by metabolic derangements. Low and high temperatures have pronounced to moderate effects on the pheromone system, but their influence in the soil habitat is probably minor except for drastic seasonal changes.

Pheromone communication is strongly influenced by pH conditions. The loss of activity may be related to the insolubility of the compounds at neutrality if the substances are charged. Alternatively, two compounds may be present and be produced at different levels, depending on pH. The lowered response of males when the medium was buffered at pH 6 suggests that pH alterations by the females, probably through acidic endproducts of metabolism, should also be considered.

Continued investigation of *Heterodera* may yield basic information concerning the nematode's reproductive physiology and represent a background for eventual development of a biocontrol scheme. The possible application of pheromone-based control to the genus collectively, based on cross-specific attraction, is intriguing.

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ALLELOPATHIC EFFECTS OF *Polygonum aviculare* L. I. VEGETATIONAL PATTERNING

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Abstract—*Polygonum aviculare* was observed to spread rapidly into heavy stands of *Cynodon dactylon* (L.) Pers. resulting in death of the latter. This indicated a strong interference against *Cynodon dactylon*. Measurements of selected soil minerals and physical factors indicated that competition was probably not the chief cause of that interference. Soil collected under dead *Polygonum* was very inhibitory to all test species except *Sporobolus pyramidatus* (Lam.) Hitchc., suggesting the presence of inhibitory compounds. Tops and roots of *Polygonum*, root exudates, and leachate of the tops inhibited seed germination and seedling growth of most test species. Therefore, allelopathy apeared to be the dominant component of the interference, with competition probably accentuating its effects. *Polygonum aviculare* was inhibitory to *Gossypium barbadense* L. and *Sorghum bicolor* (L.) Moench, indicating that allelopathy is an important component of the interference by *Polygonum* against crop yields.

Key Words—Allelopathy, interference, patterning, biological control, *Polygonum*, *Cynodon*, *Chenopodium*, *Sorghum*, *Gossypium*.

INTRODUCTION

Several workers have shown that allelopathy may play an important part in weed–weed interactions (Wilson and Rice, 1968; Rasmussen and Rice, 1971; Newman and Rovira, 1975) and weed–crop interactions (Bell and Koeppe, 1972; Tames et al., 1973; Colton and Einhellig, 1980). Such interactions may lead to the reduction or elimination of associated plants.

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Polygonum aviculare (prostrate knotweed) is a serious, principal or common weed in crops in many parts of the world (Holm et al., 1979) resulting in marked reductions in their yields. It is also a pernicious weed in lawns in many countries, including the USA.

The junior author observed a rapid encroachment of prostrate knotweed into bermudagrass (*Cynodon dactylon*) lawns in Norman, Oklahoma. bermudagrass dies in the patches of prostrate knotweed while bermudagrass at the edges of the knotweed patches turns yellow.

The rapid invasion of heavy bermudagrass sod by *Polygonum* and the existence of *Polygonum* in pure stands after a few months suggests that an allelopathic mechanism may be involved in its interference in addition to competition. Therefore, the present study was conducted primarily to determine whether *Polygonum aviculare* is allelopathic to bermudagrass, other commonly associated species, and selected crop plants. Another goal was to obtain preliminary data concerning the feasibility of using prostrate knotweed in the biological control of bermudagrass and other weeds in certain crops.

METHODS AND MATERIALS

Selection of Test Species. Cynodon dactylon and *Sporobolus pyramidatus* were chosen as test species because they were found to be associated with *Polygonum aviculare. Chenopodium album* L. was included because it is a serious weed in cultivated lands and is sometimes associated with *P. aviculare* (Bhomik and Doll, 1979; Holm et al., 1979). Sorghum (*Sorghum bicolor*) and cotton (*Gossypium barbadense*) were chosen as test species because they are important crops in which bermudagrass is often a noxious weed (Jordan, 1977).

Rate of Invasion of Bermudagrass Sod by Prostrate Knotweed. Stakes were placed around the perimeters of several prostrate knotweed stands in bermudagrass lawns in April 1980. Measurements were made in November 1980 to determine the rate of spread, and it was found that *Polygonum* stands increased in diameter at an average rate of 1.5 m in this growing season even though they were surrounded by a heavy sod of bermudagrass.

Physical and Mineral Properties of Soil. Selected physical and mineral properties of soil were analyzed to determine if *P. aviculare* causes changes in those soil properties which could account for the alteration of vegetational patterns in the field.

Ten soil samples minus litter were collected to a depth of 30 cm within a *Polygonum* stand and 10 similar samples were taken in the adjacent areas where only *Cynodon dactylon* was growing. The samples were air-dried, passed through a sieve with 2-mm openings, and analyzed for pH by the glass electrode method of Piper (1942). Soil texture was determined by mechanical

analysis using a modified Bouyoucos hydrometer method (Bouyucos, 1936; Piper, 1942). The remaining soil was ground to pass through a 0.5-mm sieve and analyzed for total nitrogen by the macro-Kjeldahl method of Bremner (1965), and for total phosphorus by the method of Shelton and Harper (1941). Easily extractable potassium, magnesium, calcium and zinc were determined by atomic absorption after extraction of 5 g of each soil sample with 20 ml of a 0.075 N acid mixture consisting of equal volumes of 0.05 N HCl and 0.025 N H_2SO_4 (Perkin-Elmer, 1976). All calculations were based on the oven-dry weight of the soil.

Calcium was the only soil factor tested which differed significantly betweeen the *Polygonum* stand and the surrounding bermudagrass stand, and it was higher in the *Polygonum* area (Table 1).

RESULTS

Effects of Field Soils on Test Species. The following experiment was designed to eliminate possible competition due to differential ion uptake and to determine if allelopathic compounds are released by prostrate knotweed into the soil and remain stable long enough to affect plant growth. Soil minus litter was collected under a *Polygonum* stand and placed in 10-cm-diameter glazed pots. A similar collection was made under *Cynodon dactylon* and used as a control, because we were interested in comparing growth of selected species in these soils. Soil collections were made in July during active growth of *Polygonum* and in March, four months after *Polygonum* completed its life cycle and died. Each collection was treated as a separate experiment because of possible changes in the control soil and pronounced differences in greenhouse conditions at the two dates.

Twenty-five seeds of all test species except *Sporobolus pyramidatus* were planted in their respective pots. Large numbers of *S. pyramidatus* seeds had to be planted in all experiments to obtain a sufficient number of seedlings for the tests. Ten test and ten control pots were used per test species in this and all subsequent experiments described below, and all experiments were run under greenhouse conditions except the U-tube experiment. Germination percentages were recorded two weeks after the seeds were planted, at which time the plants were thinned to the three largest per pot and allowed to grow another two weeks. All plants were harvested, separated into roots and tops and compared on the basis of oven-dry weights.

Soil collected under *Polygonum* in July did not affect seed germination and seedling growth of any of the test species significantly as compared with germination and growth in soil collected under *Cynodon* (Table 2). Soil collected in March, however, markedly inhibited seed germination of all test species except *Sorghum bicolor*. Seedling growth of all species, except *Sporobolus pyramidatus*, was significantly inhibited by the soil collected in

TABLE 1. COMPARISON OF SOIL FACTORS WITHIN A *Polygonum* STAND AND OUTSIDE IT

Test[a]	Inside *Polygonum* stand	Outside *Polygonum* stand
pH	7.58	7.72
Sand (%)	80.03 ± 1.25	78.14 ± 1.12
Silt (%)	6.53 ± 1.00	6.09 ± 1.00
Clay (%)	14.44 ± 0.96	15.76 ± 0.39
Total N (%)	0.57 ± 0.04	0.58 ± 0.03
P (ppm)	14.10 ± 0.29	14.30 ± 1.58
K (ppm)	129.40 ± 0.85	128.34 ± 1.58
Mg (ppm)	370.32 ± 48.26	328.00 ± 14.24
Ca (ppm)	521.80 ± 30.33[b]	474.20 ± 12.41
Zn (ppm)	8.32 ± 2.10	7.00 ± 1.13

[a] Each value is average of 10 replicates.
[b] Difference significant at 0.05 level.

TABLE 2. EFFECTS OF FIELD SOILS FROM AND ADJACENT TO *Polygonum* STANDS ON GERMINATION AND SEEDLING GROWTH OF SELECTED SPECIES

Test species	Date Soil Taken	Mean dry weight ± standard error (mg)[a]						Germination (% of control)
		Control			Test			
		Root	Shoot	Whole plant	Root	Shoot	Whole plant	
Gossypium barbadense	July 30	313 ± 44	832 ± 36	1145 ± 52	324 ± 43	772 ± 97	1096 ± 69	91
	March 20	95 ± 17	263 ± 18	358 ± 19	67 ± 17	217 ± 26[b]	284 ± 28[c]	54
Sorghum bicolor	July 30	573 ± 43	497 ± 32	1070 ± 71	597 ± 42	540 ± 37	1137 ± 56	96
	March 20	257 ± 12	287 ± 18	544 ± 22	147 ± 37[b]	219 ± 16	366 ± 50[c]	96
Chenopodium album	July 30	279 ± 19	404 ± 49	683 ± 70	234 ± 27	240 ± 22	474 ± 45	92
	March 20	107 ± 20	532 ± 40	639 ± 40	44 ± 5[c]	490 ± 76	534 ± 82[b]	66
Sporobolus pyramidatus	July 30	167 ± 18	202 ± 20	369 ± 21	204 ± 22	173 ± 9	377 ± 23	
	March 20	16 ± 4	39 ± 6	55 ± 14	10 ± 1	36 ± 6	46 ± 8	
Cynodon dactylon	July 20	149 ± 21	293 ± 47	442 ± 58	141 ± 23	324 ± 40	465 ± 34	97
	March 20	47 ± 5	116 ± 6	163 ± 12	12 ± 1[d]	36 ± 5[b]	48 ± 5[c]	53

[a] Each value is average of 30 replicates. No comparisons valid between dates—greenhouse temperature and humidity very different.
[b] Dry weights significantly different from control at 0.05 level.
[c] Dry weights significantly different from control at 0.01 level.
[d] Dry weights significantly different from control at 0.001 level.

March from *Polygonum* stands. In some species, the inhibition was primarily due to a retardation in root growth and in others primarily to a retardation in top growth. Both root and top growth of bermudagrass were significantly inhibited, however. These results indicated a phytotoxic effect of soil closely associated with prostrate knotweed, and several types of experiments were run subsequently to determine the source of the toxins in the soil under *Polygonum*. These are described below.

Effects of Leaf Leachate. Artificial rain in the form of a fine spray of cistern water was allowed to fall on fresh mature plants of *Polygonum*. The leachate which dripped from the leaves was collected and used to water test pots each containing a substrate consisting of two parts of greenhouse potting soil and one part of sand. Each control pot contained a similar substrate plus 25 seeds of a given test species but was watered with an equal amount of cistern water that had not been sprayed over *Polygonum*. Germination percentages were recorded two weeks after planting, after which the plants were thinned to the four largest per pot, allowed to grow for two weeks and compared on the basis of oven-dry weights.

The leachate reduced germination percentages slightly in all test species (Table 3). *Chenopodium album* was significantly inhibited in growth while *Cynodon dactylon* was significantly stimulated. The leachate did not affect growth of other test seedlings.

Effects of Decaying Shoots. Based on clipped quadrats in a *P. aviculare* stand, it was found that mature plants of *Polygonum* produce an average of 4.1 g of air-dried tops per kilogram of soil to a depth of 10 cm (average depth of rooting of *P. aviculare*). To test the possible phytotoxicity of *Polygonum* tops on test species, 25 seeds of each test species were planted in separate glazed pots containing 4.1 g of air-dried *Polygonum* tops per kilogram of a 2:1 soil–sand mixture. An equal concentration of milled peat moss was added to the soil–sand mixture in the control posts to keep the organic matter content the same. All pots in the experiment received equal amounts of cistern water when necessary. The percentage of seed germination was determined 14 days after planting, after which the seedlings were thinned to the four largest per pot, allowed to grow an additional 14 days, then harvested. The biomass of the test species was determined on the basis of oven-dry weights.

Decaying *Polygonum* tops drastically inhibited germination of cotton and *Chenopodium album* (Table 4), and germination of other species was appreciably reduced also. Decaying tops of *Polygonum* significantly reduced seedling growth of all test species also.

Effects of Decaying Roots. Field sampling of *Polygonum* at the end of July when the plant was mature revealed an average of 1.9 g air-dried roots per kilogram of soil to a depth of 10 cm. To test the possible effects of decaying *Polygonum* roots on the test species, 25 seeds of each test species were planted in separate pots containing either 1.9 g of air-dried roots per kilogram of a 2:1 soil–sand mixture, or an equal concentration of milled peat.

TABLE 3. EFFECTS OF *Polygonum* LEAF LEACHATE ON SEED GERMINATION AND SEEDLING GROWTH OF SELECTED SPECIES

Species	Mean dry weight ± standard error (mg)[a]		Germination (% of control)
	Control	Test	
Gossypium barbadense	1200 ± 67	1270 ± 67	81
Sorghum bicolor	1260 ± 101	1254 ± 40	96
Chenopodium album	467 ± 10	417 ± 36[c]	90
Sporobolus pyramidatus	76 ± 10	64 ± 8	
Cynodon dactylon	87 ± 17	109 ± 17[b]	93

[a] Each value is average of 40 replicates.
[b] Dry weights significantly different from control at 0.05 level.
[c] Dry weights significantly different from control at 0.01 level.

TABLE 4. EFFECTS OF DECAYING *Polygonum* SHOOTS ON SEED GERMINATION AND SEEDLING GROWTH OF SELECTED SPECIES

Species	Mean dry weight ± standard error (mg)[a]		Germination (% of control)
	Control	Test	
Gossypium barbadense	890 ± 23	563 ± 53[c]	25
Sorghum bicolor	804 ± 47	696 ± 37[c]	61
Chenopodium album	190 ± 12	51 ± 19[c]	19
Sporobolus pyramidatus	30 ± 7	16 ± 2[c]	
Cynodon dactylon	31 ± 9	24 ± 2[b]	78

[a] Each value is average of 40 replicates.
[b] Dry weights significantly different from control at 0.05 level.
[c] Dry weights significantly different from control at 0.01 level.

Germination percentages were determined two weeks after planting. The plants were thinned to the four largest per pot and allowed to grow for another two weeks, at which time they were harvested and oven-dry weights were determined.

The decaying roots reduced the germination percentages of *Cynodon dactylon* and *Chenopodium album* appreciably (Table 5). There was virtually no effect on germination of other test species. Seedling growth of *Gossypium barbadense* and *Cynodon dactylon* was significantly reduced, but the other test species were not affected.

Effects of Different Periods of Decomposition of Residues. Twenty-five seeds of each test species were placed in separate pots containing either 6 g of *Polygonum* (2 g of air-dried roots and 4 g of air-dried tops) per kilogram of a 2:1 soil–sand mixture (tests) or an equal concentration of milled peat moss (controls). After germination, the plants were thinned to three plants per pot, and 10 test and 10 control pots were harvested after 20 days, and 10 of each after 40 days. The goals were to determine if there was a delay in inhibition caused by decaying residue and/or a recovery effect after any early inhibition.

Root biomass of cotton, sorghum, and *Chenopodium* was significantly reduced by decomposing *Polygonum* residues at the end of 20 days and root biomass of all species, except sorghum, was still significantly reduced after 40 days (Table 6). It is notable that root biomass of bermudagrass was significantly reduced after 40 days but not after 20 days. Shoot biomass of all test species except sorghum was significantly reduced at the end of 20 days but only shoot biomass of cotton and bermudagrass was still reduced at the end of 40 days. Thus in affected species, inhibition of roots and shoots occurred at an early stage of growth except for root growth of bermudagrass. In all affected species, except cotton and bermudagrass, shoot growth recovered during the last 20 days, suggesting a stimulating effect on shoot growth during that period. Whole plant biomass of all test species except *Sporobolus pyramidatus* was significantly reduced after 20 days, and this reduction in whole plant biomass persisted for 40 days in all affected species except sorghum.

Effects of Root Exudates on Test Species. P. aviculare plants about 5 inches tall were transferred from the field to pots filled with washed quartz sand. The experiment was designed to eliminate competition for light, water, and minerals between *Polygonum* and the test species. Pots containing seeds of the test species and pots of *Polygonum* were placed on alternate steps of a staircase device (Bell and Koeppe, 1972). A control series consisted of pots of test species alternating with pots of washed sand. Complete nutrient solution (Hoagland and Arnon, 1950) was pumped from a reservoir at the bottom of each series. The nutrient solution dripped from pot to pot down to the bottom reservoir where it was recycled every 12 hr. One week after germination, seedlings of the test species were thinned to the two largest per pot, allowed to grow another four weeks and compared on the basis of oven-dry weights.

TABLE 5. EFFECTS OF DECAYING *Polygonum* ROOTS ON GERMINATION AND SEEDLING GROWTH OF SELECTED SPECIES

Species	Mean dry weight ± standard error (mg)[a]		Germination (% of control)
	Control	Test	
Gossypium barbadense	723 ± 20	611 ± 26[c]	96
Sorghum bicolor	404 ± 50	430 ± 42	94
Chenopodium album	147 ± 34	126 ± 15	81
Sporobolus pyramidatus	23 ± 4	15 ± 2	
Cynodon dactylon	32 ± 4	24 ± 2[b]	73

[a] Each value is average of 40 replicates.
[b] Dry weights significantly different from control at 0.05 level.
[c] Dry weights significantly different from control at 0.01 level.

TABLE 6. EFFECTS OF DIFFERENT PERIODS OF DECOMPOSITION OF *Polynonum* PLANTS ON GROWTH OF SELECTED SPECIES

Species	Treatment	Mean dry weight ± standard error (mg)[a]					
		After 20 days			After 40 days		
		Root	Shoot	Whole plant	Root	Shoot	Whole plant
Gossypium barbadense	Control	115 ± 7	410 ± 16	525 ± 16	1371 ± 43	350 ± 16	1721 ± 52
	Test	102 ± 7[b]	309 ± 13[d]	411 ± 11[d]	1003 ± 29[d]	233 ± 8[d]	1236 ± 122[d]
Sorghum bicolor	Control	423 ± 36	282 ± 44	705 ± 42	820 ± 71	1324 ± 138	2144 ± 151
	Test	182 ± 14[d]	232 ± 27	414 ± 56[c]	846 ± 71	1094 ± 117	1940 ± 127
Chenopodium album	Control	31 ± 5	60 ± 6	91 ± 2	636 ± 56	1013 ± 179	1649 ± 212
	Test	15 ± 3[b]	31 ± 6[b]	46 ± 9[b]	336 ± 40[d]	748 ± 79	1084 ± 107[b]
Sporobolus pyramidatus	Control	8 ± 1	18 ± 1	26 ± 1	115 ± 8	225 ± 22	340 ± 26
	Test	8 ± 1	13 ± 2[b]	21 ± 2	241 ± 34	111 ± 16	352 ± 23
Cynodon dactylon	Control	9 ± 2	11 ± 1	20 ± 2	95 ± 12	225 ± 29	320 ± 23
	Test	7 ± 1	8 ± 1[b]	15 ± 1[b]	41 ± 5[c]	128 ± 26[b]	169 ± 2[c]

[a] Each value is average of 30 replicates.
[b] Dry weights significantly different from control at 0.05 level.
[c] Dry weights significantly different from control at 0.01 level.
[d] Dry weights significantly different from control at 0.001 level.

The root exudate significantly inhibited root growth of sorghum, top growth of bermudagrass, and root and top growth of *Chenopodium album* (Table 7). Seedling growth of other test species was not significantly affected.

To eliminate possible effects from algal growth on the sand surface of some pots in the staircase experiment, a U-tube experiment was designed to determine if the root exudate of *Polygonum aviculare* was toxic to the same test species (Tubbs, 1973).

U-tubes made from Pyrex tubes 2.5 cm in diameter and 56 cm long, were painted with aluminum paint to exclude light. These were filled with aerated Hoagland's nutrient solution. Ten test and 10 control tubes were used for each test species. In the test series, test seedlings of uniform shoot and root length were placed in one end of the U-tubes, one seedling per tube, and a *Polygonum* seedling was placed in the other end of each tube. In the control series, one test seedling was placed in each end of each U-tube. Roots of all seedlings were inserted through a one-hole rubber stopper and held in place with nonwetting cotton. The nutrient solution was aerated for 10 min each day. Test seedlings were allowed to grow for three weeks after being placed in the U-tubes, at which time they were harvested and compared on the basis of oven-dry weights. All plants in these experiments were grown on a 14-hr photoperiod (1500 ft-c) at 28°C and a 10-hr dark period at 20°C.

Root exudates of *Polygonum* significantly reduced seedling growth of *Chenopodium album* and *Cynodon dactylon* but no other species (Table 8). The reduction in *Cynodon dactylon* was primarily in shoot growth. It is noteworthy that these two species were significantly retarded in growth in the staircase experiments also, thus substantiating results of those experiments. It is possible that autotoxicity could have been present in some of the control tubes which could have masked some of the allelopathic effects of *Polygonum* in these tests.

To test whether inhibitory activity of the exudate was related to a particular period during the growth and development of *Polygonum*, root length of *Cynodon dactylon* was measured every four days during the 20-day growing period in the U-tube experiment. Inhibition in root growth started at the beginning of the experiment and continued throughout the growing period (Figure 1). The inhibition was significant ($P < 0.05$) on all days of measurement except day 12.

DISCUSSION

Interference among plant species can be due to competition or competition plus allelopathy in certain cases (Hull and Muller, 1977; Anderson et al., 1978; Rice, 1979). Such interference often leads to a superiority of a particular species and failure of a second under natural conditions.

TABLE 7. EFFECTS OF ROOT EXUDATES OF *Polygonum* ON GROWTH OF SELECTED SPECIES

Species	Treatment	Mean dry weight ± standard error (mg)[a]			Weight as % of control
		Root	Shoot	Whole plant	
Gossypium barbadense	Control	228 ± 32	1317 ± 245	1545 ± 381	
	Test	196 ± 26	1643 ± 125	1839 ± 132	113
Sorghum bicolor	Control	911 ± 78	3137 ± 356	4048 ± 404	
	Test	651 ± 82[b]	2997 ± 437	3648 ± 497	90
Chenopodium album	Control	66 ± 8	280 ± 16	352 ± 21	
	Test	35 ± 5[c]	171 ± 22[c]	206 ± 25[d]	59
Sporobolus pyramidatus	Control	49 ± 7	50 ± 5	99 ± 1	
	Test	37 ± 2	37 ± 1	74 ± 18	75
Cynodon dactylon	Control	24 ± 2	64 ± 12	88 ± 13	
	Test	20 ± 2	43 ± 4[c]	63 ± 5	72

[a] Each value is average of at least 16 replicates.
[b] Dry weights significantly different from control at 0.05 level.
[c] Dry weights significantly different from control at 0.01 level.
[d] Dry weights significantly different from control at 0.001 level.

TABLE 8. EFFECTS OF ROOT EXUDATES OF *Polygonum* ON GROWTH OF SELECTED SPECIES IN U-TUBE EXPERIMENTS

Species	Treatment	Mean dry weight ± standard error (mg)[a]			Weight as % of control
		Root	Shoot	Whole plant	
Gossypium barbadense	Control	140 ± 30	1000 ± 18	1140 ± 10	
	Test	120 ± 26	1112 ± 22	1232 ± 11	108
Sorghum bicolor	Control	305 ± 27	860 ± 99	1165 ± 112	
	Test	274 ± 43	840 ± 261	1114 ± 299	96
Chenopodium album	Control	26 ± 1	176 ± 6	202 ± 18	
	Test	24 ± 1	148 ± 2	172 ± 6[b]	85
Sorobolus pyramidatus	Control	12 ± 1	41 ± 4	53 ± 5	
	Test	10 ± 1	32 ± 3	42 ± 4	79
Cynodon dactylon	Control	18 ± 1	117 ± 7	135 ± 8	
	Test	18 ± 2	75 ± 13[c]	93 ± 10[b]	69

[a] Each value is average of 10 replicates in test series and 20 replicates in control series.
[b] Dry weights significantly different from control at 0.05 level.
[c] Dry weights significantly different from control at 0.01 level.

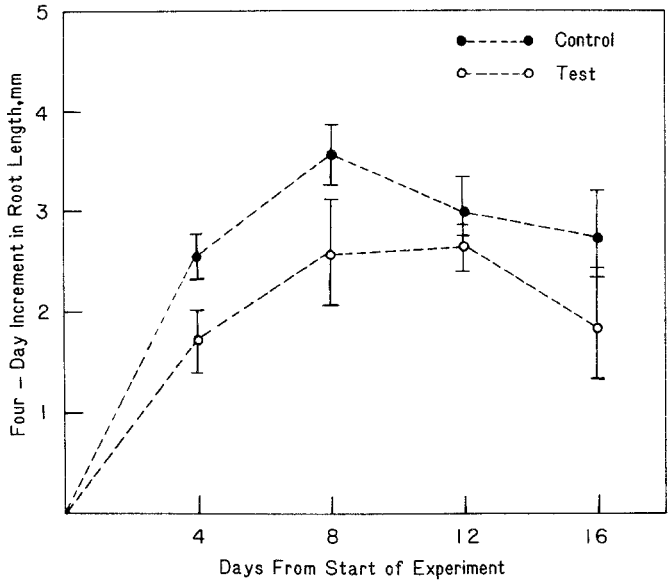

FIG. 1. Timing of release of inhibitor(s) from roots of *Polygonum aviculare* as indicated by growth in length of *Cynodon datylon* roots.

Field studies indicated that the rapid spread of *Polygonum* into a solid stand of bermudagrass is apparently not due to changes in pH, minerals, and/or soil texture. However, soil collected from *Polygonum* stands four months after death of *P. aviculare* plants drastically reduced seed germination and seedling growth of all test species except *Sporobolus pyramidatus*. It is possible also that the soil under bermudagrass which was used as control soil had some allelopathic activity, and this could have masked some of the allelopathic activity of the soil under *Polygonum* (Horowitz and Friedman, 1971). Subsequent experiments demonstrated that toxins are released from the *Polygonum* plants through leaching of the leaves with rain, exudates of the roots, and decomposition of both root and shoot residues. Toxins were present in residues and in soil under them for at least 4 months after death of the plants. Moreover, half of the test species were significantly inhibited in growth for at least 40 days after addition of prostrate knotweed residues to soil.

The yellowing of bermudagrass adjacent to patches of *Polygonum* suggests that there is a pronounced chemical effect prior to actual invasion. *Polygonum* grows especially well in the border area and possibly produces sufficient root exudates to cause marked physiological changes in the bermudagrass. Small amounts of dead leaves of *Polygonum* begin to accumulate in a few weeks after invasion, and it is likely that the residues begin to add at least small amounts of toxins in a month or two. Large amounts of

Polygonum residues accumulate on the soil surface in the fall when the *Polygonum* plants die and apparently kill any remaining bermudagrass. Most other species are probably prevented from growing in the *Polygonum* patches the following spring due to the toxins released from the residues. This scenario seems logical based on our observations and experimental evidence, but there are obvious gaps in our evidence. Tests of soil toxicity from the border area and in patches of *Polygonum* in several other months would have been helpful.

It is significant that *P. aviculare* was inhibitory to bermudagrass in all tests, whereas it inhibited *Sporobolus pyramidatus* in only two tests, and both of these included decaying shoots. Thus, it is obvious that the toxins produced by *P. aviculare* are much more inhibitory to growth of bermudagrass than to *Sporobolus pyramidatus*, and this probably explains why the latter continues to grow nicely in association with *Polygonum* after bermudagrass has been completely eliminated. It is noteworthy that *Sporobolus pyramidatus* was previously found to be allelopathic to bermudagrass also (Rasmussen and Rice, 1971).

It is clear from the evidence that allelopathy was the basic phenomenon responsible for the patterning of vegetation observed in this study. However, once seed germination and growth of a species are inhibited by allelopathic compounds, competition undoubtedly accentuates the growth inhibition. It is also clear that allelopathy is an important component of the interference exhibited by *P. aviculare* against crop yields. In our experiment, however, the allelopathic effects against cotton and sorghum were not as pronounced generally as they were against *Chenopodium album* and bermudagrass but more than they were against *Sporobolus pyramidatus*.

It appears possible that residues or living plants of *Polygonum aviculare* may prove useful in the control of bermudagrass and other weeds in certain row crops. It is premature at present, however, to suggest any specific methodology.

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ALLELOPATHIC EFFECTS OF *Polygonum aviculare* L. II. ISOLATION, CHARACTERIZATION, AND BIOLOGICAL ACTIVITIES OF PHYTOTOXINS

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Abstract—In earlier work, we found that *Polygonum aviculare* had pronounced allelopathic effects against several test species. Four inhibitors were isolated from living *Polygonum* plants, three of which were glucosides. Four different inhibitors were isolated from *Polygonum* residues and soil under *Polygonum* stands, and none of these occurred in soil from *Cynodon dactylon* (L.) Pers. stands. Three of these were glycosides containing both fructose and cellobiose as the sugars. Color reactions of all the inhibitors indicated that they are phenolic in nature. All the inhibitors reduced seed germination and/or seedling growth of *Chenopodium album* L. Moreover some of them inhibited growth of different strains of *Rhizobium* and *Azotobacter*.

Key Words—Allelopathy, inhibitors, phenols, glycosides, *Chenopodium album*, *Rhizobium*, *Azotobacter*, *Cynodon dactylon*.

INTRODUCTION

Numerous kinds of secondary chemical compounds have been isolated from plants, and their inhibitory action upon plants, animals, and microorganisms has been documented (Naqvi and Muller, 1972; Lodhi, 1976; Harborne, 1977; Horsley, 1977; Newman and Miller, 1977; Rice et al., 1981). The ecological significance of such compounds lies in their ability to affect species composition, rate of succession, plant productivity, and microbial populations in soil (Rice, 1965a,b; Muller, 1966; Wilson and Rice, 1968; Chou and Lin, 1976).

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In an earlier paper (AlSaadawi and Rice, 1982), we demonstrated that much of the interference (competition plus allelopathy) of *Polygonum aviculare* against several test species was due to allelopathy. Moreover, the ways in which the chemical inhibitors are released from *Polygonum* into the environment were determined. The nature and characteristics of the inhibitory compounds responsible for inhibition of test species were not described, however. Therefore the goals of the present project were to isolate and characterize the phytotoxic compounds from *Polygonum* and to determine their biological activities.

METHODS AND MATERIALS

Isolation and Characterization of Inhibitors. Since the inhibitory activity of *Polygonum* was associated with both roots and tops (AlSaadawi and Rice, 1982), 10 g of air-dried roots and 10 g of air-dried tops were boiled separately in distilled water for 10 min, ground in a Waring blender for 10 min and allowed to stand for 30 min. The extracts were filtered through cheesecloth and then centrifuged at 27,000 g for 10 min. The filtrate was acidified to pH 2 with HCl and extracted three times with two half volumes of diethyl ether. The ether and water fractions were evaporated to dryness under vacuum at 40°C and were made up to 10 ml with absolute ethanol and 10 ml of 50% aqueous methanol, respectively.

One milliliter of the ether and water extracts was streaked separately on acid-washed Whatman No. 3MM chromatographic paper, and the papers were developed by the descending technique in butanol–acetic acid–water (63:10:27 v/v, BAW). The developed papers were examined with short (2537 Å) and long (3360 Å) UV light with and without NH_3. All fluorescent bands were cut out and eluted with 50% aqueous ethanol. The eluates were streaked on Whatman No. 3MM paper and developed in 6% aqueous acetic acid, 6% AA. The fluorescent bands were cut out and eluted again with 50% aqueous ethanol. This method was used to enhance the purification of the isolated compounds.

The eluted compounds were spotted on acid washed Whatman No. 1 paper and the papers were developed in four different solvent systems: 6% AA, BAW, isopropanol–ammonia–water (200:10:20 v/v, IAW); and isopropanol–*n*-butanol–water (70:10:20 v/v, IBW). The R_f in these solvent systems, colors under UV light, and reactions of the different compounds with diazotized *p*-nitroaniline, diazotized sulfanilic acid, and ferric chloride–potassium ferricyanide were determined. Absorption spectra of the eluted compounds were determined in appropriate solvents.

All the compounds were hydrolyzed by refluxing in 1 N HCl for 30 min to determine if these compounds were glycosides. The hydrolysate was extracted with two half volumes of diethyl ether. The ether fraction was evaporated to

dryness and taken up in absolute ethanol. The water fraction was evaporated three times in vacuo to eliminate the HCl and was taken up in 50% aqueous methanol.

The ether fractions were chromatographed in the same solvent systems as the original compounds and colors were determined under short and long UV light. The water fractions were chromatographed with BAW in the first dimension followed by the 1BW in the second direction. The papers were dipped in a benzidine reagent (Smith, 1960, p. 250) to determine the sugar locations.

Since our previous studies (AlSaadawi and Rice, 1982) revealed that soils taken from *Polygonum* stands were very inhibitory to test species, an experiment was designed to isolate and characterize the inhibitory compounds in soil in which *Polygonum* had grown and in *Polygonum* residue. Residue was collected at several places in *Polygonum* stands on March 20, 1981, and was ground to pass a sieve with 1-mm openings. Ten grams of the ground material were extracted in 200 ml of acetone for 24 hr in a Soxhlet extractor (Rice and Pancholy, 1974), and the extract was reduced to 20 ml in a flash evaporator.

Soil samples were collected on March 20, 1981, from the top 10 cm at several places in areas occupied by *Polygonum*. Similar collections were made in an adjacent area where only *Cynodon dactylon* was present. The soil from each vegetation type was mixed thoroughly and air dried. Ten grams of the air-dried soil were then extracted in a Soxhlet apparatus as described above. The methods used in the isolation and characterization of suspected inhibitors in the residue and soil were the same as those used for the air-dried plant materials.

Effects of Isolated Compounds against Chenopodium album. The fluorescent bands resulting from chromatographic procedures in the previously described experiments were eluted with 50% aqueous ethanol, evaporated to dryness, and taken up in 12 ml of 0.05 M phosphate buffer (pH 5.5). The solution of each compound was placed in two 5-cm Petri dishes containing washed quartz sand and 30 seeds of *Chenopodium album*, one of the species used in the previous study (AlSaadawi and Rice, 1982). A paper developed in BAW without application of the extract was subjected to the same procedure and used as a control. The Petri dishes were kept in the growth chamber on a 14-hr protoperiod (1000 ft-c) at 29° C and a 10-hr dark period at 21° C. Germination, hypocotyl, and epicotyl length were recorded 7 days after planting.

Effects of Isolated Compounds on Nitrogen-Fixing Bacteria. Rhizobium leguminosarum, American Type Culture (ATC) strain 10314; *R. meliloti*, ATC strain 4400; *R. japonicum*, ATC strain 10324; *R. lupini*, ATC strain 10319; *Azotobacter vinelandii*, ATC strain 9104; and *A. chroococcum*, ATC strain 9043, were used to test the effects of isolated compounds on nitrogen fixing organisms. A yeast extract–mannitol medium (Society of American

Bacteriologists, 1957, p. 113) was used for all strains of *Rhizobium*, and a soil extract-mannitol medium (Society of American Bacteriologists, 1957, p. 109) was used for *Azotobacter* strains.

The eluted fluorescent bands from living *Polygonum* plants and from residues were evaporated to dryness and dissolved in 10 ml of an appropriate solvent. Each compound was tested for antibacterial activity using the diffusion technique on solid media (Rice, 1965b). A sterilized sensitivity disk was saturated with a given dissolved compound. The solvent was allowed to evaporate, and the disk was placed in a Petri plate seeded with 0.2 ml of a 48-hr liquid inoculum of *Rhizobium* or *Azotobacter*. Control disks were saturated with the same solvent as the treated disks. All plates were incubated at 30°C, and zones of inhibition were measured three days after inoculation.

RESULTS

Isolation and Characterization of Inhibitors. Four inhibitors were isolated from living *Polygonum* plants (Table 1). The same isolated inhibitors were associated with both roots and tops and were found in appreciable amounts in the water fraction. Color reagents revealed that the isolated inhibitors were apparently phenolic in nature.

After hydrolysis, the water fractions of inhibitors 1, 3, and 4 yielded glucose as indicated by the R_f values and the benzidine sugar test (Table 2). The ether fraction of each compound gave three spots or more, indicating that the original molecules were rather complex phenolic glucosides. No success was achieved in identifying the various aglycones resulting from hydrolysis. Absorption spectra of the original molecules (Table 2) did not help in identifying these compounds.

Four inhibitors were isolated from *Polygonum* residues and four from soil under *Polygonum* stands. Our evidence indicated that the inhibitors from the residue and soil were identical (Table 3). However, all these inhibitors were different from inhibitors present in living plants (Tables 1, 3). All the inhibitors from the residue and soil gave color reactions characteristic of phenols, and all had different absorption spectra.

Chromatograms of water fractions of the hydrolysates revealed that all the inhibitors except A yielded fructose and cellobiose (Table 4). No distinct spots were found on the chromatograms of the ether fraction, indicating that acid hydrolysis apparently destroyed the aglycones. Thus we were not successful in identifying any of the inhibitors from the residues or soil under *Polygonum*. All four were apparently phenolic compounds, with three of them being phenolic glycosides. None of these inhibitors occurred in soil from the stands of *Cynodon dactylon*.

Effects of Isolated Compounds against Chenopodium album. All the inhibitors isolated from living *Polygonum* plants significantly inhibited the

TABLE 1. INHIBITORY COMPOUNDS FROM LIVING *Polygonum* PLANTS[a]

Compound	R_fs on Whatman No. 1[b]				Fluorescence[c]		Reagent colors[d]		
	BAW	IBW	6% AA	IAW	Long UV	Short UV	*p*-Nit.	Sulfan. acid	$FeCl_3$-$K_3Fe(CN)_6$
1	0.75	0.79	0.37	0.13	abs	abs	bn	Yellow	bl
2	0.82	0.80	0.43	0.50	bl	bl	bn	none	bl
3	0.21	0.29	0.35	0.04	abs	abs	bn	none	bl
4	0.64	0.43	0.47	0.17	pink	pink	bn	none	bl

[a]Characteristics of the four inhibitors isolated from tops were same as characteristics of the four inhibitors isolated from roots so they were not repeated.
[b]See text for solvent system. R_fs are average of three runs.
[c]Abbreviations: bl, blue; abs. absorption; bn, brown.
[d]Diazotized *p*-nitroaniline (Bray et al., 1950), diazotized sulfanilic acid (Bray et al., 1950), ferric chloride-potassium ferricyanide (Smith 1960, p. 324).

TABLE 2. ABSORPTION SPECTRA OF PHYTOTOXINS FROM LIVING *Polygonum* PLANTS AND SUGAR MOIETIES AFTER HYDROLYSIS

Compound	R_fs on Whatman No. 1[a]		Benzidine sugar test	Absorption spectra[b]	
	BAW	IBW		μm	Solvent
1	0.23	0.27	brown	235,348	distilled water
2	none	none	none	248	ethanol
3	0.22	0.27	brown	165,250	distilled water
4	0.23	0.27	brown	315	acetone
Known glucose	0.23	0.27	brown		

[a]See text for solvent systems.
[b]Before hydrolysis.

hypocotyl and epicotyl growth of *Chenopodium album* (Table 5). Seed germination was appreciably reduced by inhibitors 1 and 4 and drastically reduced by inhibitors 2 and 3.

Compounds isolated from *Polygonum* residue and soil under *Polygonum* significantly inhibited hypocotyl and epicotyl growth of *Chenopodium album*, except C (Table 6). Compound D had little impact on seed germination, whereas A and B appreciably reduced seed germination. Inhibitor C drastically reduced seed germination even though it did not have significant effects on epicotyl and hypocotyl growth.

Effects of Isolated Compounds on Growth of Nitrogen-Fixing Bacteria. Strain 4400 was only test strain of *Rhizobium* inhibited by any of the compounds isolated from living *Polygonum* plants, and it was inhibited by all compounds (Table 7). Compound 2 was most inhibitory to this strain.

Strain 9043 was the only test strain of *Azotobacter* inhibited by any of the compounds isolated from living *Polygonum* plants and only compound 3 affected its growth.

All compounds isolated from *Polygonum* residue and soil under *Polygonum* stands were inhibitory to *Rhizobium* strains 10314 and 4400 (Table 8). Growth of *Rhizobium* strain 10324 was inhibited only by compound D, and *Rhizobium* strain 10319 was not inhibited by any of the isolated compounds.

Compound D inhibited growth of *Azotobacter* strain 9104, but no other isolated compounds inhibited the test strains of *Azotobacter*.

DISCUSSION

Results described in our previous paper (AlSaadawi and Rice, 1982) indicated that the phytotoxicity of *Polygonum* was associated with both the

TABLE 3. INHIBITORY COMPOUNDS FROM *Polygonum* RESIDUES AND SOIL UNDER *Polygonum* STANDS[a]

	R_fs on Whatman No. 1[b]				Fluorescence[c]				Reagent color[d]		
					Long UV		Short UV				
Compound	BAW	IBW	6% AA	IAW	$-NH_3$	$+NH_3$	$-NH_3$	$+NH_3$	*p*-Nit.	Sulfan. acid	$FeCl_3$-$K_3Fe(CN)_6$
A	0.89	0.85	0.63	0.84	v bl	v bl	bl	bl	tan bn	yel	bl
B	0.84	0.83	0.47	0.48	l bl	l bl	l bl	l bl	d bn	none	bl
C	0.87	0.72	0.40	0.63	br yel	yel	yel	yel gr	none	yel	bl
D	0.81	0.77	0.08	0.35	br bl	br bl	br bl	br bl	tan bn	yel	bl

[a]Characteristics of the four inhibitors isolated from soil were same as those of the four inhibitors from residues and thus were not repeated in the table.
[b]See text for solvent system. R_fs are average of three runs.
[c]Abbreviations: v, violet; bl, blue; l, light; br, bright; yel, yellow; gr, green; bn, brown; d, dark.
[d]Diazotized *p*-nitroaniline (Bray et al., 1950), diazotized sulfanilic acid (Bray et al., 1950), ferric chloride-potassium ferricyanide (Smith, 1960, p. 324).

TABLE 4. ABSORPTION SPECTRA OF PHYTOTOXINS FROM *Polygonum* RESIDUES AND SOIL UNDER *Polygonum* STANDS AND SUGAR MOIETIES AFTER HYDROLYSIS

Compound	Sugar moieties	R_fs on Whatman No. 1[a]		Benzidine sugar test	Absorption Spectra[b]	
		BAW	IBW		μm	Solvent
A		none	none	none	311	acetone
B	a	0.23	0.47	gold-brown	235	butanol
	b	0.13	0.23	brown		
C	a	0.23	0.47	gold-brown	238	butanol
	b	0.13	0.23	brown		
D	a	0.22	0.45	gold-brown	312	acetone
	b	0.12	0.23	brown		
Known fructose		0.25	0.45	gold-brown		
Known cellobiose		0.14	0.25	brown		

[a]See text for solvent systems.
[b]Before hydrolysis.

roots and tops. The current study revealed that the same active compounds were present in both roots and tops.

The results also demonstrated that allelopathic compounds isolated from living *Polygonum* plants were different from those present in *Polygonum* residue. *Polygonum aviculare* is an annual, and it either synthesizes different allelopathic compounds during its senescent period toward the end of its life cycle, or biological degradation of the phenolic inhibitors present in the actively growing *Polygonum* plant occurs during senescence or after death. Other studies have shown that phenolic compounds in plants and the soil can change markedly under different environmental stresses (Wang et al., 1967; Koeppe et al., 1969; Rice, 1974). Also, several phytotoxic compounds were reported to be converted by methylation, demethylation, hydroxylation, and ring fusion to other kinds of compounds by many facultative fungal parasites (Van Etten and Smith, 1975; Hargreaves et al., 1976; Ingham, 1976).

Our results demonstrated clearly that the allelopathic compounds present in the soil under *Polygonum* definitely came from *Polygonum* residue. None of these inhibitors was found in adjacent soil under *Cynodon dactylon*. These results were particularly striking from an ecological standpoint since inhibitory compounds from *Polygonum* were present in the soil for at least 4 months after death of *Polygonum* in the field. No attempt was made to isolate the allelopathic compounds from soil after that time.

Inhibitory compounds in living *Polygonum* may also play an important

TABLE 5. EFFECTS OF COMPOUNDS FROM LIVING *Polygonum* ON SEED GERMINATION AND SEEDLING GROWTH OF *Chenopodium album*

Compound	Hypocotyl[a]		Epicotyl[a]		Germination (% of control)
	Mean length (mm)	% of control	Mean length (mm)	% of control	
Control	12.6 ± 1.9	100	8.2 ± 0.6	100	100
1	6.0 ± 1.2[b]	47.8	3.7 ± 0.5[b]	45.3	71.3
2	6.8 ± 1.0[b]	53.6	4.6 ± 0.4[b]	56.5	47.6
3	5.3 ± 1.8[b]	42.3	4.8 ± 0.5[b]	57.8	52.4
4	5.1 ± 1.4[b]	40.2	4.2 ± 0.5[b]	51.4	76.2

[a]Average of at least 25 seedlings. Only extruded epicotyls and hypocotyls included in averages.
[b]Mean lengths significantly different from control at 0.01 level or better.

TABLE 6. EFFECTS OF INHIBITORY COMPOUNDS FROM *Polygonum* AND SOIL UNDER *Polygonum* STANDS ON SEED GERMINATION AND SEEDLING GROWTH OF *Chenopodium album*

Compound	Hypocotyl[a]		Epicotyl[a]		Germination (% of control)
	Mean length (mm)	% of control	Mean length (mm)	% of control	
Control	13.7 ± 1.9	100	16.2 ± 1.0	100	100
A	9.4 ± 3.0[b]	68.9	9.5 ± 3.0[d]	58.7	82.5
B	5.5 ± 1.1[d]	40.1	8.6 ± 1.4[d]	53.2	75.0
C	12.4 ± 2.0	90.2	12.9 ± 1.6	79.5	40.0
D	10.0 ± 1.1[b]	72.6	11.0 ± 1.1[c]	68.2	90.0

[a]Average of at least 25 seedlings.
[b]Mean lengths significantly different from control at 0.05 level.
[c]Mean lengths significantly different from control at 0.01 level.
[d]Mean lengths significantly different from control at 0.001 level.

TABLE 7. EFFECTS OF COMPOUNDS ISOLATED FROM LIVING *Polygonum* PLANTS ON GROWTH OF NITROGEN-FIXING BACTERIA[a]

	Compound			
Test organism[b]	1	2	3	4
R 10314	0.00[c]	0.00	0.00	0.00
R 4400	1.33	2.00	1.66	1.33
R 10319	0.00	0.00	0.00	0.00
R 10324	0.00	0.00	0.00	0.00
A 9104	0.00	0.00	0.00	0.00
A 9043	0.00	0.00	1.00	0.00

[a]Controls had no inhibition.
[b]Symbols: R, *Rhizobium*; A, *Azotobacter*. Numbers are ATC strain designations.
[c]Each figure is mean radius (mm) of inhibited zone of three trials.

role in inhibiting the associated species since these compounds can be released into the environment through leaching of the tops during rain, root exudation, and decomposition of the materials left in the field after mowing (AlSaadawi and Rice, 1982). Several other scientists have reported that a variety of chemical compounds are released from different plant species in similar ways (Rovira, 1969; Tubbs, 1973; Rice, 1974; Horsley, 1977; Young, 1979).

We did not test for synergistic effects of the eight inhibitors which were isolated, but it is certainly possible that such effects may occur and result in much greater total inhibition than the cumulative effects of individual

TABLE 8. EFFECTS OF COMPOUNDS ISOLATED FROM *Polygonum* RESIDUES AND SOIL UNDER *Polygonum* STANDS ON GROWTH OF NITROGEN-FIXING BACTERIA[a]

	Compound			
Test organism[b]	A	B	C	D
R 10314	0.66[c]	1.00	0.33	0.50
R 4400	2.66	2.00	1.16	1.33
R 10319	0.00	0.00	0.00	0.00
R 10324	0.00	0.00	0.00	0.80
A 9104	0.00	0.00	0.00	1.50
A 9043	0.00	0.00	0.00	0.00

[a]Controls had no inhibition.
[b]Symbols: R, *Rhizobium*; A, *Azotobacter*. Numbers are ATC strain designations.
[c]Each figure is mean radius (mm) of inhibited zone of three trials.

inhibitors (Wilson and Rice, 1968; Rasmussen and Einhellig, 1977; Einhellig and Rasmussen, 1978).

Our results indicated that all eight phytotoxins isolated inhibited seed germination and/or seedling growth of a plant species commonly associated with *Polygonum aviculare* in the field. Moreover, several of them inhibited growth of some of the test strains of *Rhizobium* and *Azotobacter*. Thus, *Polygonum aviculare* can exert a direct allelopathic effect against associated higher plants and indirect allelopathic effects against them through a reduction in the rate of both asymbiotic and symbiotic nitrogen fixation (Rice, 1979).

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IRIDOID GLYCOSIDES IN THE NECTAR OF *Catalpa speciosa* ARE UNPALATABLE TO NECTAR THIEVES

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Abstract—The floral nectar of *Catalpa speciosa* has a chemical mechanism that limits thievery. A bioassay employing sphingid larvae, *Ceratomia catalpae*, shows that catalpa iridoid glycosides are present in the floral nectar. When potential nectar thieves are fed nectar, a sucrose solution of identical concentration, or a sucrose solution plus 0.4% catalpol and 0.4% catalposide (catalpa iridoids), the thieves drink significantly more of the pure sucrose solution than either of the other two sugar sources. Those thieves that drink either the nectar or the sucrose solution plus catalpa iridoids develop behavioral abnormalities including regurgitation and loss of locomotion. The response of the potential nectar thieves to nectar or the sucrose solution plus catalpa iridoids cannot be distinguished by the amount consumed or by their behavior. The legitimate diurnal pollinators of *C. speciosa* are not affected by the iridoid glycosides.

Key Words—*Catalpa speciosa*, catalpol, catalposide, iridoid glycosides, nectar thieves, pollination, toxic nectar.

INTRODUCTION

Floral visitors which remove the nectar but fail to effect pollination are often called nectar thieves. Most commonly, nectar theft results from a mismatch between the morphology of the thief and the morphology of the flower (Inouye, 1980). For example, a floral visitor (e.g., an ant or small bee) may be too small to contact the stamens and stigma upon entering or leaving a large flower. It is commonly thought that closed, zygomorphic flowers, dense hairs that present a physical impediment to small foragers, sticky exudates on the peduncle or corolla tube, and the lack of landing platforms on hummingbird-pollinated flowers are morphological adaptations that deter nectar thieves

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(Faegri and van der Pijl, 1979; Guerrant and Fiedler, 1981; Kerner, 1878). Recently, Baker and Baker (1975) and Janzen (1977) have hypothesized that some floral nectars contain toxic or unpalatable substances that deter nectar thieves. This hypothesis has been strengthened by the mounting evidence which shows that the nectars of many species contain various secondary chemicals (Baker, 1978; Baker and Baker, 1975; Guerrant and Fiedler, 1981).

The flowers of *Catalpa speciosa* (Warder ex Barney) Engel. (Bignoniaceae) are pollinated during the day by bumblebees and carpenter bees and at night by large moths, especially sphingids (Stephenson and Thomas, 1977; Stephenson, personal observation). Although *C. speciosa* receives frequent visitations from these legitimate pollinators, in seven field seasons studying various aspects of the reproductive biology of catalpa, I have observed very few nectar thieves. The lack of nectar thieves on *C. speciosa* is surprising because (1) the floral tube is large and unobstructed; (2) the unvisited flowers contain a large nectar reward compared to other bee-pollinated species (Stephenson and Thomas, 1977); and (3) catalpa produces a large floral display over a short period of time (the floral rewards are clustered in time and space) (Stephenson, 1979). These characteristics should make catalpa very attractive to nectar foragers that are not large enough to effect pollination.

Previously, I reported (Stephenson, 1981) that potential nectar thieves drank less *C. speciosa* nectar than a sugar solution of identical concentration. In addition, the thieves developed behavioral abnormalities such as erratic movements, regurgitation, loss of balance, or loss of locomotion after drinking the nectar but had none of the abnormalities after drinking the sugar solution. The findings suggested that *C. speciosa* has a chemical mechanism to limit thievery.

In this paper, I present evidence from field and laboratory experiments which shows that the nectar of *C. speciosa* contains iridoid glycosides, that the iridoid glycosides are unpalatable to potential nectar thieves, and that bumblebees and carpenter bees (legitimate pollinators) are unaffected by these iridoids.

METHODS AND MATERIALS

The leaves, fruits, and bark of *Catalpa speciosa*, along with its close relatives *C. bignonioides*, *C. ovata*, and *C. bungei*, have been shown to contain a mixture of about 15 iridoid glycosides (Bobbitt et al., 1961, 1966, 1967; Lunn et al., 1962; Nayar and Fraenkel, 1963; and references therein). Nayar and Fraenkel (1963) reported that catalpa iridoids are necessary in order to elicit a feeding response from catalpa's host-specific herbivore, the larva of *Ceratomia catalpae* (Sphingidae). They showed that these larvae

would not feed on the leaves of any species other than catalpa (25 species from 12 families were tested), even when faced with starvation. However, the leaves of various plants when smeared or coated with a water extract of catalpa leaves were readily consumed. In addition, the larvae consumed an artificial diet when it contained either powdered catalpa leaves or a sample of purified catalpa iridoids.

In order to determine if the nectar of *C. speciosa* contained catalpa iridoids, I performed the following bioassay. I made an artificial diet similar to the one used by Nayar and Fraenkel (1963), which consisted of 21 ml of water, 1 g cellulose powder, 0.25 g casein, 0.125 g yeast, 0.25 g glucose, and either 4 ml of *C. speciosa* nectar or 4 ml of a sucrose solution of identical concentration to that of the nectar. The nectar of *C. speciosa* is sucrose dominant (H.G. Baker, personal communication). The components were mixed together in a beaker, heated to 80°C in a water bath, and poured into a Petri dish while hot. After the diet had cooled and hardened, 1.0-cm-diameter disks were punched out with a cork borer. Two disks were placed in a Petri dish lined with filter paper. Each Petri dish contained two disks of the same diet (nectar or sucrose solution) and two third instar *Ceratomia catalpae* larvae that had been starved for 3 hr. Sixteen replicates were run for each diet, and each test ran for 24 hr. The diets were evaluated on the basis of the number of fecal pellets deposited during the test period.

RESULTS

The results of this experiment are summarized in Table 1. After 3 hr there were no significant differences in the number of fecal pellets in the Petri dishes with the nectar diet or the sucrose solution diet. However, I noticed that the

TABLE 1. FEEDING RESPONSES OF *Ceratomia catalpae* TO ARTIFICIAL DIET WITH ADDED SUCROSE SOLUTION OR *C. speciosa* NECTAR[a]

Artificial diet	Total fecal pellets after 3 hr	Mean number of fecal Pellets per replicate after 3 hr[b]	Total fecal pellets after 24 hr	Mean number of fecal pellets per replicate after 24 hr[c]
With sucrose Solution	47	2.9 ± 1.7	54	3.4 ± 1.9
With *C. speciosa* nectar	41	2.6 ± 1.9	120	7.5 ± 3.3

[a] Sixteen replicates of two third-instar larvae were offered each type of artificial diet. The larvae were starved for 3 hr prior to the experiment.
[b] Not significant; $0.3 > P > 0.20$; $t = 0.59$; $df = 30$.
[c] $P < 0.001$; $t = 43.8$; $df = 30$.

nectar diet was partially consumed after 3 hr, whereas the sucrose solution diet did not appear to have been eaten. Consequently, the fecal pellets that appeared during the first 3 hr were probably the result of feedings prior to the initiation of the experiment. The larvae were not starved long enough. After 24 hr, however, there was a mean of 7.5 ± 3.3 (SD) pellets per dish with the nectar diet and only 3.4 ± 1.9 pellets per dish with the sucrose solution diet. These differences are highly significant. Only seven new fecal pellets were deposited in the 16 dishes containing the sucrose solution diet during the final 21 hr of the experiment. In addition, the diets with nectar were either partially or totally consumed after 24 hr while the sucrose solution diets were not eaten.

Because catalpa iridoids have been shown to be a necessary phagostimulant for *Ceratomia catalpae*, I conclude from this experiment that catalpa iridoids are present in the nectar of *C. speciosa*.

About nine months after the above bioassay was performed, I received purified samples of the two most common catalpa iridoids (catalpol and catalposide) from Professors J.S.D. Bacon, J.M. Bobbitt, and J.T. Edward. The samples were run on thin-layer chromatography (0.4% of each sample in a 36% sucrose solution) alongside *C. speciosa* nectar (which had been frozen since extraction) with *n*-butanol–ethanol–water (40:11:19) as the solvent (Kooiman, 1970). The dried chromatograms were sprayed with *p*-anisidine phosphate reagent and heated for 1 min at 105°C. UV light was used to aid in the detection of the resultant spots. The nectar produced two spots that were identical in color and R_f to the catalposide and catalpol. The nectar also produced a third, unknown spot. These data support the conclusion drawn from the bioassay.

I then wanted to determine if catalpa iridoids are responsible for deterring the potential nectar thieves of *C. speciosa*. That is, do these iridoids limit the amount of nectar consumed by the thieves, and do they cause the behavioral abnormalities that I reported previously (Stephenson, 1981)? In order to answer these questions, I collected approximately 1 ml of nectar using microcapillary tubes in June 1981. The sugar concentration of the pooled nectar was determined to be 36% sucrose-equivalents, using a temperature-compensated refractometer. It was then necessary to identify potential nectar thieves. I established three criteria: A potential thief must avidly gather nectar; it must be active at the same time and location that *Catalpa speciosa* flowers; and it must be too small to be a pollinator. The ants that visit the extrafloral nectaries on the leaves of *C. speciosa* (Stephenson, 1982) and the common skipper butterfly (*Poanes hobomok* Harris, Hesperiidae), which was actively visiting flowers near the stand of catalpa, fit these criteria. The ants that visit the extrafloral nectaries are the workers of *Camponotus noveboracenis* (Fitch), *C. nearcticus* Emery, *Prenolepis imparis* (Say), *Formica lasioides* Emery, *F. pallidefulva nitidiventris* Emery, and *Crematogaster cerasi* (Fitch), Hymenoptera, Formicidae. No attempt was made to identify the ants as they

participated in the experiment. These potential nectar thieves are the same ones that I used previously (Stephenson, 1981).

The palatability of catalpa iridoids to ants was determined by placing 20 μl of nectar, 20μl of sucrose solution of identical concentration, and 20μl of the sucrose solution plus 0.4% catalpol and 0.4% catalposide approximately 8 cm apart on the bark of a catalpa tree trunk. This concentration of iridoids was chosen because (1) it had a taste similar to that of nectar and (2) Bobbitt et al., (1961) reported that the leaves of catalpa contain 0.8% catalpa iridoids. As the ants that were moving up the tree trunk to the extrafloral nectaries discovered one of these sugar sources, I recorded the amount of time each visitor spent drinking it. Every 30 min I replaced each sugar source with a fresh supply.

The palatability of catalpa iridoids to the other potential thief, the skipper *Poanes hobomok*, was determined by the methods I used previously (Stephenson, 1981), except the butterflies were fed either nectar, sucrose solution or sucrose solution plus 0.4% catalpol and 0.4% catalposide. Basically, the method consists of placing a butterfly's proboscis into a calibrated microcapillary tube filled with one of the sugar sources and recording the amount that the butterfly drinks.

The results of these experiments are summarized in Tables 2 and 3. There are no significant differences in the amount of time the ants spent drinking the nectar and sucrose solution plus catalpa iridoids, whereas the ants spent significantly more time drinking the sucrose solution than either the nectar or the sucrose solution plus catalpa iridoids. In addition, many of the ants that drank the nectar or sucrose solution plus iridoids exhibited behavioral abnormalities: they fell from the tree; vigorously wiped their heads and antennae, or ran in circles. None of the ants that drank the sucrose solution exhibited these abnormalities.

TABLE 2. PALATABILITY OF *C. speciosa* NECTAR, SUCROSE SOLUTION, AND SUCROSE SOLUTION PLUS CATALPA IRIDIODS (0.4% CATALPOSIDE; 0.4% CATALPOL) TO ANTS (MEAN ± SD)

	N	Time spent drinking (sec)[a]
Nectar	58	25 ± 41
Sucrose solution	38	118 ± 73
Sucrose solution plus iridoids	52	33 ± 49

[a] Nectar vs. sucrose solution: $t = 7.85$; $df = 94$; $P < 0.001$. Nectar vs. sucrose solution plus iridoids: $t = 0.92$; $df = 108$; $P > 0.10$. Sucrose solution vs. sucrose solution plus iridoids: $t = 6.62$; $df = 88$; $P < 0.001$.

TABLE 3. PALATABILITY OF *C. speciosa* NECTAR, SUCROSE SOLUTION, AND SUCROSE SOLUTION PLUS CATALPA IRIDOIDS (0.4% CATALPOSIDE; 0.4% CATALPOL) TO *Poanes hobomok*, THE COMMON SKIPPER (MEAN ± SD)

	N	Amount consumed (μl)[a]	Behavioral abnormalities
Nectar	14	3.2 ± 1.4	3 were comatose
Sucrose solution	21	9.5 ± 6.3	none
Sucrose solution plus iridoids	22	3.7 ± 3.2	7 were comatose

[a]Nectar vs. sucrose solution: $t = 3.7$; $df = 33$; $p < 0.001$. Nectar vs. sucrose solution plus iridoids: $t = 0.50$; $df = 34$; $0.40 > P > 0.30$. Sucrose solution vs. sucrose solution plus iridoids: $t = 3.8$; $df = 41$; $P < 0.001$.

There are also no significant differences in the amount of nectar and sucrose solution plus catalpa iridoids that the skippers drank (Table 3). However, the skippers drank significantly more sucrose solution than either nectar or sucrose solution plus iridoids. In addition, many of the skippers that drank the nectar or sucrose solution plus iridoids exhibited behavioral abnormalities: most could not reroll their proboscides; others could not walk or fly upon completion of the experiment and appeared to be comatose. All of the skippers that were fed the sucrose solution could reroll their proboscides and could walk and fly upon completion of the experiment.

From these data I conclude that catalpa iridoids are the unpalatable substances to the potential nectar thieves of *C. speciosa*.

Because nectar is a primary reward for the legitimate pollinators of *C. speciosa*, any advantage associated with nectar that is unpalatable to thieves would be negated if the nectar is also unpalatable to the legitimate pollinators. In order to examine the palatability of *C. speciosa* nectar to bumblebees and carpenter bees, I captured bees (*Bombus bimaculatus*, *B. fervidus*, *B. impatiens*, *B. vagans*, and *Xylocopa* sp.) that were foraging on or near *C. speciosa*, placed them into a screen-covered cage, and starved them for 2 hr. I then permitted the bees to forage on an artificial flower-board.

Wells 3 mm deep and 4 mm in diameter were drilled 10 cm apart into a 30-cm × 20-cm × 6-mm clear Plexiglas sheet. This produced two rows of three evenly spaced wells. A blue cardboard square (4 cm × 4 cm) was centered and affixed under each well. The flower-board was then placed over a piece of green cardboard. Next, every well on the board was filled with 20 μl of either *C. speciosa* nectar, sucrose solution, or sucrose solution plus catalpa iridoids. All of the wells on any given board were filled with the same sugar source. After filling the wells, the board was slipped into the cage and the foraging behavior of individual bees was monitored. After a bee foraged on the board, the board was removed and the amount of the sugar source

remaining in the first two wells visited by the bee was determined by using calibrated microcapillary tubes. The bee was observed for 5 min and then removed from the cage. The board was cleaned and filled with a different sugar source, and the procedure was repeated.

In most cases, a bee began to forage on the flower-board within 10 min of its placement into the cage. Usually, only one bee at a time foraged on the board and usually the bee removed the sugar source from all six wells (although only the first two wells were analyzed). When two bees foraged simultaneously, the data were counted if the bees visited two wells prior to encountering each other. If, however, the bees attempted to forage at the same well, or if a bee visited a well previously emptied by the other bee, the data were discounted and the bees were removed from the cage.

The results of this experiment are summarized in Table 4. Most bees removed all or nearly all of the sugar source in the first two wells. Furthermore, there were no significant differences in the amounts of the three sugar sources the bees removed (see Table 4). Even though many of the bees visited all six wells, none appeared to behave abnormally after consuming any of the sugar sources. From these data, I conclude that all three of these sugar sources are palatable to the legitimate daytime pollinators of *C. speciosa.*

DISCUSSION

The floral nectar of *Catalpa speciosa* is a large and potentially exploitable resource for nectar thieves. However, potential nectar thieves consume only limited amounts of catalpa nectar and appear to become intoxicated after doing so (Stephenson, 1981). The data presented here suggest that the nectar contains iridoid glycosides and that these iridoids are unpalatable to potential thieves but not to bumblebees and carpenter bees, the legitimate diurnal pollinators. The unpalatable nectar of *C. speciosa* can be viewed as a mechanism that reduces both the amount of resource per flower that can be

TABLE 4. PALATABILITY OF *C. speciosa* NECTAR, SUCROSE SOLUTION, AND SUCROSE SOLUTION PLUS CATALPA IRIDOIDS (0.4% CATALPOSIDE AND 0.4% CATALPOL) TO BUMBLEBEES AND CARPENTER BEES (MEANS ± SD)

Reward	Number of Wells	Amount consumed per well (μl)[a]
C. speciosa nectar	50	19.3 ± 2.4
Sucrose solution	54	19.7 ± 1.4
Sucrose solution plus iridoids	20	19.5 ± 1.4

[a] $P \cong 0.20$; $H = 0.43$; $df = 2$; Kruskal-Wallis test.

taken and the number of flowers that can be visited per unit time by the thieves. This would effectively shrink the size of the resource from the perspective of the thieves (but not the pollinators). Consequently, alternative nectar sources, such as other flowering species and extrafloral nectaries, may then be more attractive to thieves than the floral nectar of *C. speciosa.*

Secondary chemicals have been reported in the floral nectars of many species (Baker, 1978; Baker and Baker, 1975; Clinch et al., 1972; Deinzer et al., 1977; Guerrant and Fiedler, 1981; Pryce-Jones, 1944). In some cases, these chemicals in the nectar have been shown to adversely affect various aspects of commercial honey production or pose potential health hazards to humans who consume honey made from these nectars (Clinch et al., 1972; Deinzer et al., 1977; Pryce-Jones, 1944). Ecologists have only begun to explore the role of toxic nectars in the pollination biology of plants (Feinsinger and Swarm, 1978; Guerrant and Fiedler, 1981). To my knowledge, this is the first report of iridoid glycosides in nectar.

The toxic or unpalatable effect of iridoids on insects is well known. They are often referred to as bitter substances (Thomas, 1961; Hegnauer, 1966). Hagnauer (1973) reported that iridoids are toxic to generalist insects and that some plant species with iridoids (including species containing catalpol) have been used as insecticides. Iridoids have also been reported in the defensive secretions of many arthropods (see Cavill, 1969; Harborne, 1977; Roth and Eisner, 1962). In addition, some iridoids are antimalarial and antimicrobial (Merck Index, 1976). Finally, iridoids have been implicated in the unpalatability defenses of the checkerspot butterfly, *Euphydryas* spp. (Bowers, 1981), which suggests that they may also affect vertebrates.

This study makes no attempt to understand the mechanisms by which the bumblebees and carpenter bees circumvent the toxic effects of the catalpa iridoids. Given the rather large percentage of floral nectars that contain secondary chemicals (Baker, 1978; Baker and Baker, 1975; Guerrant and Fiedler, 1981), it is reasonable to assume that bees frequently encounter these chemicals and must have well-developed mechanisms for dealing with them (Rhoades and Bergdahl, 1981). It should also be noted that some of the bees in this study consumed up to 120 μl of nectar or sucrose solution plus catalpa iridoids over a very short period of time without showing any adverse effects; this is probably very close to the maximum amount that a large bee will take on any one foraging trip. Real (1981) noted that bumblebees usually consume only 60–80 μl of nectar per trip when foraging on an artificial flower-board.

This study did not examine the effects of catalpa iridoids on the nocturnal pollinators of *C. speciosa.* However, one of the principal nocturnal pollinators is the sphingid, *Ceratomia catalpae*, whose larvae eat the leaves of catalpa. It is reasonable to speculate that the adult moth has retained the capacity to detoxify catalpa iridoids.

From the data presented here, it appears that the extrafloral nectar of *C. speciosa* differs from the floral nectar because the same ants that avidly

gather extrafloral nectar (Stephenson, 1982) find the floral nectar to be unpalatable. This suggests that the extrafloral nectar either lacks or has very low concentrations of iridoid glycosides. In other species, it has been shown that floral and extrafloral nectar from the same plant differ in sugar concentration (Vansell, 1939), in the complement of sugars (Elias et al., 1975; Elias and Gelband, 1975; Keeler, 1977) and in the amount and types of amino acids (Baker et al., 1978). Baker et al., (1978) suggest that these differences occur because the two types of nectaries function to attract different insects with different nutritional requirements and preferences.

In conclusion, it is thought that floral morphology functions to both attract some floral visitors and exclude others (Faegri and van der Pijl, 1979). Recent studies suggest that the concentration and amount of lipids, amino acids, and sugars in floral nectar also have adaptive significance in attracting and excluding floral visitors (see Baker and Baker, 1975; Heinrich, 1975; Heinrich and Raven, 1972). This study provides evidence that secondary chemicals in floral nectars may also have adaptive significance in excluding some floral visitors.

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VOLATILE HYDROCARBONS IN THE DUFOUR'S GLAND OF THE PARASITE *Nemeritis canescens* (GRAV.) (HYMENOPTERA; ICHNEUMONIDAE)

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Abstract—The Dufour's gland of the parasitic wasp *Nemeritis* (= *Venturia*) *canescens* (Grav.) was shown by gas chromatography and mass spectrometry to contain a mixture of C_{21}, C_{23}, and C_{25} saturated and monounsaturated hydrocarbons. The main component (62%) was (*Z*)-10-tricosene. The biological activity of the components is discussed.

Key Words—Dufour's gland, volatile components, *Nemeritis canescens* (Grav.), hydrocarbons, Hymenoptera, Ichneumonidae, oviposition.

INTRODUCTION

Larvae of *Ephestia kuehniella* (Zeller) already parasitized by *Nemeritis canescens* (Grav.) appear less liable to subsequent attack (Fisher, 1961; Ganesalingam, 1974), probably because, like many other parasites, *N. canescens* (Fisher, 1961; Greany and Oatman, 1973; Lockey, 1978; Price, 1974; Robertson, 1978; Rogers, 1972) marks its host with an oviposition deterrent after parasitizing it. The deterrent has been shown to be in Dufour's gland of *N. canescens*, because untreated larvae are more readily attacked than those treated with extracts of the gland (Fisher, 1961; Johnson, 1980). We have therefore examined the volatile components of the Dufour's gland.

METHODS AND MATERIALS

Individual Dufour's glands, freshly dissected from *N. canescens*, were analyzed. Larger quantities were stored in hexane, sealed under nitrogen at −20°.

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Gland extracts (hexane) were chromatographed on 0.25-mm-thick precoated silica plates (Merck silica gel 60F-254) developed in hexane–ether–acetic acid (90:10:1). Components were detected by UV (254 nm) and then iodine vapor.

For gas chromatography, a Pye Unicam Series 204 chromatograph was used with a 2-m × 4-mm (ID) glass column of 1% SE-30 on 100–120 mesh Gas Chrom Q and a helium flow rate of 30 ml/min. Aldehydes produced by ozonolysis of unsaturated components were analyzed on a similar column packed with 2.5% Carbowax 20M on 80–100 mesh Diatomite MQ. The (*Z*)- and (*E*)-tricosenes were separated on a 2-m × 2-mm (ID) glass column with a nematic liquid crystal stationary phase, 4-(*p*-methoxy cinnamyloxy)-4′-methoxyazobenzene (5%) on 100–120 mesh Gas-Chrom Q (Lester and Hall, 1980). The column was programmed from 120–180°C at 1°C/min with a nitrogen flow rate of 30 ml/min. For combined gas chromatography and mass spectrometry (GC-MS), the above chromatograph was linked via a glass jet-separator (200°C) to a mass spectrometer (VG Micromass 70-70F with VG2025 Data System) operated at 70 eV with a source temperature of 200°C. Chemical ionization mass spectra were determined using isobutane (5×10^{-5} Torr).

Hexane extracts of the Dufour's glands were chromatographed on a short column packed with silica (60–200 mesh) impregnated with 20% silver nitrate (HI-FLOSIL-AG, Applied Science Laboratories, Inc.), giving saturated hydrocarbons by elution with hexane and unsaturated hydrocarbons with hexane–ether (9:1).

The unsaturated hydrocarbons were further separated by microscale preparative gas chromatography using a modified Pye 105 chromatograph with the analytical 1% SE-30 column described above. Separated components were collected in Pasteur pipetes.

Infrared spectra were determined in microdisks of freshly fused potassium bromide using a beam condenser with Perkin-Elmer Infracord spectrometer. Unsaturated components were ozonized in carbon disulfide (Beroza and Bierl, 1969).

Individual, freshly dissected Dufour's glands from *N. canescens* were analyzed by applying them to the sample wire of a solids injector (Hamilton, ss-60) then placed in the cold port of the gas chromatograph. To avoid pyrolysis peaks from septum fragments thrust into the injection zone by the thick needle of the injector, the septum and cap were first placed on the needle which was then cleaned and "flamed" before applying the sample. After insertion of the needle into the cold injection port, the septum cap was screwed down and helium passed for several minutes to flush the injection port and column. The sample was then volatilized by heating the injection port and swept onto the cold (50°C) column. When the temperature of the injection port reached 350°C, the column oven was programed from 50 to 280°C at 6°/min.

The (*E*)- and (*Z*)-10-tricosenes were prepared from 1-bromotridecane and 1-decanal by a Wittig reaction following the procedure of Horiike and Hirano (1980). The olefinic products were isolated by column chromatography on alumina giving (*E*)- and (*Z*)-10-tricosene (16% and 4%, respectively) after rechromatography on silver nitrate (20%) impregnated silica. Their structures were established by chemical ionization mass spectrometry (M^+322) and ^{13}C nuclear magnetic resonance spectroscopy (Jeol-PFT-100) in $CDCl_3$, Me_4Si as standard ($\delta = 0.00$ ppm) peaks at 129.8 (C_{10} and C_{11}), 32.0 (C_3 and C_{21}), 29.7 (C_4–C_8 and C_{13}–C_{20}), 22.8 (C_2 and C_{22}), 14.0 (C_1 and C_{23}) for both compounds and at 32.6 (*E*) or 27.3 (*Z*) for C_9 and C_{12}. (*Z*)-9-Tricosene was obtained from Farchan Division, Story Chemical Corporation, Willoughby, Ohio.

The responses of *Nemeritis* were observed when doses of the principal components of the Dufour's gland were presented to an active female wasp in a small plastic arena.

Two methods were used. In the first, healthy *Ephestia* larvae were dipped into a hexane extract of one of the main components, allowed to dry, and then exposed, in pairs with a healthy untreated larva, to an "experienced" *Nemeritis* in a plastic Petri dish (9 cm diameter) inverted over a filter paper.

In the second method, 5 μl of a hexane extract of each component was deposited on a filter paper from a micropipete and presented to a single *Nemeritis* as the floor of the test arena.

The wasps were observed continuously for 5 min from introduction, and their behavior was noted in each 1-min period.

The principal components were diluted so that each was presented to the parasites at the level of one gland equivalent per trial. For (*Z*)-10-tricosene, heneicosane, tricosane, and pentacosane, the naturally occurring concentrations in each Dufour's gland are 8.7, 2.8, 1.5, and 0.7 μg, respectively. The tests were conducted under indirect lighting at 24°C (±1°C). All *Ephestia* larvae were incubated at 25°C for 48 hr after each experiment and then dissected to record the distribution of eggs where oviposition had occurred.

Because newly emerged *Nemeritis* vary considerably in their searching activity, only 2-day-old females which had previously been given the opportunity to attack *Ephestia* larvae were used. Such "experienced females" will readily search an empty arena within 2 min of introduction.

RESULTS AND DISCUSSION

Extraction with ether yielded approximately 14 μg material per gland, although GC analysis of single glands showed that this quantity varied considerably from insect to insect (10–16 μg) as did the relative proportions of the components in the Dufour's glands (Table 1, Figure 1). Thin-layer chromatography of individual gland extracts showed that they contained

TABLE 1. COMPOSITION OF DUFOUR'S GLAND HYDROCARBONS OF *Nemeritis canescens*

	(% ± SD)
1. (*Z*)-8-, 9-, and 10-heneicosenes	1.2 ± 0.4
2. Heneicosane	20.3 ± 2.1
3. (*Z*)-10-Tricosene	62.0 ± 5.7
4. Tricosane	11.1 ± 3.8
5. (*Z*)-10-Pentacosene	1.1 ± 0.4
6. Pentacosane	4.7 ± 1.8

almost exclusively hydrocarbons (R_f-0.72) sometimes with minute traces of a more polar material (R_f-0.20).

The saturated hydrocarbons of the Dufour's gland (Table 1) were isolated by argentation chromatography and each identified by comparison with authentic compounds [GC retention time, peak enhancement, electron impact (EI) and chemical ionization (CI) mass spectra]. All gave $(M-H)^+$ ions as the base peak in their chemical ionization mass spectra.

The unsaturated hydrocarbons isolated from the Dufour's gland by argentation chromatography were identified by GC-EI-MS and GC-CI-MS

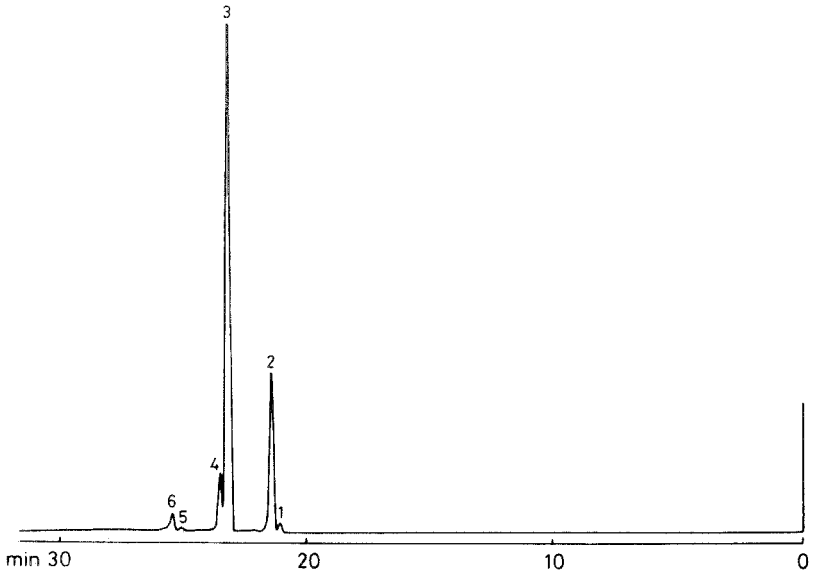

FIG. 1. GC of volatile material from a single Dufour's gland of *Nemeritis canescens* (Grav.) by solid injection. The column was programed from 50 to 280° at 6°/min. Peak numbers correspond to those in Table 1.

as a mixture of C_{21}, C_{23}, and C_{25} monoenes; their isobutane chemical ionization mass spectra all showed strong M^+ and $(M + H)^+$ ions. Double-bond configurations could not be deduced from the intensities of the $(M + C_4H_9)^+$ and $(M - H)^+$ ions because they varied strongly with source temperature, neither were their positions indicated by an enhanced abundance of ions arising from allylic activation (Budzikiewicz and Buser, 1980) under these conditions. Therefore each component of the unsaturated hydrocarbon fraction was separated by preparative gas chromatography and each component was ozonized. Tricosene, the main component (Table 1) of the Dufour's gland, produced equal amounts decanal and tridecanal (identified by comparing retention times and mass spectra with authentic aldehydes), establishing that the double bond was at C-10. The infrared spectrum of the tricosene showed no band at 969 cm^{-1} indicating a *cis* double bond. Synthetic (*Z*)- and (*E*)-tricosene were well separated (retention times 43.4 and 48.2 min, respectively) on a packed column with a liquid crystal stationary phase (Lester and Hall, 1980). The configuration of the double bond in the natural tricosene was confirmed by peak enhancement on this column with authentic (*Z*)-10-tricosene. The GC-EI-MS and GC-CI-MS of the tricosene were identical with those of synthetic (*Z*)-10-tricosene.

Ozonolysis of the minor components (pentacosene and heneicosene) produced insufficient aldehydes for identification, and single ion monitoring (*m/z* 29) under normal conditions (resolution of 1000) was impractical because of bleed from the Carbowax 20M column. However, at a resolution of approximately 2000, the doublet *m/z* 29.0027 ($H-(C=O^+$, aldehyde) and *m/z* 29.0991 ($C_2H_5^+$, column bleed) was well separated and the $H-C=O^+$ ion could be monitored without background interference. Dodecanal and tridecanal were thus detected in the ozonolysis products of the pentacosene, establishing the double-bond position at C-10. Similarly, using single ion monitoring at a resolution of 2000, a mixture of C_8, C_9, C_{10}, C_{11}, C_{12}, and C_{13} aldehydes were detected as ozonolysis products of the C_{21} unsaturated fraction showing that a mixture of heneicosenes with double bonds at C-8, C-9, and C-10 were present.

Satisfactory infrared spectra were not obtainable on the small amounts of the pentacosene and heneicosenes available, but the absence of a band at 969 cm^{-1} in the total unsaturated fraction (300 μg) suggests that all the unsaturated components have the *cis* configuration.

n-Alkenes have been found in many insect species, but double-bond positions have been determined in relatively few. In C_{21}, C_{23}, and C_{25} alkenes, unsaturation is often found at C-9, bur rarely at C-10. A (*Z*)-10-pentacosene has been reported in *Musca autumnalis* (Diptera) (Uebel et al., 1975), and a mixture of 9- and 10-pentacosenes of unspecified configurations have been found (Lockey, 1977) in the cuticular lipids of *Tenebrio obscurus* (Coleoptera). However, no naturally occurring 10-tricosene (or 10-heneicosene) has been reported.

The biological activity of the major hydrocarbon components isolated from the Dufour's gland of *Nemeritis* is currently under detailed investigation and will be published separately. Only the preliminary observations are reported in this paper which is primarily concerned with the isolation and chemical identification of the components. Of the four major components, heneicosane, (*Z*)-10-tricosene, tricosane, and pentacosane, only the first (heneicosane) had an immediate and detectable effect on the behavior of the parasitoid wasp. Each *Nemeritis* enclosed in an arena with heneicosane-treated *Ephestia* larvae immediately became very active. The host larvae were rapidly palpated with the antennae and briefly probed with the ovipositor but without oviposition taking place. The parasite then became extremely active, running around the arena and repeatedly attempting to fly. Between the flight attempts the wasp ran round the arena rapidly, repeatedly visiting each larva in turn, palpating it, and leaving it without probing with the ovipositor. *Nemeritis* introduced into arenas containing only a spot of 2.8 μg heneicosane on a filter paper also became very active. Searching behavior commenced and palpation of the heneicosane spot was immediately followed by flight attempts, which continued at intervals throughout the test period. No probing behavior was seen.

The remaining components tested [(*Z*)-10-tricosene, tricosane, and pentacosane] elicited no such responses. *Nemeritis* females equally palpated, probed, and oviposited in treated and control *Ephestia* larvae. In tests with spots of components at single gland equivalent concentrations, *Nemeritis* females searched the whole arena, probed the perimeter, and crossed the component spots repeatedly without stopping or palpating them. In no case were the repeated attempts to fly observed.

These preliminary observations suggest that heneicosane is the only component that causes a marked change in the behavior of the wasp. If heneicosane acts as a pheromone, stimulating active running and flight in larger arenas, it is probable that it is functioning as an external marker substance that results in the wasp leaving the area, with the increased chance of finding another patch or group of hosts.

Various accessory reproductive glands have been suggested as the source of host-marking chemicals (Greany and Oatman, 1972; Jackson, 1969; Robertson, 1968). The Dufour's gland has been shown to be the source of host marking pheromones in the ichneumonid *Campoletis perdistinctus* (Viereck) (Guillot and Vinson, 1974) and the braconids *Microplitis croceipes* (Cresson) and *Cardiochiles nigriceps* (Viereck) (Vinson, 1972). In the latter case the activity of the Dufour's gland extract was attributed to hydrocarbon components (Guillot et al., 1974), but these were not identified.

Heneicosane, which accounted for 20% of the Dufour's gland secretion in *Nemeritis*, has been found as a component in the trail pheromones of ants (*Formica* spp.) by Bergström and Löfqvist (1973), in *Oecophylla longinoda* (Bradshaw et al., 1979), and in a weevil, *Callosobruchus*, by Oshima et al.

(1973). The major component, (*Z*)-10-tricosene (62%), has no apparent effect on the behavior of *Nemeritis*, and it is suggested that this oily substance may be functioning primarily as a lubricant for the passage of eggs down the ovipositor and secondarily as a carrier for the heneicosane, which is a solid (mp 40.4°C) at room temperature.

Löfqvist (1976) has suggested that some of the hydrocarbons found in the Dufour's gland of the ant *Formica rufa* may act as alarm pheromones and stimulate fast running behavior and that they may have a combined additive effect.

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COMPOSITION, QUANTIFICATION, AND PERIODICITY OF SEX PHEROMONE GLAND VOLATILES FROM INDIVIDUAL *Heliothis virescens* FEMALES

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Abstract—Sex pheromone gland volatiles from individual *Heliothis virescens* (F.) females were collected and analyzed on an SP-2330 capillary gas–liquid chromatography column for identification and quantification of the compounds emitted. Only four of the seven compounds previously reported as pheromone components appeared consistently in the volatile collections: 14:Ald, Z9–14:Ald, 16:Ald, and Z11–16:Ald. The female glands did not emit the same amounts of these compounds throughout a 24-hr period; they emitted maximum quantities between 6 and 11 hr after the onset of scotophase with the remainder of the photoperiod having minimal emission rates. Although the absolute quantities fluctuated, the percent compositions of the compounds remained about the same throughout the 24-hr period.

Key Words—*Heliothis virescens*, Lepidoptera, Noctuidae, Z11–16:Ald, 16:Ald, Z9–14:Ald, 14:Ald, sex pheromone emission, gland volatiles, blend composition, pheromone emission rates.

INTRODUCTION

(*Z*)-9-Tetradecenal (Z9–14:Ald) and (*Z*)-11-hexadecenal (Z11–16:Ald) were first identified from the female pheromone gland of *Heliothis virescens* by Roelofs et al. (1974), utilizing methylene chloride extracts of glands, and by Tumlinson et al. (1975), using ether washes of the glands. These two compounds in a 1:16 synthetic mixture were highly stimulatory to male *H. virescens* in laboratory tests (Roelofs et al., 1974). Tumlinson et al. (1975)

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suggested that other compounds may be behaviorally significant; equivalent amounts of the crude ether wash from female glands attracted more males in cage tests than did purified natural pheromone or the synthesized material. In 1980, Klun et al. reported the isolation and identification of five new compounds from hexane gland washings: tetradecanal (14:Ald), hexadecanal (16:Ald), (*Z*)-7-hexadecenal (*Z*7–16:Ald), (*Z*)-9-hexadecenal (*Z*9–16:Ald), and (*Z*)-11-hexadecenol (*Z*11–16:OH) along with the two known pheromone components and suggested that all seven compounds comprised the complete pheromone blend of *H. virescens*. Field-trapping experiments (Sparks et al., 1979) suggested that at least some of the five newly identified compounds were indeed components of the pheromone, but which of the five were behaviorally active was not demonstrated.

It was our intention to perfect the collection system developed by Baker et al. (1981) to allow for accurate, quantitative measurements of volatiles collected from individual female glands with extremely low rates of emission. Using this technique, we examined the individual variation in *H. virescens* emission rates as well as the ratios of compounds and compared these to those previously reported from extracts. We felt insight could be gained into the importance of these compounds in this species' communication system by examining how precisely their ratios were regulated in different females. We investigated the effect of photoperiod on the ratios and quantities emitted. A comparison was also made between lab-reared females and feral females from Bolivia.

METHODS AND MATERIALS

Heliothis virescens larvae were reared on a pinto bean media modified from Shorey and Hale (1965). The pupae were collected, separated by sex, and placed in a Percival® controlled environment chamber at 26°C, 30–70% relative humidity, on a 14:10 light–dark photoperiod.

Females in their fourth photoperiod were individually prepared for collection as described by Baker et al. (1981), with the following modifications. Each female was injected with ca. 1 μl of 0.5 g/ml tetrasodium ethylenediaminetetraacetate (EDTA-Na_4) by passing the tip of the syringe needle through the cavities of the thorax and abdomen to the vicinity of the last two abdominal segments. This effectively prevented the females from retracting the gland once it had been artificially extruded. Each abdomen preparation was placed for 10 min in the collection device (Baker et al., 1981) in a stream of molecular sieve-filtered N_2 (type A, 8–12 mesh beads), flowing at 120 ml/min. The collection apparatus itself was modified by shortening the distance between the gland and the glass wool used for adsorbing gland volatiles and reducing the diameter of the glass tip of the nitrogen exit a few

millimeters downstream from the glass wool. The glass wool needed for capture of volatiles was therefore reduced to ca. 0.005 g and CS_2 rinse reduced to ca. 200 μl. However, the efficiency of recovery of synthetic candidate aldehydes, alcohols, and acetates remained at 85–100%, levels no different than for the original unmodified system (Baker et al. 1981). For both the unmodified and modified systems no significant breakthrough occurred with the 14- and 16-carbon aldehydes tested or with the 16-carbon alcohol, and nearly 100% mass balance was achieved. Eight ng of octadecanal (18:Ald) was added to each sample as an internal standard. The collection rinse plus internal standard was reduced to ca. 0.5–1.0 μl under a gentle stream of N_2 and analyzed immediately on a Varian model 3700 gas chromatograph equipped with a 45-m SP-2330 glass capillary column (direct injection, injector temperature = 180°, FID temperature = 180°, isothermal oven temperature = 150°C, N_2 flow rate = ca. 30 cm/sec). GLC peak areas were calculated by a Hewlett-Packard 3380A integrator.

For some preparations (e.g., after the gland volatiles had been collected for 10 min in the N_2 stream), the ovipositor was clipped off at its base and extracted in a 10-μl solution of 1 ng/μl 18:Ald in CS_2. The extract was analyzed on a Hewlett-Packard 402 gas chromatograph equipped with a 3-m × 4-mm OD glass column of 4.706 g 10% Silar 10C on acid washed 100–120 Chromasorb W (oven temperature = 170°C; N_2 flow rate = 30 ml/min). GLC peak areas were calculated using peak height × retention time.

Females one generation removed from a feral field population collected in Bolivia by Dr. J. Greenblatt and D. Kelly of Albany International were received as pupae. These second-generation pupae were sexed and each female placed in a small cup with a water-soaked dental wick and placed in a Percival® controlled environment chamber on a 14:10 light–dark photoperiod until emergence. Females in their fourth photoperiod were prepared and their volatile compounds collected as described above.

Synthetic standards of all the compounds (14:Ald, *Z*9-14:Ald, 16:Ald, *Z*7-16:Ald, *Z*9-16:Ald, *Z*11-16:Ald, *Z*11-16:OH, and 18:Ald) were injected on SP-2330 to get relative retention times for comparison with female volatiles. Retention times of female volatiles were also compared with synthetic standards on two other GLC columns. Volatiles from a pool of five collections were injected on a 1-m × 4-mm OD column packed with 5% SF-96 on Chromasorb W AcW-DMCS at 150°C and 20 ml/min N_2 flow rate, and glass capillary–dry ice collections made at 1.75–2.75 min (14-carbon aldehyde region) and 4.50–6.0 min (16-carbon aldehyde region). The capillaries were rinsed with ca. 30 μl CS_2 to which 50 ng dodecyl acetate (12:Ac) internal standard was added and which was then concentrated under a nitrogen stream. Each fraction was injected onto a second 3-m × 4-mm OD column of 10% XF-1150 on ChW AcW-DMCS at 150°C and with the N_2 flow rate at 25 ml/min.

RESULTS

Collections from the individual female glands of *H. virescens* indicated that only four compounds were emitted by nearly every female during scotophase (Table 1, Figure 1). The retention times of these four compounds relative to 18:Ald on SP-2330 at 150°C were 0.686, 0.555, 0.402, and 0.333, which corresponded to the standards of *Z*11–16:Ald, 16:Ald, *Z*9–14:Ald, and 14:Ald, respectively, which had retention times relative to 18:Ald of 0.688, 0.557, 0.404, and 0.334, respectively. The presence of these four compounds in female emissions was also confirmed by the injection onto SF-96 of a pooled sample of five females' emissions and the subsequent glass capillary collection of two pairs of peaks from the 14-carbon and 16-carbon aldehyde regions. There were two small peaks in the 14-carbon aldehyde region at 2.1 and 2.3 min (synthetic 14:Ald and *Z*9–14:Ald were 2.1 and 2.3 min, respectively), and reinjection of this region on XF-1150 resulted in two peaks with relative retention times to 12:Ac of 1.39 and 1.64 (synthetic 14:Ald and *Z*9–14:Ald relative retention times of 1.39 and 1.65). There were two larger peaks on SF-96 at 5.1 and 5.6 min in the 16-carbon aldehyde region (synthetic 16:Ald and *Z*11–16:Ald were 5.1 and 5.6 min, respectively), and reinjection of this fraction onto XF-1150 produced two peaks with relative retention times of 2.90 and 3.48 (synthetic 16:Ald and *Z*11–16:Ald relative retention times were 2.93 and 3.46, respectively).

Examination of the temporal pattern of volatile release throughout the photoperiod indicated that exposure of the gland surface only resulted in significant volatile emission between 6 and 11 hr after the onset of scotophase (Figure 2). This suggests that there could have been some sort of biochemical regulation of compound production and release by the gland cells apart from the physical regulation of evaporation by gland retraction. *Z*11–16:Ald was emitted at a rate of ($\bar{X} \pm$ SE) 3.26 ± 0.36 ng/min, 16:Ald at 0.58 ± 0.08 ng/min, *Z*9–14:Ald at 0.25 ± 0.04 ng/min, and 14:Ald at 0.32 ± 0.05 ng/min (Table 1). Minimum quantities were evaporated between 1 and 3, and 14 to 24 hr after scotophase onset with means of only 0.40 ± 0.13 ng/min for *Z*11–16:Ald, 0.08 ± 0.03 ng/min for 16:Ald, 0.02 ± 0.01 ng/min for *Z*9–14:Ald, and 0.04 ± 0.01 ng/min for 14:Ald (Table 1). Therefore, about eight times as much of each compound was emitted during the maximum period as during the minimum period of emission. The emission rate of *Z*11–16:Ald from Bolivian females was slightly lower, 1.85 ± 0.49 ng/min, than that of lab-reared females, although this may be due in part to the smaller sample size (Table 1).

Other differences also distinguished the periods of maximum and minimum pheromone release. The maximum period was characterized by 98–100% of the females releasing detectable amounts of 14:Ald, *Z*9–14:Ald, 16:Ald, and *Z*11–16:Ald, whereas during the minimum periods 100%

TABLE 1. QUANTITIES OF COMPOUNDS IN VOLATILE EMISSIONS AND GLAND EXTRACTS FROM *H. virescens* FEMALES

	Emitted volatiles, lab females (ng/min ± SE)[a]		Gland extracts, lab females (ng/gland ± SE)[b]		Emitted volatiles, Bolivian females (ng/min ± SE)[a]
	Max[c] (N = 40)	Min[d] (N = 24)	Max[c] (N = 7)	Min[d] (N = 4)	Max[c] (N = 10)
*Z*11-16 : Ald	3.26 ± 0.36	0.40 ± 0.13	129.46 ± 50.17	8.19 ± 3.01	1.85 ± 0.49
16 : Ald	0.58 ± 0.08	0.08 ± 0.03	33.04 ± 11.47	1.26 ± 0.56	0.10 ± 0.03
*Z*9-14 : Ald	0.25 ± 0.04	0.02 ± 0.01	0.48 ± 0.33	0.00 ± 0.00	0.09 ± 0.02
14 : Ald	0.32 ± 0.05	0.04 ± 0.01	0.80 ± 0.37	0.11 ± 0.11	0.06 ± 0.02
*Z*7 and/or *Z*9-16 : Ald	0.03 ± 0.01	0.00 ± 0.00	3.71 ± 3.71	3.54 ± 1.66	0.04 ± 0.01
*Z*11-16 : OH	0.00 ± 0.00	0.00 ± 0.00	7.40 ± 4.07	7.69 ± 4.21	0.00 ± 0.00

[a]Individual compounds which were less than the lower limit of detection (0.01 ng/min) were counted as 0.
[b]Individual compounds which were less than the lower limit of detection (0.15 ng/gland) were counted as 0.
[c]Maximum period volatile emission, 6–11 hr after the onset of scotophase.
[d]Minimum period of volatile emission, 1–3 and 14–24 hr after the onset of scotophase.

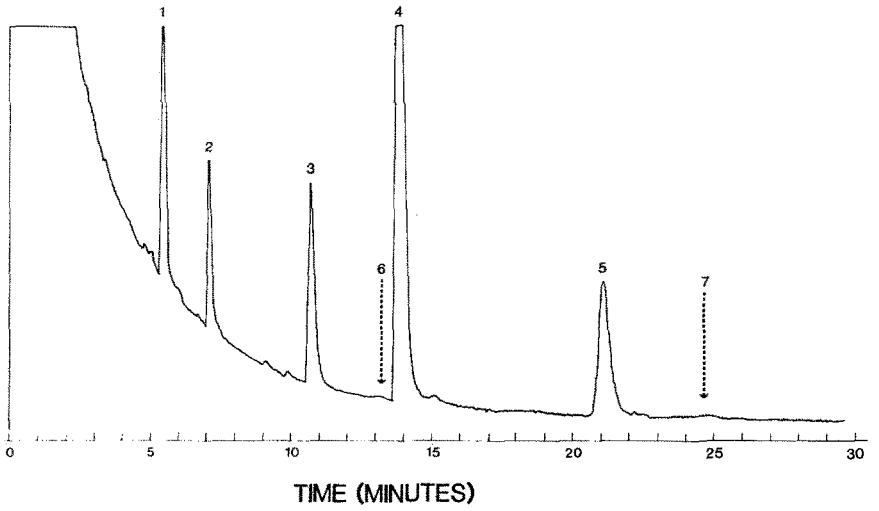

FIG. 1. Sample SP-2330 chromatogram of volatile emissions from 10-min collection of *H. virescens* female. 1 = 14: Ald; 2 = Z9-14:Ald; 3 = 16:Ald; 4 = Z11-16:Ald; 5 = 18:Ald (internal standard). Retention times of Z7- and Z9-16:Ald (6) and Z11-16:OH (7) are indicated by broken arrows.

released Z11-16:Ald, while only 79% released 16:Ald, 42% released Z9-14:Ald, and 58% released 14:Ald (Table 2). The maximum emission period corresponds to the peak hours of calling (Sparks et al., 1979), and the emitted compound ratios likely are more biologically meaningful than those from the minimum period, in which female ovipositors would not normally be extruded.

Relative retention times of 0.612 and 0.628 corresponded to standards of Z7-16:Ald and Z9-16:Ald, respectively, on SP-2330 at 150°C. Only 75% of the females during the maximum period and only 13% during the minimum period released detectable amounts of one or both of these compounds (Table 2). Additionally, Z11-16:OH with a retention time relative to 18:Ald of 1.165 was not detected in the emissions of any of the females during either the maximum or minimum periods of evaporation.

The mean percent compositions (calculated using the percent composition of each compound emitted from each individual female) of Z11-16:Ald, 16:Ald, Z9-14:Ald, and 14:Ald for emissions during the maximum period were 74.48, 12.65, 5.04, and 6.58, respectively; for the extracts, the mean percent compositions were 70.40, 19.90, 0.13, and 0.34, respectively (Table 3). Although Z11-16:OH was never found in any of the emission collections, it made up 2.76% of the compounds found in the gland extracts. Z7-16:Ald and/or Z9-16:Ald also made up a greater percentage of the compounds found in the gland extracts (6.47%) than in the emission

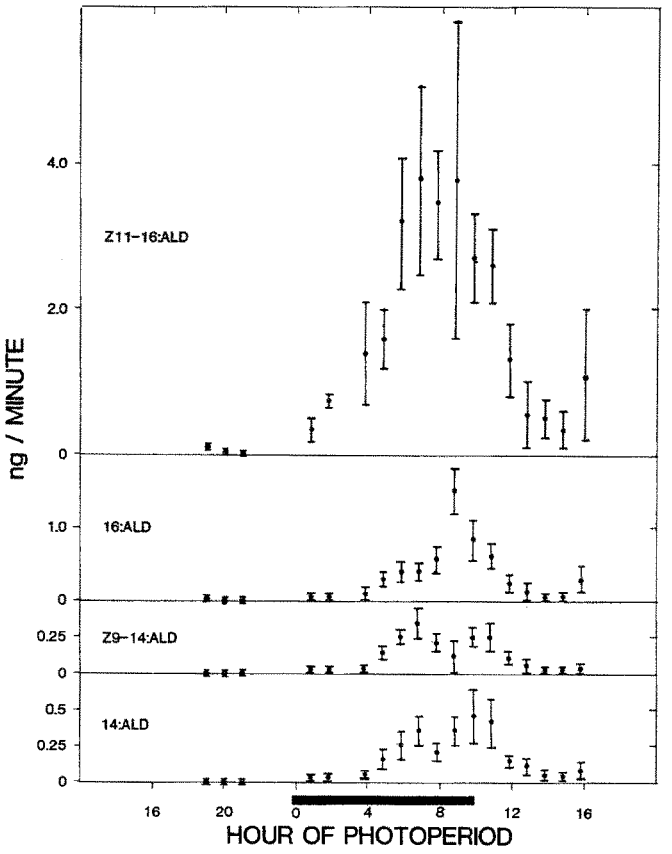

FIG. 2. Mean emission rates (±SE) of compounds from *H. virescens* females over 24-hr period. Darkened area on *x* axis indicates scotophase. N = 3, 3, 3, 8, 8, 8, 6, 3, 7, 6, 10, 3, 4, 3, 3, 4, 3, 2 for hours 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19, 20 and 21, respectively.

collections (0.91%) (Table 3). Females from Bolivia appeared to emit slightly lower percentages of 16:Ald, Z9–14:Ald and 14:Ald, and slightly higher percentages of Z11–16:Ald and Z7–16:Ald and/or Z9–16:Ald than laboratory-reared females, although part of this difference may be due to their small sample size (Table 3).

The mean ratios of the four compounds from the maximum emission period were nearly identical to those from the minimum period (Table 3). A plot of the percent composition of each compound in the maximum period of emission against the total ng/min produced by the individual gland (Figure 3) resulted in insignificant r values, demonstrating that none of the ratios of the four compounds was dependent on the total amount of volatile compounds emitted by the gland. Therefore, even when the gland emitted low levels of

TABLE 2. PERCENTAGE OF *H. virescens* FEMALES EMITTING DETECTABLE AMOUNTS OF EACH COMPOUND OR CONTAINING DETECTABLE AMOUNTS OF EACH COMPOUND IN GLAND EXTRACTS

	Emitted volatiles, lab females		Gland extracts, lab females		Emitted volatiles, Bolivian females
	Max[a] ($N = 40$)	Min[b] ($N = 24$)	Max[a] ($N = 7$)	Min[b] ($N = 4$)	Max[a] ($N = 10$)
*Z*11-16 : Ald	100	100	100	100	100
16 : Ald	98	79	100	100	100
*Z*9-14 : Ald	98	42	29	0	90
14 : Ald	98	58	57	25	100
*Z*7 and/or *Z*9-16 : Ald	75	13	14	75	80
*Z*11-16 : OH	0	0	43	100	0

[a]Maximum period of volatile emission, 6-11 hr after onset of scotophase.
[b]Minimum period of volatile emission, 1-3 and 14-24 hr after the onset of scotophase.

TABLE 3. PERCENTAGES[a] (± SE) OF COMPOUNDS IN VOLATILE EMISSIONS AND GLAND EXTRACTS FROM *H. virescens* FEMALES

	Emitted volatiles, lab females		Gland extracts, lab females		Emitted volatiles, Bolivian females
	Max[b] ($N = 40$)	Min[c] ($N = 24$)	Max[b] ($N = 7$)	Min[c] ($N = 4$)	Max[b] ($N = 10$)
*Z*11–16 : Ald	74.48 ± 1.66	71.77 ± 4.86	70.40 ± 6.78	38.37 ± 1.12	85.2 ± 1.35
16 : Ald	12.65 ± 1.08	16.30 ± 2.87	19.90 ± 5.50	5.72 ± 0.83	5.60 ± 1.03
*Z*9–14 : Ald	5.04 ± 0.45	3.49 ± 1.14	0.13 ± 0.08	0.00 ± 0.00	3.75 ± 0.65
14 : Ald	6.58 ± 0.76	8.62 ± 3.04	0.34 ± 0.12	0.28 ± 0.28	3.12 ± 0.47
*Z*7 and/or *Z*9–16 : Ald	0.91 ± 0.17	0.32 ± 0.22	6.47 ± 6.47	23.90 ± 8.38	2.35 ± 0.70
*Z*11–16 : OH	0.00 ± 0.00	0.00 ± 0.00	2.76 ± 1.55	31.74 ± 6.46	0.00 ± 0.00

[a]Percentages (± SE) were calculated using the percent compositions of each compound emitted from each individual female. If a compound was less than the lower limit of detection (0.01 ng/min or 0.15 ng/gland), it was counted as contributing 0% to the composition.
[b]Maximum period of volatile emission, 6–11 hr after the onset of scotophase.
[c]Minimum period of volatile emission, 1–3 and 14–24 hr after the onset of scotophase.

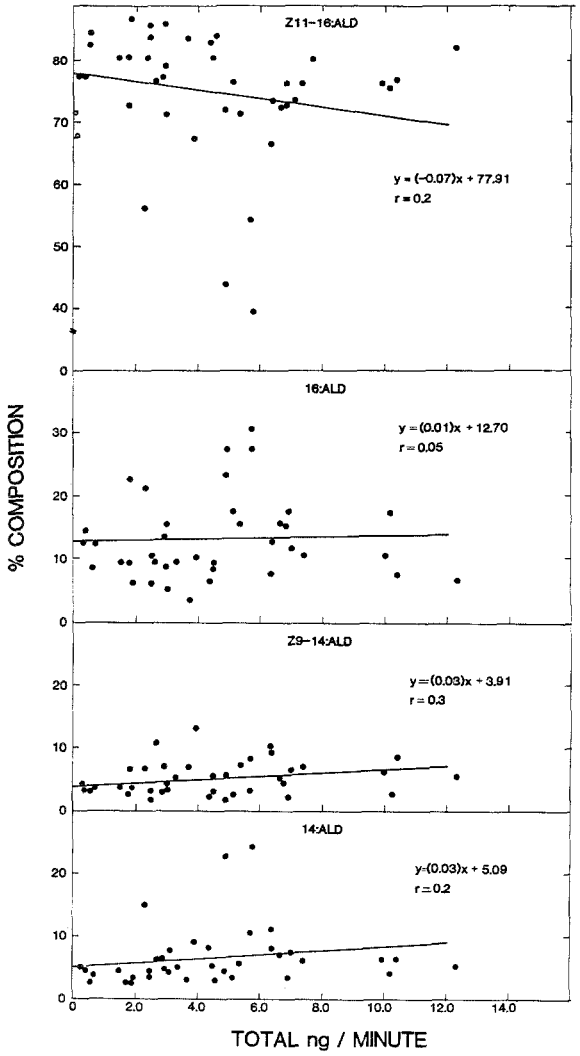

FIG. 3. Change in percent composition of each emitted compound as the sum of the four emitted compounds increased. Each point represents a reading from an individual female during the maximum release period.

volatile compounds, i.e., during photophase, the ratio of the four compounds changed very little.

DISCUSSION

Analysis of the volatile collections established that only four compounds, Z11-16:Ald, 16:Ald, Z9-14:Ald, and 14:Ald, were released consistently by females during the period of maximum pheromone release. Z7-16:Ald, Z9-16:Ald, and Z11-16:OH, on the other hand, appeared inconsistently or not at all in the collections. The lack of detectable amounts of Z7- and Z9-16:Ald by 25% of maximally emitting females and Z11-16:OH by all females implies that these compounds may be relatively unimportant for mate-finding in this species. It is difficult to understand how selection pressures would permit compounds crucial to mate-finding to be omitted from a significant proportion of females' emitted blends, although the possibility remains that females may be emitting them at levels undetectable by GLC, and their extremely low percentages in the blend ratios may significantly affect male behavior. A trapping experiment (Hartstack et al., 1980) appeared to indicate that Z11-16:OH increased capture of males when added to the two-component Z11-16:Ald and Z9-14:Ald blend or as part of a seven-compound blend. However, in the same study several other experiments resulted in no significant increase, and sometimes a decrease, in capture when seven compounds were used compared to the two-components alone.

The predictable occurrence of 16:Ald and 14:Ald in maximally emitting females' blends along with the pheromone components Z11-16:Ald and Z9-14:Ald suggests that these are more likely to be important for sexual communication. Their effects, along with those of Z7-16:Ald, Z9-16:Ald, and Z11-16:OH, should continue to be examined carefully in male behavioral experiments to determine whether they, too, should be considered to be pheromone components. Z7- and Z9-16:Ald and Z11-16:OH were found in greater quantities in excised gland extracts than in emissions, and this discrepancy for Z11-16:OH is consistent with its behavior on glass surfaces (Baker et al., 1981) from which it is emitted very slowly compared to similar quantities of Z11-16:Ald. We conclude from the emission data that of the five new compounds implicated as being part of *H. virescens* sexual communication (Klun et al., 1980), 16:Ald and 14:Ald may be the only ones that are behaviorally important. Tumlinson et al. (1982) also have consistently detected 16:Ald and 14:Ald in *H. virescens* female emissions, substantiating these results.

The blend ratios of the four compounds always emitted during peak calling were not significantly affected by the total emission rate (Figure 3).

Furthermore, although the absolute amounts of the four compounds varied markedly between the hours of maximum and minimum emission (Table 1), the ratios at these two different time periods did not (Table 3). Blend quality, therefore, was maintained despite changes in blend quantity, and although variations in pheromone blend quality have been found through individual gland extract analysis in other species (Miller and Roelofs, 1980; Klun et al., 1979), this is the first study to examine variations in emitted blends from individual females. It remains to be determined whether or not the blend constancy of 16:Ald and 14:Ald is related to their use as pheromone components or whether they are biosynthetic excess baggage evaporated along with the two known components *Z*11–16:Ald and *Z*9–14:Ald. Their signal value to other species should not be ignored, either, and perhaps they will turn out to be antagonistic to species that share part of the same chemical communications system.

The hours during which the maximum amounts of the pheromone were emitted from the gland and the largest amounts of pheromone were extracted from the gland coincide with the natural calling period for this species which is 2330–0230 hr, the greatest male response occurring at ca. 0130 hr (Sparks et al., 1979). As photophase approached, however, the amount of pheromone evaporating from the gland started to decrease until it reached a minimum level which remained low for the duration of the photoperiod. Further studies may clarify whether synthesis at the beginning of scotophase, evaporation during scotophase, and then resorption of unemitted compounds at the end of scotophase could account for the relative amounts found on the gland compared to those emitted.

Interestingly these emission rate changes occurred despite the constant degree of extrusion of glands in our apparatus. This implies that an underlying biosynthetic fluctuation was causing the periodicity of pheromone emission, not merely the physical actions of gland extrusion and retraction. Coffelt et al. (1978) found more pheromone on the gland surfaces of female *Plodia interpunctella* (Hübner) during their calling period than from glands rinsed at other hours of the photoperiod, again implying an underlying biochemical fluctuation. Further studies of individual female emissions using our collection apparatus should provide new insight into regulation of pheromone blend ratios and rates of release in a variety of species.

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SPECIFICITY OF LABORATORY TRAIL FOLLOWING BY THE ARGENTINE ANT, *Iridomyrmex humilis* (Mayr), TO (*Z*)-9-HEXADECENAL, ANALOGS, AND GASTER EXTRACT

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Abstract—In laboratory trail-following bioassays of Argentine ant workers, *Iridomyrmex humilis* (Mayr), the geometric isomer, (*E*)-9-hexadecenal, of the trail pheromone component (*Z*)-9-hexadecenal elicited insignificant trail following as did the potentially more stable formate analogs, (*Z*)-7-tetradecenyl formate, (*E*)-7-tetradecenyl formate, and tetradecyl formate. Further, in direct choice tests, workers showed no preference for gaster extract trails (0.002 ant equiv/cm) over trails of (*Z*)-9-hexadecenal (0.2 ng/cm). Moreover, a 10-fold increase in synthetic trail concentration to 2.0 ng/cm caused (*Z*)-9-hexadecenal trails to be significantly preferred over gaster extract trails by trail-following ants.

Key Words—Argentine ant, *Iridomyrmex humilis* (Mayr), Hymenoptera, Formicidae, trail-following bioassay, (*Z*)-9-hexadecenal, geometric isomer, analogues, choice tests.

INTRODUCTION

Recently, (*Z*)-9-hexadecenal (*Z*9-16:Ald) was isolated and identified from the ventral glands of Argentine ants, *Iridomyrmex humilis* (Mayr) and, from preliminary tests of its "aggregating" effects of workers, implicated as part of this species' trail pheromone (Cavill et al., 1979, 1980). Van Vorhis Key and Baker (1981) demonstrated that intense and prolonged trail following is elicited by this compound alone, and not by several analogs. Although trail following levels to Z9-16:Ald were equivalent to crude gaster extract trails, direct choice tests between natural and synthetic trails were not performed.

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Because of the possibility of using *Z*9–16:Ald to modify *I. humilis* trail-following behavior in urban settings and in the field where this ant is a pest of citrus, we sought to determine its competitiveness with gaster extract trails and to see whether several formate analogs, potentially more stable than the aldehyde pheromone, would elicit comparable levels of trail following.

METHODS AND MATERIALS

Chemicals. *Z*9–16:Ald and (*Z*)-7-hexadecenal (*Z*7–16:Ald) were obtained from the Controlled Release Division of Albany International Corporation. Purity of these compounds was determined by gas–liquid chromatography (GLC), using 10% XF-1150 (50% cyanoethyl methyl silicone) on Chromosorb W, AW-DMCS, 100/120 mesh (2.5 × 2 mm), at 150° C and a carrier flow rate of 25 ml/min. The *Z*7–16:Ald was greater than 98%, and the *Z*9–16:Ald greater than 97% free of other volatile impurities. (*Z*)-7-tetradecenyl formate (*Z*7–14:Form), (*E*)-7-tetradecenyl formate (*E*7–14:Form), and tetradecyl formate (14:form) were provided by W. Roelofs and M. Gieselmann at the Geneva, New York, Agricultural Experiment Station. The *Z*7–14:Form was greater than 94%, the *E*7–14:Form greater than 91%, and the 14:Form greater than 93% free of other volatile impurities, determined by GLC, using 3% OV-101 (methyl silicone) on 100/120 mesh, acid-washed Chromosorb W-DMCS on a 2-m × 2-mm column at 170° C and a carrier flow rate of 40 ml/min. (*E*)-9-Hexadecenal was provided by L.K. Gaston and M.M. Pope (University of California at Riverside) and was greater than 96% free of other volatile impurities (<0.2% *Z*9–16:Ald; <0.2% 16:Ald), determined by GLC using 10% Silar 10C (3-cyanopropyl silicone) on acid-washed Chromosorb W, 100/120 mesh, on a 2.8-m × 2-mm column. The retention time of *E*9–16:Ald was 8.07 min at 170° C and a 25 ml/min carrier flow rate.

General. Colonies of the Argentine ant, *Iridomyrmex humilis* (Mayr), collected near Riverside, California, were maintained in the laboratory as described previously (Van Vorhis Key et al., 1981). Immediately prior to bioassay periods, colonies were provided with new food sources in enclosed dishes attached to the nest boxes by flexible tubing. Once recruitment had been initiated through the tubing, individual unfed ants which had been recruited to the food source but had not yet arrived were redirected and introduced onto the experimental trail directly from the natural trail inside the tubing.

Analog Activities. Application of trails, preparation of gaster extracts, and bioassay procedure were performed as described previously (Van Vorhis Key et al., 1981). Circular trails (50.7 cm circumference, 2 mm wide) were constructed by siphoning diluted solutions onto revolving filter paper disks

(Whatman No. 1, 24 cm diam). These disks were then placed under a glass plate held 3 mm above the disk by a spacer ring. The time each ant spent within 15 mm of the center of the trail and the number of times it entered this area (approaches) were recorded during a 2-min period after introduction of a single recruited worker onto the trail. Trail-following continuity (sec following/approaches) was then calculated for each ant and mean trail-following continuities were compared using Duncan's new multiple-range test on $\log_{10}$-transformed data.

Choice between Synthetic and Gaster Extract Trails. Two circular trails, 14 cm in diameter with their centers 6.8 cm apart, were applied to a 24-cm-diam filter paper disk. The trails were 44 cm long and contained dosages equivalent to the previously used 50.7-cm trails (Van Vorhis Key et al., 1981; Van Vorhis Key and Baker, 1981) (Figure 1).

Trails of 100 ng-eq (2.0 ng/cm) Z9–16:Ald were paired with trails of either 10 ng/eq (0.2 ng/cm) or 1 ng/eq of (0.02 ng/cm) Z9–16:Ald, 0.1 ant-eq of gaster extract trails (0.002 ant-eq/cm), or solvent trails. Trails of 10 ng-eq Z9–16:Ald were also paired with either 1 ng-eq trails of Z9–16:Ald, with gaster extract trails or with another 10 ng-eq trail. Trail-following ants choosing the dashed-lined paths in Figure 1 would be making turns of approximately: (a) 60°, (b) 60°, (c) 120°, and (d) 0°.

Recruited, unfed ants were individually introduced randomly in equal numbers onto one of the two trails in each pair (except solvent trails) and, from video tapes of the assays, the choice each ant made at each choice point was determined. Trail pairs were assayed in a complete-block design, and individual ants contributed no more than 3 replicates of choice to the experiment. To be scored as trail following, ants had to be within 0.25 cm of the trail center for 2 cm before and after the choice point. Trail preference was determined using chi-square comparisons between actual choices and expected values from ants choosing between two 10-ng trails. The numbers of

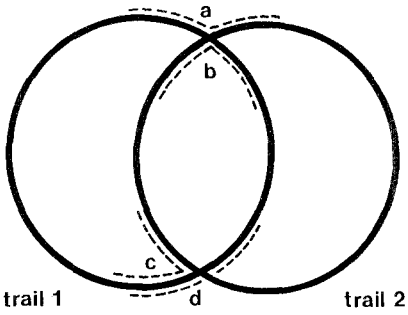

FIG. 1. Schematic of the arrangement of circular trails for trail choice tests. Dashed lines indicate possible paths of trail-following ants; required turning angles upon taking these routes are (a) 60°, (b) 60°, (c) 120°, and (d) 0°.

TABLE 1. FREQUENCIES OF TRAIL SWITCHING AT CHOICE POINTS BETWEEN TRAILS OF DIFFERENT CONCENTRATIONS OF *Z*9–16:ALD AND GASTER EXTRACT TRAILS OF 0.1 ANT-EQUIVALENT

Trail 1	Trail 2	% switching from trail 1 to trail 2 (*N*)	% switching from trail 2 to trail 1 (*N*)
100 ng-eq	solvent	0.4%[a] (1/256)[b]	100% n.s. (1/1)
100 ng-eq	1 ng-eq	7.8% (10/129)	95.4% (21/22)
100 ng-eq	10 ng-eq	6.3% (7/111)	86.7% (13/15)
100 ng-eq	0.1 ant-eq gaster extract	0% (0/47)	100% n.s. (2/2)
10 ng-eq	1 ng-eq	8.7% (21/242)	97.8% (44/45)
10 ng-eq	0.1 ant-eq gaster extract	42.8% n.s. (24/56)	52.4% n.s. (22/42)

[a] Asterisk indicates a significant deviation from the expected frequency of trail choice based on actual choices between two identical 10 ng-eq trails, determined using chi-square analysis ($P < 0.005$).

[b] Numbers in parentheses refer to fraction of ants following one trail which switched to following the other. Equal numbers of ants were introduced to each trail.

choices observed for each trail pair are listed in Table 1 and range from 49 to 187.

Validity of the experimental design and of the analysis technique was assessed by comparing the frequency with which ants switched from following one 10 ng-eq trail to following the other (41%: a + b + c in Figure 1); 59% remained following the same trail (d in Figure 1; $N = 226$). These percentages were then used to calculate the estimated values for chi-square comparisons with data from other pairs of trails.

RESULTS

Analog Activity. Among the compounds tested, only one, the known trail pheromone component, *Z*9–16:Ald, elicited significant trail following, and the activity of 10 ng-eq trails was equivalent to that of 0.1 ant-eq of gaster extract (Figure 2). Interestingly, the geometric isomer, *E*9–16:Ald, evoked no significant trail following at this dosage, and therefore behavioral and probably receptor specificity extends to geometric as well as positional isomers (Van Vorhis Key and Baker, 1981). Although ants tended to spend more time near *Z*7–16:Ald trails than near formate or solvent trails, *Z*7–16:Ald also did not elicit significant trail following in this experiment. Perhaps most surprising was the lack of trail following to the formates, especially to *Z*7–14:Form, the closest structural mimic to *Z*9–16:Ald we

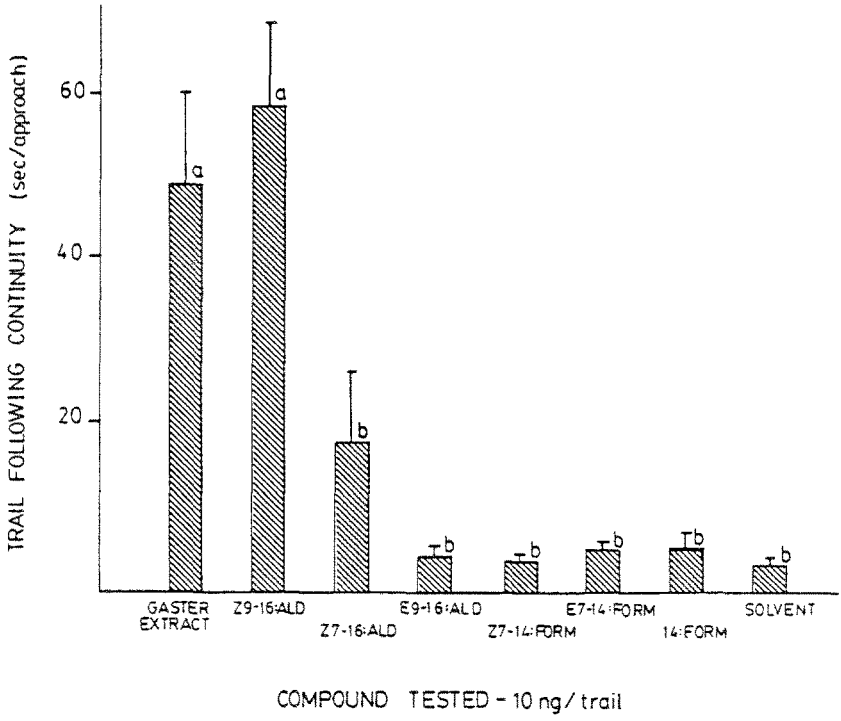

FIG. 2. Trail-following continuities elicited by 10 ng-eq trails of various analogs of Z9-16: Ald, 0.1 ant-equivalents of gaster extract and solvent trails. Means not followed by the same letter are significantly different according to Duncan's new multiple-range test ($P < 0.01$; $N = 20$).

tested. This demonstrates further the high specificity of the trail-recruitment system of this ant.

Trail Choice. When at the intersections of two Z9–16: Ald trails, Argentine ant workers consistently chose the more concentrated of the two in reciprocal choices (Table 1). These preferences deviated significantly from the expected 59%:41% choice frequencies of ants either remaining on, or switching to, respectively, identical 10 ng-eq trails. As expected from earlier work (Van Vorhis Key and Baker, 1981), 10 ng-eq Z9–16: Ald and 0.1 ant-eq of gaster extract trails were chosen equivalent numbers of times, and at frequencies not significantly different from those expected between two 10 ng-eq trails. However, a 10-fold increase to 100 ng-eq in the Z9–16: Ald trail allowed it to outcompete the gaster extract trails in choice tests with workers always choosing the synthetic over the gaster extract trails. The equality of the 10 ng-eq Z9–16: Ald and 0.1 ant-eq gaster extract trails was demonstrated by the equivalent reciprocal crossing from one trail to the other. That such

crossings were equally likely implies that the higher concentration of Z9-16:Ald in the synthetic trail compared to gaster extract (Van Vorhis Key and Baker, 1981) can compensate for the greater chemical diversity of the gaster extract trails (Cavill et al., 1979, 1980).

DISCUSSION

Argentine ant workers follow trails of Z9-16:Ald, and not other positional isomers of aldehyde analogs of different chain length, although in a previous experiment using a less discriminating bioassay Z7-16:Ald did show substantial activity (Van Vorhis Key and Baker, 1981). The lack of following to the opposite geometric isomer, E9-16:Ald, now demonstrates further the specificity of trail following to only Z9-16:Ald. Similar specificity of response by ants is known for trail-following *Atta texana* workers (Sonnet and Moser, 1972, 1973; Caputo et al., 1979), and for alarm responses by *A. texana* and *Atta cephalotes* (Riley et al., 1974), *Pogonomyrmex barbatus*, and *P. badius* (Benthuysen and Blum, 1974; Blum et al., 1971).

Perhaps even more interesting than the lack of trail following after changes in chain length, position of the double bond, or geometric configuration of that bond, was the lack of following to Z7-14:Form, probably the compound most structurally similar to Z9-16:Ald of all those we tested. Here, the substitution of an oxygen atom for a carbon at a single point in the chain was enough to eliminate trail-following response. The other tetradecenyl formates tested were also behaviorally inert, but this was expected due to their similarities to the inactive E9-16:Ald and 16:Ald.

Interest in formates as behaviorally active substitutes for aldehydes has been shown by workers in the field of moth sex pheromones (Mitchell et al., 1975, 1976; Cross et al., 1980; Caro et al., 1980). Formates should be more stable than aldehydes when applied as mating disruptants in slow-release field formulations and, therefore, could be more efficacious over longer time spans than similarly applied aldehydes. The behavioral inactivity of Z7-14:Form, however, indicates that this formate will not be acceptable as substitute for Z9-16:Ald in Argentine ant field-behavior-modification programs.

The Z9-16:Ald trails were competitive with gaster extract trails in trail-choice experiments, and the ants even showed a significant preference for 100 ng-eq Z9-16:Ald trails over trails of 0.1 ant-eq of gaster extract. That trail following ants "choose" to leave tubing containing naturally deposited trails to follow experimental trails of Z9-16:Ald is further evidence of this compound's activity. These results are encouraging for possible field use of this compound in bait-finding and pick-up schemes. Further work is now being conducted to determine the competitiveness of Z9-16:Ald in the field with naturally deposited trails.

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Letter to the Editor

ON THE NATURE OF CHEMICAL COMMUNICATION BY CRAYFISH IN A LABORATORY CONTROLLED FLOW-THROUGH SYSTEM

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A recent article by Itagaki and Thorp (1981) examined the prospects of chemical communication in the Southern crayfish *Procambarus clarkii* (Girard, 1852). These authors had investigated pheromonal communication by both males and females, rather than the males only as in most of the crustacean pheromone literature. Although the experimentation and analysis appear sound there are, perhaps, misconceptions concerning the nature of pheromones and intraspecific communication and misinterpretations of results within the paper. These will be addressed in this letter.

Their experiments can be outlined as follows: subject animals were acclimated to a flow-through system and then, during separate tests, subjected to a variety of stimulus waters. The stimulus waters was from a flow-through tank containing either no conspecific animals, a solitary male, a pair of males, a solitary female, or a pair of female conspecifics. Each experimental situation was given an alphabetic designation (Table 1 = Table 2 from Itagaki and Thorp, 1981). Behavior was recorded on videotape and analyzed with respect to a number of parameters.

The following is a list of reinterpretations and/or points in need of clarification:

1. Communication (Webster's Dictionary, Neilson et al., 1951) is defined in terms of the imparting or conveying of information. If we are to accept such a definition as universal, viz., as applying equally well to the realm of animal communication, we must also accept that signals, produced by one animal and received by another animal constitute a form of communication.

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TABLE 1*[a]

TRT	Observation tank	Head tank	TRT	Observation tank	Head tank
A	1 ♂	1 ♂	F	1 ♀	0 (control)
B	1 ♂	1 ♀	G	1 ♂	2 ♂♂
C	1 ♂	0 (control)	H	1 ♂	2 ♀♀
D	1 ♀	1 ♂	I	1 ♀	2 ♂♂
E	1 ♀	1 ♀	J	1 ♀	2 ♀♀

Comparisons[b]	Significant behaviors[c]	Rank order	Total *DF*	*F* ratio	Prob. > *F*
ABC, ACG, EFJ, AG, BH, DI, HJ, AB, GH, and IJ	All nonsignificant ($P > 0.05$)				
DEF	Chela wave	(E>F>D)	62	3.90	0.026
CGH	Chela(e) in upstream baffle	(H>G>C)	60	3.73	0.030
FIJ	Gross body movement	(I>J>F)	62	4.72	0.013
	Climbing	(I>J>F)	62	3.51	0.036
	Chela(e) in upstream baffle	(I>J>F)	62	3.22	0.047
BCH	Chela(e) in upstream baffle	(H>B>C)	59	3.42	0.040
DFI	Gross body movement	(I>D>F)	61	4.01	0.023
	Grooming	(F>I>D)	61	3.34	0.042
	Chela(e) in upstream baffle	(I>D>F)	61	3.38	0.041
ACDF	Grooming	(F>A>C>D)	81	2.78	0.046
	Chelae up	(D>F>A>C)	81	3.08	0.032
BCEF	Chela(e) in upstream baffle	(B>F>E>C)	83	3.10	0.031
CFGI	Digging	(I>G>F>C)	83	3.20	0.270
	Chelae in upstream baffle	(I>G>F>C)	83	5.78	0.001
CFHJ	Grooming	(F>J>C>H)	82	3.27	0.025
	Chelae up	(J>F>H>C)	82	2.69	0.051
	Chela(e) in upstream baffle	(H>J>F>C)	82	2.58	0.059
EJ	Time in upstream zone	(J>E)	40	5.77	0.021
AD	Chelae up	(D>A)	38	3.79	0.059
BE	Chela wave	(E>B)	40	8.13	0.007
	Time in upstream zone	(B>E)	40	9.77	0.003
GI	Chela(e) in upstream baffle	(I>G)	40	5.03	0.031
CF	Chelae up	(F>C)	42	4.35	0.043
	Chela(e) in upstream baffle	(F>C)	42	4.23	0.046
	Average chelae up per trail	(F>C)	39	4.94	0.032
DE	Chela wave	(E>D)	39	6.07	0.018
	Time in upstream zone	(D>E)	39	4.87	0.034
A-J	Grooming		203	1.97	0.044
	Chelae up		203	2.04	0.036
	Chela(e) in upstream baffle		203	1.97	0.044

[a]Those tested were chelae up, chela(e) in upstream baffle, chela waving, climbing, digging, grooming, gross body movement, meral spread, presence in upstream zone, and mean of the quantity "average duration per trial" for chelae up, chela wave and meral spread.
[b]Grouped letters indicate that means of these experiments were compared statistically.
[c]Behaviors described in Table 1†. Probability values listed only for significant behaviors.

*Table 1: Table 2 of Itagaki and Thorp (1981), Results of general linear model procedures for comparing various treatment effects on duration of specified crayfish behaviors.[a]
†Refers to Table 1 of Itagaki and Thorp (1981).

Detection and recognition are then subsets of communication. If the communication is mediated via chemicals produced and released by one animal and received by another animal of the same species, then such communication is said to be conspecific (intraspecific) and pheromonal. If such communication occurs between members of the same sex, and the information transferred is the sex of a conspecific, the phenomenon can be described in terms of sexual recognition. With the above in mind, it seems that these authors might have selected a broader definition for their examination of chemical communicatory behavior. Additionally, the concept that communication must have a "real or perceived advantage to the signaler" (page 116, lines 2–3) for the initial encoding of the signal is erroneous and is not what was written by Wilson (1970), whose definition is quoted. If we consider a simple example such as the scent trails left by humans that are readily perceived by dogs (cf. Shorey, 1976), it is easy to see that in this situation communication occurs—and is not due to volition and has no "real or perceived advantage" to the human.

2. In their discussion section one finds that toxic waste products are listed separately from pheromones (p. 123, l. 37, 40; p. 124. l. 5). This suggests that such wastes have been arbitrarily excluded from possibly being communicatory chemicals. Ryan (1966) and Christofferson (1978) have shown that urine from premolt female crabs contains a pheromonal substance which is perceived by adult males. The implication that toxic chemicals cannot be communicatory does not seem justified.

3. The arbitrary zones, upstream, midstream, and downstream thirds of the apparatus, also raise questions (p. 119, l. 1–3). The male crayfish used in their experiments weighted 13–44 g (wet weight). I measured 11 male *P. clarkii*. Mean weight was 33.4 g $\pm$ 7.5 SD (wet weight); carapace length was 5.13 cm $\pm$ 0.30 SD; overall body length (excluding antennae) in the nonsubmissive posture, i.e., with extended abdomen, telson, and uropods, and with the chelae lying in a nonaggressive attitude in front of the body, was 15.25 cm $\pm$ 0.74 SD; width with appendages in normal postures was 5–8 cm. If we consider then that an animal commands roughly 100 cm^2 (length $\times$ width) while the total available area of their chamber is only 375 cm^2 (25 $\times$ 15 cm, length $\times$ width), we see that an animal commands nearly an entire zone. It is therefore unlikely that the animal is ever within a single zone. As the authors do not give their criteria for determining which zone an animal was in, it is difficult to evaluate their results.

4. Complications arise concerning their interpretation of statistically significant data. They report that only one behavior, out of a possible 28 in tests where the response of solitary males or females is tested with respect to conditioned water from other solitary individuals of both sexes, differs statistically significantly from the blank at the $P < 0.05$ level. They interpret this to mean that sexual recognition and indeed any form of conspecific recognition does not occur (p. 125, l. 23–15; see also l. 29–31). Concerning this point: (1) Their methods section does not detail the 28 behaviors monitored—

only 16 behavioral parameters are discussed or alluded to. (2) Presumably, although not stated, their argument is that one would expect one out of 20 behaviors to appear significant, when it actually was not, on purely statistical grounds when the significance is set at the $P < 0.05$ level (type I error; see p. 122, l. 4–8). This argument would apply only if one were dealing with data from a series of experimental replicates or independently occurring events within an experiment. This is not the case in the experiments under discussion: many of the behaviors monitored are mutually exclusive (see Table 1 of Itagaki and Thorp 1981, p. 119). (3) Additionally, it appears that they have assumed equality of the behaviors that they chose to monitor, at least with respects to the ability of those behaviors to indicate recognition (however, in their Table 1 it is acknowledged that one behavior, climbing, is a subset of another behavior, gross body movement). The assumption that all behaviors of this organism which were monitored are of equal predictive value (at least statistically) in detecting the occurrence of a physiological event (viz., reception of some chemical signal) is unwarranted. There is no a priori reason to believe that any or all of the behaviors studied bear any relation to the phenomenon under study. It must be remembered that what appears to be a relevant item (stimulus, behavior, and the like) to man is not necessarily relevant to other species; irrelevant parameters may be monitored while relevant ones are overlooked.

It seems more appropriate to argue that (1) any statistically significant observable change is the expression of a real phenomenon given the constraints of the experimental regime, and (2) the behaviors showing such statistically significant differences are of particular import, at least when compared to the others monitored (this apparent neglect for type II errors seems justified considering the last sentence of the preceding paragraph).

5. It is stated that, if the stimulus waters from solitary animals are replaced by those from consexual pairs, the behavior is altered with respect to a number of parameters (p. 122, l. 8–13). The authors, however, do not concede recognition or even communication (p. 122, l. 17–18), but rather suggest that such a response is perhaps no different from that which might be elicited by some other relevant chemical stimulant, e.g., "fish" (=food? p. 123, l. 20). Why this was not tested, as McLeese (1973) has done, is not mentioned. In discussing this result they state that increasing the number of animals in the tank from which stimulus water is drawn tends "to enhance the response" (p. 123, l. 16) in their subject animals. This implies that a further increase from two to, say, three would have a similar enhancing effect. Thorp and Ammerman (1978) have shown, using an apparatus similar to the one in the present study, that water drawn from the tanks of two solitary individuals and then mixed and used as a stimulus source does not produce the same effect as water drawn from a tank occupied by a pair of animals. Those authors interpreted their findings as indicating the presence of a stress pheromone.

Itagaki and Thorp seem no longer to accept this previous finding in the interpretation of their results.

6. The authors cite three aspects of flow-through systems which make them preferable to static systems: (1) Flow-through systems "provide better directionality for chemical cues than do static systems" (p. 123, l. 34). Henson and Wilkens (1979) have shown that detection of current direction is quite possible, at least on physical grounds, for the crayfish, *P. clarkii.* Unless the half-life of the pheromones in question is of the order of 1–2 min, there would be essentially an equal concentration of the substance throughout the Itagaki and Thorp flow-through experimental tank (current flow rate = 1.5 cm/sec; p. 118, l. 3). Ameyaw-Akumfi and Hazlett (1975) obtained positive results for the presence of pheromone in crayfish using "aged" stimulus waters. We are not told the age of the stimulus waters used; but it seems reasonable to assume that they were more than a few minutes old, meaning that deterioration of at least one crayfish hormone could take considerably longer. It is likely that the only "directionality" in this system is provided by the current and not by the chemicals themselves. (2) Itagaki and Thorp maintain that dispersal rates for chemicals in a flow-through system are more similar to those found in "natural lotic conditions" (p. 123, l. 35–36) than in static systems. They provide, however, neither data nor references to support this claim. Using so-called static systems, Rose and Casper (unpublished manuscript) have shown differences in dye dispersion within such systems; actual properties appeared dependent on the quantity (and perhaps microlocation) of substance present. (3) Itagaki and Thorp's final point is that the dynamic situation prevents the accumulation of pheromones and/or toxic wastes (p. 123, l. 37–39; see above comment on whether these should be so distinguished). Published reports indicate that high levels of pheromones are not ignored by normally receptive animals—rather the receiving animals may become frenzied as opposed to oblivious (Jacobson, 1972) or produce a different behavior rather than intensifying the response seen at lower concentrations (Roelofs, 1978).

7. Reported intrasexual (≅homo- or bisexual, see Beach, 1944, for a discussion of these terms) and sodomic behavior is also cited as evidence against the pheromonal communication hypothesis (p. 124, l. 39–43; p. 125, l. 1). Counter examples include man and the rat—an animal where both intrasexuality (Stone, 1924; Beach, 1938, 1942, 1945) and pheromones (Leon, 1980; Vandenbergh, 1980) have been documented.

In summary the results and methodology of this paper appear solid. The interpretation, however, appears hampered by overrestrictive definition of the phenomena under consideration and incorrect evaluation of statistical analyses. A reinterpretation of the data (Table 1) indicates that (1) the stress pheromone produced by males is similar in its effects to that produced by females when perceived by males (their comparisons GH vs. CGH) or females (IJ vs. FIJ); (2) females are aggressive toward other females but submissive

toward males (comparison DEF, subjective behavioral classifications follow Ameyaw-Akumfi and Hazlett, 1975); (3) both males and females detect stressed conspecifics upstream (comparisons BCH, DFI, EJ; terminology of Thorp and Ammerman, 1978); (4) males and females can be distinguished on the basis of their behavior alone (at least in this experimental regime, quantitative comparison CF; see also AD, BE, and GI where males and females responded differently to similar upstream stimuli); (5) females recognize the sex of upstream males (DE, DEF). The conclusion that long-distance (>0.5 m) chemical communication does not occur in crayfish does not seem justified (p. 125, l. 23–25; see also l. 29–31).

Furthermore, one must be extremely careful in affirming the hypothesis that a phenomenon does not occur. Fraenkel and Gunn (1961) wrote "It is inconceivable that there are specific receptors for electric currents, and the experiments [the early experiments demonstrating electroreception by fish] tell us nothing about normal behavior," but time has proved them wrong and shown us animals that live in a world where electric currents provide a nearly total description of the environment (Lissman, 1963).

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Letter to the Editor

VERIFICATION VERSUS FALSIFICATION OF EXISTING THEORY
Analysis of Possible Chemical Communication in Crayfish

A disturbing feature in science is the frequent emphasis on verification of popular theories rather than on falsification of hypotheses. As Dayton and Oliver (1980) stressed recently "The verification of ideas may be the most treacherous trap in science, as counter-examples are over-looked, alternate hypotheses brushed aside, and existing paradigms manicured. The successful advance of science and the proper use of experimentation depend upon rigorous attempts to falsify hypotheses." While all disciplines of science suffer from this problem, the reliance of behavioral research on observational techniques requires that one exercise extreme caution in data interpretation. To avoid compromising the conclusions of field and laboratory studies, it is necessary to test rigorously alternative hypotheses and to rely on valid statistical techniques.

In his recent review of a 1981 paper by Itagaki and Thorp, Rose (1982) concluded that the earlier paper contained ". . . misconceptions concerning the nature of pheromones and intraspecific communication and misinterpretations of results within the paper." From our perspective the only potentially significant criticism concerned our general approach in evaluating experimental results. The opposite approach advocated at least de facto by Rose is illustrative of the problem mentioned previously. The specific criticisms by Rose and our opposite approaches to data interpretation are discussed below.

Although theoretically it takes only one case to reject a "properly framed" hypothesis, one must be sure that the results of a test are real (with regard to type I errors), exclusive of alternative hypotheses, and directly applicable to the overall question. The overall null hypothesis (H_0) in our study was that long-distance chemical communication of sexual identity, agonistic state, and stress condition does not occur among adult crayfish. To falsify the overall null hypothesis, it was necessary to show that (1) statistically significant results led to rejection of H_0, (2) these results were consistent with other data, and (3) the data were not equally well explained by alternative hypotheses. Two alternative hypotheses were that (1) the number of

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comparisons declared statistically significant could have been explained by chance alone (type I error), and (2) similar results would have been obtained if we had employed novel stimuli (e.g., another taxa such as various fish species, etc.) rather than conspecifics. Because we did not conclude in our paper that "chemical detection" was equivalent to reception of species-specific pheromones, we did not proceed with contingency plans for employing novel stimuli. Unfortunately, proponents of the argument for pheromones in crayfish (e.g., Ameyaw-Akumfi, 1976) have failed to conduct the mandatory tests of this alternative hypothesis.

Significance levels are difficult to interpret in multitreatment experiments involving several simultaneous comparisons (Kirk, 1968, p. 82). Assigning a significance level is ambiguous because ". . . the conceptual unit can be the individual comparison, hypothesis, family of comparisons, or experiment. . . . The error rates become more divergent as the number of comparisons and hypotheses evaluated in an experiment are increased" (Kirk, 1968, pp. 82–83). In our study we tested 364 specific hypotheses based on 26 a priori comparisons (treatment groups, e.g., ABC, Table 2) for 14 "behaviors" (duration of 8 behaviors + average duration per trial for 3 behaviors + position in observation tank + right or left handedness = 14 total "behaviors"). Each of five basic experiments was replicated with 20 male and 20 female observation crayfish. We were aware that strict adherence to an alpha level of 0.05 for 364 hypotheses would produce a number of hypotheses falsely declared significant (type I error). The expected number of tests incorrectly declared significant would be approximately 18, which differed only slightly from the 30 statistically significant tests in our original Table 2 (for brevity the 334 nonsignificant results were deleted from the table). Of the 30 statistically significant hypotheses, only a few were a priori tests of communication of sexual identity; the remaining hypotheses were related to chemical detection (not communication) and communication of agonistic state and stress condition. Two multitreatment comparisons (ABC and CGH, Table 2) tested whether an observation tank male could distinguish between stimulus water from either controls (0 animals), 1 or 2 males, or 1 or 2 female crayfish. Only 1 of 28 hypotheses (14 behaviors each for the comparisons among ABC and CGH) was statistically significant compared to an expected 1.4 by chance alone. These results and others described in the 1981 paper led us to conclude that sexual recognition over long distances does not occur in crayfish through chemical communication.

The internal inconsistency of the results also lead us to reject the overall null hypothesis. For example, "Although females waved their chelae more often (DE) to solitary females than to solitary males, there were no differences in response to water conditioned by either two males or two females (IJ)" (Itagaki and Thorp, 1981). One would expect these internal inconsistencies if

the "statistically significant" results were random rather than real. Please note that we are not advocating deleting significant results from the publication; rather, we are recommending a conservative approach of not rejecting the overall null hypothesis (H_0) without strong and consistent results. In our case, we would have preferred to be able to falsify H_0 since that would have allowed us to pursue additional questions in this area, but the results did not justify rejecting H_0.

An alternative approach to the one advocated above is to accept all significant comparisons as real without regard to type I errors and to other problems mentioned previously. The effective result of this approach, whether intended or not, is to decrease the probability of falsifying H_0 and, thus, to increase the number of theories verified (just the opposite of the valid approach). If an investigator requires only one significant comparison to verify an hypothesis, it is almost impossible to falsify a theory. As an example of this nonconservative approach, Rose (1982) stated, "It seems more appropriate to argue that (1) *any statistically significant* [our italics] observable change is the expression of a real phenomenon given the constraints of the experimental regime, and (2) the behaviors showing such statistically significant differences are of *particular import*, [our italics] at least when compared to the others monitored . . ." We never stated or implied, as suggested by Rose, that all behaviors monitored in our study were of equal predictive value (in fact, meral spread was most representative of agonistic behavior, and it showed no significant responses). However, we disagree that it is appropriate, a posteriori, to emphasize only those comparisons or behaviors which are statistically significant. Ignoring non-significant results and emphasizing only significant tests biases one's conclusions.

In addition to critizing our data interpretations, Rose (1982) indicated that we had ". . . misconceptions concerning the nature of pheromones and intraspecific communication . . ." In drawing conclusions from experimental results, it is important to state the applicable "boundary conditions," such as the definition of the phenomenon investigated. Rose criticized our paper for not using the definition of communication that he had extracted from a 1951 edition of Webster's Dictionary. He also stated that we misquoted or misinterpreted the definition proposed by Wilson (1970); a direct comparison of our quote with Wilson's will show that this was not the case. Our use of the term "communication" follows the strict definition proposed by Burghardt (1970): "Communication is the phenomenon of one organism producing a signal that, when responded to by another organism, confers some advantage (or the statistical probability of it) to the signaler or his group." While Rose is free to interpret the significance of our conclusions in light of his colloquial definition, we reserve the right to use an established

scientific definition. It was not the purpose of our 1981 paper to analyze alternative definitions of communication; a thorough analyses has already been published (Burghardt, 1970). However, even if one accepts the nonscientific definition suggested by Rose, the perception by dogs of scent trails left by humans (Rose, 1982), would not qualify, in our opinion, as communication.

The remaining criticisms in Rose's paper concerned minor points in our original publication and are related, we believe, to his misinterpretations of our methods and/or assumptions. An explanation of all these points would be unnecessarily redundant here, but readers who are interested in further clarification should consult our 1981 paper. One point not stated in our earlier paper was that the position of observation tank crayfish in one of three zones was determined by the position of the eyes, not by the location of the entire body. Although we have never maintained that our flow-through apparatus was without limitations, we continue to believe that it has significant advantages over most static systems. The principal advantage relates to avoidance of toxic waste accumulation. Despite Rose's claim, we never stated or implied that pheromones could not be contained within excretory fluids (the scientific literature adequately demonstrates otherwise); rather, we believe that previous studies on adult crayfish communication (Ameyaw-Akumfi, 1976; Thorp and Ammerman, 1978) were not designed to distinguish between effects produced by toxic wastes or by possible pheromones in the excretory fluids of crayfish.

In conclusion, the nonconservative approach advocated by Rose (1982) ignores type I errors, is inadvertently biased in favor of statistically significant compared to nonsignificant results, and, as a result, emphasizes the verification of popular theories rather than the falsification of hypotheses. Rose's interpretation of what constitutes communication versus chemical detection is valid only if one accepts the colloquial definition from Webster's Dictionary over an established scientific definition. Although we found evidence of "chemical detection" by crayfish, we reaffirm the conclusion of our experimental study that ". . . chemical communication between adult crayfish does not occur or is not efficient at distances greater than the effective range for visual communication."

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Letter to the Editor

IS KAIROMONE A VALID AND USEFUL TERM?

Since it was first proposed by Brown et al. (1970), the term "kairomone" has been the subject of criticisms but also has found warm supporters. Most of the debate has been summarized by Weldon (1980), who advocated its maintenance in the nomenclature of chemical ecology. However, as Weldon missed my own criticisms of the term (Pasteels, 1973, 1976), I would like to repeat them here and answer some of Weldon's arguments without necessarily developing all the issues already discussed by him.

As is the case for others (see Weldon, 1980, for detailed literature), I feel uneasy with the term for two main reasons. First, I cannot agree with its present definition, and second, I am unsure of its usefulness.

Kairomone was defined by Brown et al. (1970) as "a transpecific chemical messenger, the adaptative benefit of which falls on the recipient rather than on the emitter." They immediately added: "Kairomones are, in fact, commonly nonadaptive or maladaptive to the transmitter." I cannot subscribe to this definition and above all to the last statement, which I find unsound on biological grounds. To take a caricatural analogy, it would be equivalent to say that since mosquitoes and fleas like human blood, blood is nonadaptive or maladaptive to man. Most of the compounds used by predators or parasites to find their prey or host have obvious adaptive value, e.g., as pheromones or allomones, as already pointed out by Blum (1974, 1977, 1980). Even if the adaptive value is not obvious, as for incidental metabolites, the compounds are more than often the result of metabolic processes which are adaptive.

Besides, if maladaptive at the individual level, "kairomones" could still be adaptive at kin or any kind of supraindividual level and thus be selected through kin or other kinds of cooperative selection.

Indeed, adaptive value is very hard to measure, most of the time multidimensional, and certainly cannot be judged on the basis of a single interaction. On pure logic, I am inclined to say that so-called "kairomones" are in fact, most of the time, globally not maladaptive. If so, they would be eliminated through selection. Many predator–prey or host–parasite interactions are, however, rather stable. In particular, very specific interactions,

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which are those mediated by specific signals (i.e., "kairomones"), are characterized by great stability.

Thus if kairomone is to be kept in the nomenclature of chemical ecology, it should be defined without any reference to its "maladaptive" value to the emitter. A definition like a chemical cue used by some organisms to exploit others would be acceptable.

Even with such a definition, is kairomone an useful term? This is of course a much more subjective question. As in all kinds of classification, there are "splitters" and "lumpers." I am a lumper.

Weldon (1980) defended the usefulness of kairomone as opposed to allomone (sensu Nordlund and Lewis, 1976) for two main reasons. First, Weldon advocated that "the distinction . . . is a real and useful one since it amounts to specifying who has taken the adaptive measure, the emitter or the receiver." I am not convinced by the argument. To take another caricatural analogy, we do not feel it is necessary to adaptively differentiate a digestive tract when considering that it is adapted to digest a prey, or when considering that a parasite has been adapted to live and reproduce in it. Since this holds, of course, for any kind of structure and organ, why not for chemicals? Moreover, as recognized by Weldon, a third term now must be added ("synomone," Norlund and Lewis, 1976) for chemicals mediating mutualistic interactions. I am afraid that by adding more terms, we render chemical ecology more confusing for the nonspecialists, even biologists, without necessarily rendering it more clear for the specialists.

Secondly, Weldon found the distinction between kairomones and allomones to be heuristic since "making distinctions between them demands precise consideration of the interactions betweeen organisms and the consequences for participants." On the contrary, I believe that a narrow labeling of a compound could obscure its real ecological meaning by giving too much emphasis to a single interaction.

Duffey (1976) has listed 46 different terms already used to designate chemical interactions between organisms, and he missed 11 terms proposed by Kirschenblatt (1962). Also, Dindal (1975) listed, from the literature, 26 terms used to specify different types of interindividual relationships, many of them possessing synonyms, and he further divided these terms into subcategories with still other names. Not surprisingly, there is no simple parallelism between the two classifications!

We badly need simplicity and stability for the nomenclature of chemical ecology, and it is time to strive for some international agreement, remembering that no classification can be perfect.

Being a lumper, I am inclined to use "allomone" for all interspecific signals as opposed to "pheromone" which designates intraspecific signals. To cover both, as well as chemical signals coming from the abiotic environment, I

advocate the use of the term "*ecomone*," first coined by Florkin (1965; see also Florkin and Schoffeniels, 1969, and Pasteels, 1977). Ecomone is used here as a synonym of semiochemical (Law and Regnier, 1971), a term better known by the Anglo-Saxon scientists. My only preference for ecomone (which has priority) is that is does not need to be translated into a foreign language. After all, although English is becoming the main language for scientific literature, teaching is still performed in national languages.

As I state above, several of my arguments are more subjective than objective, and I am ready to change my mind and attitude for the sake of stability, if a general consensus could be reached.

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OLFACTORY SENSITIVITY TO GROUP-SPECIFIC SUBSTANCES IN ATLANTIC SALMON (*Salmo salar* L.)

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Abstract—The olfactory sensitivity of three groups of Atlantic salmon (*Salmo salar* L.) toward substances emanating from their own groups was studied. Thresholds were determined by electrophysiological recordings of the induced waves from the medial and lateral part of the olfactory bulb surface. The intestine contained more potent olfactory substances on a gram per liter basis than skin mucus, urine, or amino acids. Chemical fractions were obtained from a parallel study on the nature of the naturally occurring substances by Stabell et al. (1982). A retarded fraction from chromatography on a Sephadex G-25 column contained the most potent material. The most potent fractions of the intestinal content evoked responses mainly in the medial part of the olfactory bulb, whereas the lateral part responded to amino acids. The results suggest that all salmon smolts of the waterways contribute to an odor trail in the coastal currents, thus facilitating the odor-dependent migration of the mature salmon.

Key Words—*Salmo salar*, olfactory sensitivity, olfactory bulb, induced waves, pheromones, migrational cues, homing.

INTRODUCTION

When anadromous salmonid fishes return to freshwater to spawn after staying in the sea, they will home to their native river. The sense of smell has been shown to be essential for correct homing in salmon (Wisby and Hasler, 1954; Groves et al., 1968; Toft, 1975). This implies the presence of specific substances in each river.

According to the "pheromone" hypothesis proposed by Nordeng (1971,

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1977), the salmon smolts migrating to sea release population specific substances, forming an odor trail that guides the mature salmon to their native river. This hypothesis is supported by the results from behavioral experiments which have shown that adult sea char (*Salmo salvelinus* L.) is attracted to water where smolts from their own population have been kept (Selset and Døving, 1980). Physiological recordings from single cells in the olfactory bulb of sea char have given evidence of a sensory basis for discrimination between populations of char (Døving et al., 1974).

The mechanism that is used by the fish olfactory system to discriminate specific substances of its own population from others is unknown. Two possibilities can be considered: (1) the returning salmon has a higher olfactory sensitivity to substances originating from its own population; and (2) the olfactory sensitivity is the same towards all population-specific substances. In the latter case the specific effect on the behavior of the fish must rely on specific interaction in the central nervous system.

The aim of the present study was twofold: first, to investigate the olfactory sensitivity in Atlantic salmon to substances from salmon smolts of different groups; and second, to approach, by electrophysiological means, the most interesting chemical fraction of the samples with respect to population-specific substances. A short abstract of the results of this study has been published (Fisknes, 1979).

METHODS AND MATERIALS

Donor and Experimental Fishes. The fishes (*Salmo salar* L.) were raised at the Research Station for Salmonids, Sunndalsøra Unit, Western Norway and were offspring of wild fish caught in three Norwegian rivers: Driva, Namsen, and Tafjord. The fishes were thus the first generation in captivity. They were hatched in the spring of 1976. From autumn 1976 they were marked (fin clipped and freeze-branded) and mixed with other fish. They smoltified in spring 1977 and from then on they were held in culture in the sea. One to two weeks before the experiment the test fishes were transferred by air to the aquarium facilities at the University of Oslo and kept in recirculating seawater at a temperature of 10–12°C.

Handling of Experimental Fish. The experimental fishes were 2–3-year-old salmon weighing 690–2630 g. They were initially anesthetized with tricain-methane sulfonate (MS-222, Sandoz) at 1:5000 concentration and then immobilized with Alloferin (Roche) given intramuscularly. The dose of the active principle, toxiferin, injected was about 4 mg/kg body weight. The fish were wrapped in a wet sponge and placed in a holder in the experimental set-up. The gills were continuously perfused with seawater at 14–16°C through a tube inserted into the mouth, at a flow about 1.8 liter/min. The

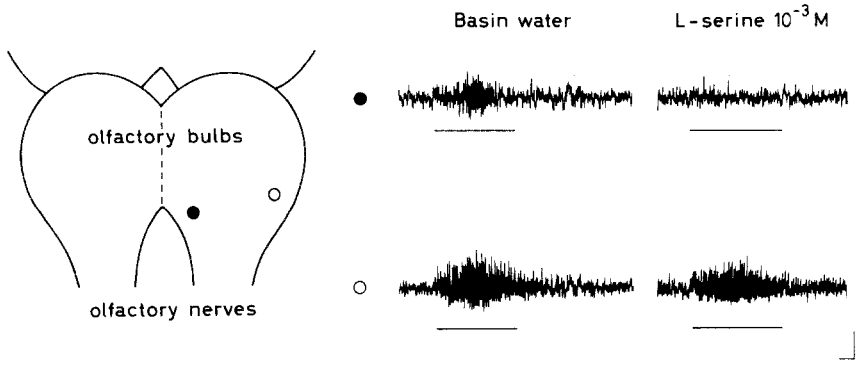

FIG. 1. Recording traces from the medial (•) and lateral (○) part of the left olfactory bulb in salmon. Stimuli were water from a saltwater basin containing salmon and L-serine at 10^{-3} M. Time bar = 1 sec; vertical bar = 100 μV.

dorsal part of the skull, together with the underlying fat and connective tissue, was carefully removed and the olfactory bulbs exposed.

Recording. The recording sites were at the lateral and medial part of the dorsal surface of the left olfactory bulb, positioned as shown in Figure 1. At each site a differential recording system was used. The electrodes were of glass and filled with Hanks' Ringer for marine fishes (Wolf and Quimby, 1969). The electrodes were inserted into electrode holders containing sintered Ag/AgCl. Tip diameter of the electrodes ranged from 10 to 50 μm. A stainless-steel wire served as a common indifferent electrode. It was connected to the fish in the connective tissue caudal to the opening made in the skull. The potentials were amplified with two differential preamplifiers (WPI, DAM-5A) and fed through a bandpass filter, 1–50 Hz. The signals were displayed on a pen recorder (Hewlett-Packard, type 7402 A).

Stimuli. The stimulus donors were hatched at the same time as the experimental fishes and some of them were siblings or half-siblings of the experimental fishes. Skin mucus, intestinal content, and urine were collected in May 1977 from two groups of smolts from each of the three rivers. Each group contained two to seven individuals that were siblings. The methods for collection of these samples and the chemical procedure, including fractioning, are described in detail by Stabell et al., (1982). Briefly, the original samples of skin mucus, intestinal content, or urine were centrifuged (5700 rpm, 15 min). The samples of skin mucus and intestinal content were treated with acetic acid to a final concentration of 5%. The precipitate after centrifugation (25,000*g*, 30 min) was separated from the supernatant. For olfactory tests the precipitate was stirred in distilled water after washing in 5% HAC and then centrifuged. The supernatants of skin mucus and intestinal content were run in a Sephadex G-25 column and separated into fractions I–VIII. Fractions IV–VIII

appeared after the salt volume and will be mentioned as retarded fractions. Aliquots for biological tests were taken out at all stages of the chemical procedure. The dry weight of all samples was determined. The samples were kept frozen at −20°C. Just prior to the experiment they were thawed and diluted with artificial sea water in concentration steps of 1:10.

Controls. As control stimuli L-serine and L-glutamine (Sigma) were made up in a series of dilution steps just prior to use. In addition "basin water," a water sample from the basin in which the fishes were kept, was used as a control stimulus.

Stimulation. The olfactory epithelium was continuously flushed with artificial sea water through a polyethylene tube inserted in the anterior nares. The waterflow was ca. 13 ml/min. The stimulus solution was introduced from a pipet via a manually operated three-way stopcock.

Procedure. All samples were applied in series of increased concentration. Each concentration was applied twice with a 30-sec interval between each stimulation. At intervals of 30 sec or more, no interaction of the stimuli was evident from observation of the response amplitudes. The time interval between two different dilution series was a minimum of 60 sec. Threshold concentration was determined as the lowest concentration of the stimulus that elicited a response (see Belghaug and Døving, 1977). The concentrations were expressed as gram per liter.

RESULTS

When an olfactory stimulus was introduced into the olfactory pit, a change in the potentials recorded from the surface of the olfactory bulb was seen (Ottoson, 1959a,b). Under present recording conditions, the response was characterized by oscillatory waves with constant frequency. The amplitude of the "induced waves" increased with increasing concentration of the stimulus. The frequency was 8–11 Hz at the temperature conditions used in the present study (cf. Døving and Belghaug, 1977). Figure 1 shows the responses to two of the control stimuli, "basin water" and L-serine, recorded from the medial and lateral part of the bulb.

Thresholds. Samples from both skin mucus and intestinal content were highly stimulatory to the salmon olfactory organ. Responses were elicited both in the medial and the lateral part of the olfactory bulb. The threshold values (g/liter) showed, however, that there exists a difference in the stimulatory effectiveness between skin mucus and intestinal content, the intestinal content being 10–1000 times more potent than the skin mucus (Figure 2). This was the case both for the original sample and the supernatant. The urine samples were at best equipotent to the skin mucus.

In the second step in the chemical fractionation procedure, samples of the

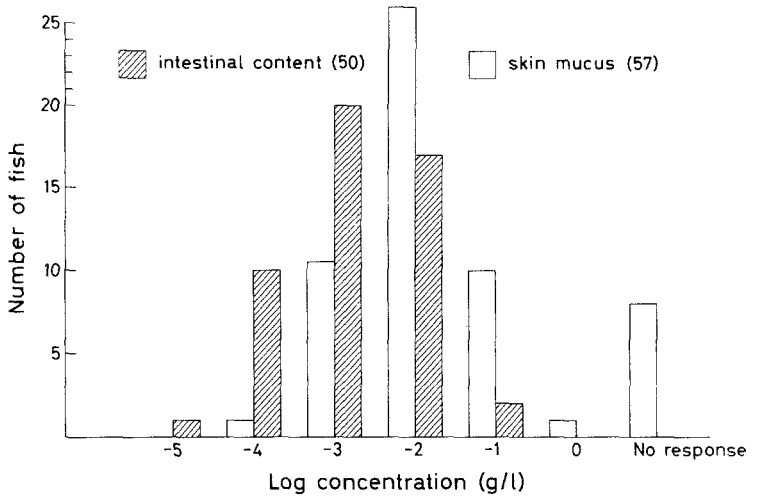

FIG. 2. Distribution of thresholds to supernatants of intestinal content and skin mucus from salmon smolts. The numbers of observations are given in parenthesis.

skin mucus and intestinal content were divided into supernatant and precipitate fractions. The stimulating efficiency of these fractions was compared with that of the respective crude material. It thus showed that the stimulating substances were not completely isolated in the supernatant, but also remained in the precipitate.

When stimulating with successive fractions from the G-25 Sephadex gel filtration, a change occurred in the response between the medial and lateral parts of the bulb. The first three fractions (I–III), which precede the salt volume of the column, elicited a response with higher amplitude from the lateral part of the olfactory bulb than the medial part. The retarded fractions (IV–VIII) were more likely to elicit the highest response in the medial part of the bulb (Figure 3). This was evident in experiments with fractions of intestinal content, but not for fractions of the skin mucus. Substances in the retarded fractions seem to have qualitative and stimulatory properties different from those in the first, unretarded fractions.

Fractions I–III, which elicited responses with highest amplitude in the lateral part of the bulb, showed an increasing stimulatory effect from I to III. The lowest threshold was found for fraction III and 1×10^{14} g/liter in four test fishes. Most effective were the first three of the retarded fractions (IV, V, and VI). The most potent of these was fraction V, with a threshold concentration observed down to 1×10^{-5} g/liter.

The amino acids used in the experiment always elicited a response in the lateral part of the bulb, but did so only sometimes in the medial part. The

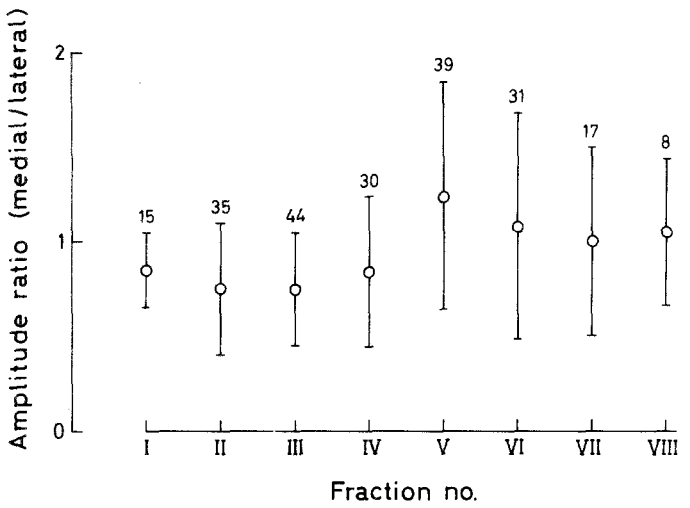

FIG. 3. The ratio between the amplitude of the responses at the medial versus the lateral part of the salmon olfactory bulb observed when stimulating with different fractions of the supernatant of the intestinal content (see text). For each fraction the mean and one standard deviation of the ratio is indicated. The number of observations is given above each bar.

threshold were found to have a mean of 4×10^{-5} M ($N = 8$) for L-serine and 8×10^{-6} M ($N = 8$) for L-glutamine. These concentrations correspond to 4×10^{-3} g/liter and 1×10^{-3} g/liter, respectively, *i.e.* about 10 to 100 times higher than those of the most potent fractions of skin mucus and intestinal content.

Cross-Comparisons. One of the objectives of the present study was to seek evidence for group-specific sensitivity to natural substances. The experimental fishes were therefore exposed to stimuli from donor fishes of the three groups. Table 1 shows an example of thresholds in three fishes, one from

TABLE 1. EXAMPLE OF A SERIES OF CROSS-STIMULATIONS[a]

Experimental fish	Donor fish		
	Driva	Namsen	Tafjord
Driva	III	II	III
Namsen	I	III	III
Tafjord	II	II	II

[a]The entries indicate the threshold values obtained when stimulating with fraction V from the intestinal content of the three smolt groups. I, threshold range 10^{-5}–10^{-4}; II, 10^{-4}–10^{-3}; III, 10^{-3}–10^{-2} g/liter.

each river, to the most potent fraction (fraction V) of intestinal content from smolts of the same three groups. In this case the Driva fish was most sensitive to the Namsen donor and the Namsen fish to the sample of Driva fish. All these samples were equally potent to the experimental fish from Tafjord. A systematic search through the experimental fishes revealed no specific sensitivity to any particular donor sample. Neither was a particular donor sample specifically potent to the experimental fish.

DISCUSSION

The results of the present experiments open perspectives in various aspects of salmon migration. First, the threshold of the amino acids versus the fractions of intestinal content indicate a potency of natural substances from the fish superior to those of the amino acids. Second, the qualitative differences in the responses of the olfactory bulb point to an important separation of nervous function toward the natural substances that will need further investigation. Third, the results of the cross-comparisons performed in the present study focus on the mechanisms that provide the salmon with their olfactory cues for homing.

In our present experiments the threshold towards the amino acids used was at best 1×10^{-3} g/liter. The samples of crude intestinal content contained substances that evoked responses in concentrations down to 1×10^{-5} g/liter. Every fraction tested contained a number of substances. Since the chemical nature and quantity of the active substances are unknown so far, we may only speculate upon the actual threshold for a single component. It is hardly probable that they are substances with molecular weights lower than the amino acids. Thus the actual threshold on a mole basis will be considerably lower than the mass per liter measurement given. A molecular weight of 500 will give a threshold of 20 nM. It should be noted that the concentrations used by Selset and Døving (1980) in the behavior studies were 1.5×10^{-9} g/liter. The methods used in the present experiments do not reflect a sensitivity in accord with that obtained in behavior experiments. The reasons for this discrepancy are at least twofold. The activity, as seen from the electrodes on the bulbar surface, does not reflect the activity changes in one single neuron in the olfactory bulb, which in theory can be responsible for the behavior of the fish. Second, there exists a possibility that there are seasonal variations in sensitivity and that the period at which the present fishes were tested (June–October) did not coincide with that of their peak sensitivity.

The most potent fraction of intestinal content evoked larger responses in the medial part of the olfactory bulb than in the lateral part. Døving et al. (1980) have shown that this part of the olfactory bulb in grayling and sea char responds to bile salts and derivatives of bile salts at concentrations below the ones found for amino acids in the lateral part of the bulb. Thus, the intestinal

sample contains substances that are qualitatively different from the amino acids, and they evoke responses in a part of the olfactory bulb that has been proposed to serve functions concerning social behavior in the fish while the lateral part of the olfactory bulb, which responds to amino acids, is supposed to serve functions concerning feeding (Thommesen, 1976, 1978). The samples that evoke reactions in the medial part of the bulb and contain the most potent substances are equivalent to those chemical fractions of sea-char smolts that attract mature migrating sea chars (Selset, 1980; Selset and Døving, 1980). Evidence from the present study concurs with the results found in other salmonid species with different techniques mentioned above.

Priesner (1968, 1969) studied the response in male antennae of saturniid moths to female abdominal gland substances. In this extensive study it was shown that different species produced specific substances. The receptors were specifically sensitive to substances produced by their own species. Between very closely related species, the specificity was absent. The present study indicates similar mechanisms. Data from Stabell et al. (1982) indicated chemical variability in the samples used. When presented to the fish, however, there was no evidence indicating that the samples from one group of donor smolts contained substances that were specifically potent to the experimental fishes of the same group.

The present results show that no single donor sample was a specifically potent odor to the siblings. In other words, all fish seem equally sensitive to all donors. These findings indicate two important points in salmonid migration. First, all the salmon smolts of the waterways will contribute to a gigantic odor trail in the fjords and coastal currents. According to Nordeng's "pheromone" hypothesis, the adult salmon return to their native river following this trail. The specificity of a current containing odors from salmon smolts of different rivers will increase as the counter-current swimming fish approaches the origin of its population. Chemical specificity is indicated by the parallel study performed by Stabell et al. (1982). Their results from thin-layer chromatography of the fractions used in the present experiments demonstrated chemical variation in the retarded fractions and in particular in the most potent fraction found, fraction V. Second, the specificity in behavior which would permit the salmon to return correctly to its spawning site will depend upon proper connections within the nervous substrate underlying the fish migration. Further progress can be made in elucidating the mechanisms behind the wiring of the nervous substrate in migratory behavior after identification of the substances that are responsible for the correct choice of spawning site. In considering the nervous mechanisms we note the results of the experiments described by Hasler et al. (1978), who have shown that returning coho salmon (*Oncorhynchus kisutch* Walbaum) are guided by artificial odors to which they have previously been exposed. Their results indicate that specific memory processes might be involved in salmonid

migration. We believe that genetic factors are important in salmonid migration because of the specificity of natural populations (Ståhl, 1981) and the variations in chemical composition of the odorant substances (Stabell et al., 1982). A combination of genetic factors and memory should also be considered in future experiments. Advances in our understanding of salmonid migration require the tools provided by the knowledge of the naturally occurring odorants.

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PREFERENCE OF CERTAIN SCOLYTIDAE FOR DIFFERENT CONIFERS
A Statistical Approach

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Abstract—Nine Scolytidae (*Cryphalus piceae*, *Cryphalus abietis*, *Pityokteines curvidens*, *Dendroctonus micans*, *Ips sexdentatus*, *Ips typographus*, *Orthotomicus erosus*, *Tomicus piniperda*, and *Phloeosinus bicolor*) were subjected to olfaction tests on ten conifer species taken two by two. These conifers were *Abies cephalonica*, *Abies nordmanniana*, *Picea abies*, *Picea orientalis*, *Pinus pinaster*, *Pinus sylvestris*, *Pinus brutia*, *Pinus laricio*, *Cupressus atlantica*, and *Cupressus sempervirens*. A statistical study of the results, by means of the factorial analysis of correspondence completed by the duo preference test, commonly used in sensory analysis, revealed a taxonomic clustering by genus of the plant species and analogous specific attraction behavior for the insects. *Pityokteines curvidens* has a behavior analogous to that of the two *Cryphalus* considered. *Phloesinus bicolor* shows a very strong specificity for *Cupressus*. The essential oils of the conifers were analyzed to determine their terpene composition and the ten odor spectra thus obtained were compared. The hierarchical classification, using a Euclidian distance, brought out similarities in the spectra, especially in the case of *Pinus*. It is shown that definitive establishment of Scolytidae is not due to the presence in the odor spectrum of any particular terpenoid. The attractive power of a species results from the synergism of the different terpenes. Moreover the definitive establishment of the insects also depends on their sensorial adaptation to volatile substances which can be wider or narrower for the species studied.

Key Words—Scolytidae, Coleoptera, conifers, terpenoids, olfactory tests, host attraction specificity, statistical methods.

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INTRODUCTION

The family Scolytidae includes several hundred species, some of which are very harmful to forests. A few examples will suffice to demonstrate the damage produced by these insects: *Dendroctonus frontalis* and *Dendroctonus ponderosae* destroy huge areas of conifer forests in North America; *Dendroctonus mexicanus* in a few years destroyed 2.5 million hectares in Honduras; *Ips typographus* caused considerable destruction of forests of *Picea abies* in Germany; and in previous papers we pointed out the role of two Scolytidae, *Ips sexdentatus* and *Dentroctonus micans* (Chararas, 1959, 1962, 1980), in the damage caused to *Picea orientalis* plantations in Turkey.

In those regions where forestry species must confront unfavorable ecological factors, for example, high temperatures and persistent summer droughts as is the case in Tunisia, certain Scolytidae, in particular *Tomicus piniperda* and *Orthotomicus erosus*, constitute a serious threat to *Pinus maritima* (Chararas, 1962, 1980). It should be noted that only conifers are infested by the Scolytidae studied here.

The typical process of infestation can be schematically described as follows: (1) initiation of attack by adult insects on different conifer species, usually on slightly weakened individuals, the osmotic pressure of which has been disturbed (Chararas, 1958, 1962, 1980); (2) selection of trees with a modified oleoresin pressure (Rudinsky, 1966); (3) burrowing into the bark by the adults, where females lay their eggs; (4) quick deterioration of the tree as a consequence of the lesions provoked by the feeding behavior of the larvae and the adults; and (5) the resulting degraded biotopes are then attacked by different Buprestidae and Cerambycidae which hasten the process of deterioration.

As the attraction of Scolytidae by conifers involves a certain specificity, the search for a coherent explanation of the chemical and biochemical processes related to that attraction has been the subject of discussion in many papers during the last two decades. From a biological point of view Scolytidae can be split into two groups, those which attack deciduous trees and those which attack conifers. For instance *Scolytus scolytus* and *Scolytus multistriatus* breed uniquely on elm trees. In this case it has been demonstrated that certain phenolic compounds such as flavone are responsible for the attractive stimulus (Baker and Norris, 1968a,b). These results were also observed for *Scolytus mediterraneus* living in fruit trees (Gurevitz and Ishaaya, 1972). However, it has also been shown that these species are never attracted by, and are unable to feed on, conifers (Chararas, 1980).

It should be underlined that no conifer-specific Scolytidae will colonize deciduous trees. We have demonstrated by experimental infestations that this specificity can be explained by the presence of terpenoids (Chararas, 1962,

1980; Chararas and Msadda, 1970). The same behavior was described by Rudinsky (1966a,b) in the case of American Scolytidae. This author was able to demonstrate a very strong attractive effect of the oleoresins of *Pseudotsuga menziesii* which contains α- and β-pinene, limonene, and camphene. In the same way a 1% solution of α-pinene exhibited a very strong attraction on many Scolytidae, in particular *Dendroctonus pseudotsugae*, under natural conditions with devices installed in open forest. Several recent papers have shown specific attractive effects of terpenes.

However, the mechanisms of that attraction are very complex because some of these compounds also produce a repulsive effect. Oksaneen et al. (1970) demonstrated that "*trans*-verbenol is attractive and has the necessary double bond and hydroxyl group, but *cis*-verbenol is repellant, so that other factors must be involved in the activity of these compounds."

Crossbreeding with trees containing repulsive or even toxic terpenes has been suggested in order to select for pest-resistant tree species. Smith (1966) measured oleoresin toxicity on *Dendroctonus brevicomis*. The antibiotic and toxic role of turpentine was first demonstrated by Callahan (1966). All the different effects of terpenes have been discussed in an extensive review (Gerhold, 1966).

American workers have demonstrated that α-pinene increased the effect of *trans*-verbenol in secondary attraction (Pitman, 1971). Thus male and female *D. brevicomis* are attracted by mixtures of a pheromone such as frontalin or brevicomin with 3-carene (Hughes and Pitman, 1970). In the case of other insects, specific parasites of conifers, such as *Hylobius abietis* (Curculionidae), the same biological role of terpenes has been reported. Since Mustaparta (1975) demonstrated by electrophysiological tests that *H. abietis* was stimulated by α-pinene, verbenone, or *trans-verbenol*, other recent research has shown the attractive effect of terpenes. Ozols et al. (1973) have demonstrated the attraction of some Scolytidae by different oleoresin fractions (*I. typographus*, *Hylurgops palliatus*) and of *Trypodendron lineatum* by α-pinene (Ozols et al., 1973; Nijholt and Schönherr, 1976).

Pheromone elaboration is influenced by several terpene compounds, as males of *D. frontalis* exposed to the vapors of α-pinene for 20 hr contained *cis*-and *trans*-verbenol and both sexes contained 4-methyl-2-pentanol. Similar exposure to β-pinene resulted in the presence of pinocarvone in males and *trans*-pinocarveol in both sexes (Renwick and Vité, 1970; Renwick et al., 1973).

The effects of terpenes at low levels are numerous: attraction and orientation of certain insect individuals, participation in pheromone formation demonstrated by the lack of attraction exhibited by the frass of Scolytidae grown on terpene-free substrates (Chararas, 1980).

The transformation of terpenes in the digestive tract is not simple,

however. As well as their oxidation as a result of digestive tube secretions, transformations of certain terpenes can be provoked by the intestinal flora of scolytid insects (Chararas, 1980).

One can therefore see that terpenes have several functions: they take part in the resistance of conifers to insect attack as well as in the primary attraction and the elaboration of certain aggregative pheromones.

It is now generally accepted that terpenic compounds play an important part in the attraction of Scolytidae and that some of these insects breed in only one particular tree species, while others may use several species within a genus or even species of different genera, despite large differences in their odor spectra.

The object of this work is to determine the degree and strictness of the specificity as a function of scolytid and conifer species.

METHODS AND MATERIALS

We selected nine species of Scolytidae, all of them conifer parasites and we evaluated their specificity towards 10 species of *Abies*, *Pinus*, *Picea*, and

TABLE 1. INSECTS AND CONIFERS STUDIED: NAMES AND CODES

	Code
Insects	
Cryphalus piceae	× 1
Cryphalus abietis	× 2
Pityokteines curvidens	× 3
Dendroctonus micans	× 4
Ips sexdentatus	× 5
Ips typographus	× 6
Orthotomicus erosus	× 7
Tomicus piniperda	× 8
Phloesinus bicolor	× 9
Conifers	
Abies cephalonica	A
Abies nordmanniana	B
Picea abies	P
Picea orientalis	O
Pinus pinaster	R
Pinus sylvestris	S
Pinus brutia	T
Pinus laricio	L
Cupressus atlantica	C
Cupressus sempervirens	K

Cupressus. The different species of insects and conifers are listed in Table 1 along with the corresponding codes used for the statistical study.

Olfaction Tests. The trials were run in 50-m^3 breeding cages made from wire netting in order to provide continuous aeration and thus avoiding any saturation of the enclosure by terpene vapors. Such a device closely resembles natural conditions and is not comparable to the ordinary Y or T olfactometers normally used for the study of insects in the presence of volatile molecules. These classical olfactometers produce no significant results as the experimental enclosure is quickly saturated by a mixture of odors. Moreover, Scolytidae naturally select their biotopes when in full flight, not while walking, and we took this into account in our bioassays.

Each test consisted in measuring the attraction specificity of 20 physiologically mature adult insects confronted with two different conifer species as represented by two 20-cm logs. After the test, the number of insects attracted by each species was counted. Statistically, those counts represent conditional frequencies for attraction. Three replications were carried out for each test. Tests were performed over 24 hr, allowing effective penetration of the insect in the trunk prior to counting. Because logs were cut, oleoresin pressure was low and no insects were rejected. However, some were not yet established when testing conditions were extreme or unfavorable, but counts of those nonestablished individuals were taken into account for statistical computation.

The pairing of 10 tree species two by two gave 45 combinations. The nine insect species were tested on each combination. In all, we ran $45 \times 9 \times 3 = 1215$ tests, each requiring 20 insects.

Chemical Analysis of Essential Oils. In order to determine the exact role of terpenes in the specificity of attraction, we took phloem fragments from the logs used in the olfaction tests for chemical analysis.

The extraction of terpenic compounds was performed by cold soaking of 20–50 g of ground phloem in pentane. Water was eliminated from the filtered extract on a molecular sieve, and the solvent was finally evaporated in a cold vacuum. The analysis was performed by gas chromatography using a 2.3-m stainless steel column (1/8 in.) packed with 10% polypropylene glycol sebacate on Chromosorb PAW 100–200 mesh. The temperature was programed from 70° C to 210° C (3° C/min).

The relative concentrations are expressed as a percentage fraction compared to the major peak. These results represent the odor spectrum of each of the conifer species. A preliminary study of the essential oils by GC-MS coupling allowed us to identify most of the terpenic compounds.

Statistical Analysis. A data base was formed using olfaction test results, in order to process them by a statistical method well suited to such large frequency tables, i.e., the factorial analysis of correspondence. This method, involves a transformation of data which usually leads to a more stable analysis

than that obtained by direct application of principal component analysis. The test responses are represented by m points C_i ($i = 1, \ldots, m$), corresponding to the results of counts, each point having as coordinates ($P_{ij}/P_i\cdot$) where $j = 1, \ldots, n$. The folowing formula was used to define the weighted distance between the two counting points C_i and C_k:

$$d^2(C_i, C_k) = \sum_{j=1}^{m} (1/p \cdot j) [(P_{ij}/P_i\cdot) - (P_{kj}/P_k\cdot)]^2$$

This expression defined the chi-square distance between two counting points. It is possible to demonstrate that it is equivalent to a Euclidian distance between two countings C'_i and C'_k The coordinates C'_i are $P_{ij}/(P_i \cdot P_j \cdot)^{1/2}$, thus the correspondence analysis applied to the cloud of m points C_i is equivalent to a principal component analysis of the m points C'_i.

Being based on a distance measure, factorial correspondence may provide a clustering of insects and conifers according to their respective distances. Computations were performed on a CII-Honeywell-Bull IRIS 80 computer; the software used was that described by Lebart and Fenelon (1973).

The next step was to define the specificity of the attraction of each insect group in relation to each conifer group. We therefore applied a classical sensory discrimination test, the paired-preference test, to the results of that first data base by means of the probability tables recently published by Roessler et al. (1978). A specific FORTRAN IV program was written in order to carry out these calculations.

A second data base was formed using the odor spectra in order to check whether the terpene composition would give the same clustering pattern as that obtained with the olfaction tests. The terpene concentrations for each species are statistical variables of a different nature to the attraction frequencies previously studied. Therefore another classical statistical method was used: the hierarchical classification. For this we used the BMDP2M program of the University of California–Los Angeles (Dixon, 1975).

RESULTS

The factorial analysis of correspondence applied to the olfaction tests can permit a simple graphical interpretation of these results. If the conditional preferences of all the insects for each conifer is projected onto the first two factorial axes, an excellent clustering of the plant species is observed. In Figure 1 we show that the conifers form four groups corresponding to the four genera considered in this study: *Cupressus*, *Abies*, *Picea*, and *Pinus*. To simplify the diagram, only the zones of clustering have been traced; the clustering of nonestablished insects has not been shown as it was of little importance.

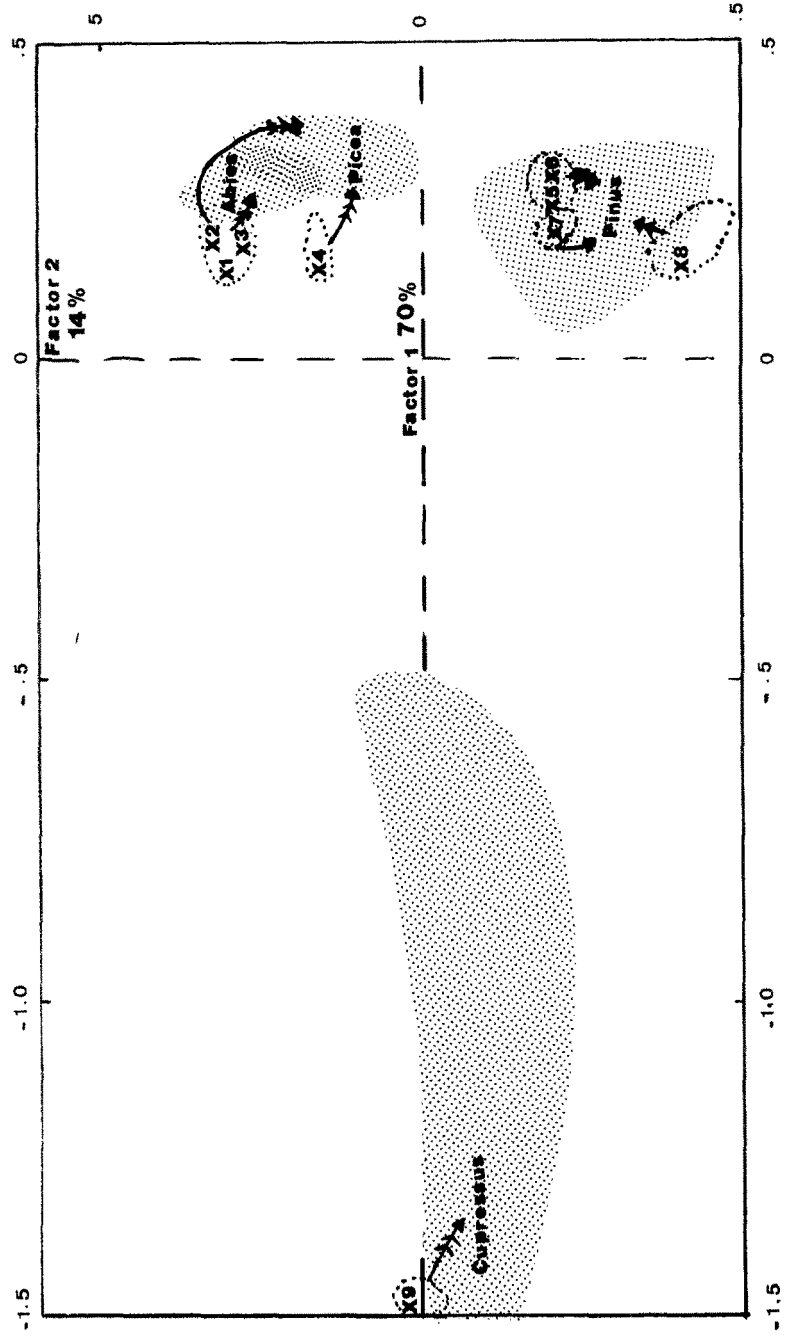

FIG. 1. Two-factorial projection of insect tree preferences following a factorial analysis of correspondence.

Abies and *Picea* overlap slightly, which expressed a poor discrimination of these two genera by the insects studied. Conversely, the separation of *Cupressus* expresses a much greater selectivity.

We projected onto the same graph the points representing the insects coded from X1 to X9. As a first approximation they form the five groups which appear in Figure 1. In order to evaluate the specificity of behavior of these insect groups, their preference probabilities with respect to the ten conifer species were calculated: the results are presented in Figures 2–7. The preference of the insect, or groups of insects, is given for the plant species represented at the top of the figure as compared to the plant species represented at the left. The darker the intersection of a line and a column, the more marked that preference.

One can thus see in Figure 3, for example, that *D. micans* (X4) prefers *Abies cephalonica* (A) to *Cupressus atlantica* (C) and that this preference is highly significant (level of confidence 0.001; represented by a very dark zone); *D. micans* also prefers, in a highly significant manner (level of confidence 0.01) *Picea abies* (P) to *Pinus sylvestris* (S); the preference is again significant

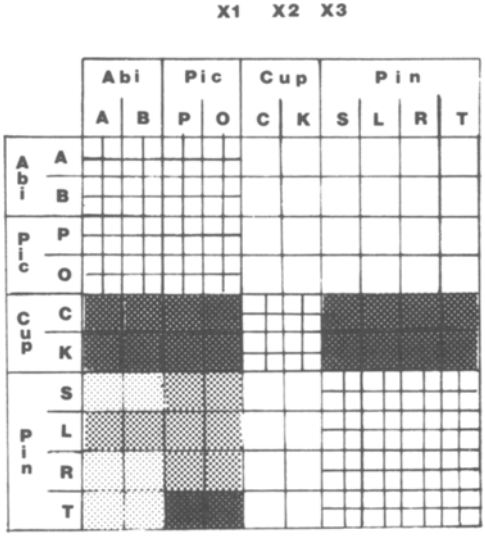

FIG. 2. Preference test results for *Cryphalus piceae*, *Cryphalus abietis*, and *Pityokteines curvidens*. Abi = *Abies*; Pin = *Pinus*; Pic = *Picea*; Cup = *Cupressus*. This figure must be read starting from the horizontal attractant species to the vertical repellent. If the square corresponding to the intersection is shaded, as in the case of the square between horizontal A and vertical C, it means that the species on the horizontal was statistically prefered to that on vertical during the pairing test. On the other hand, the square on horizontal C and vertical A remains white. When no preference was observed, the square is filled by a light cross (for more explanations see the text).

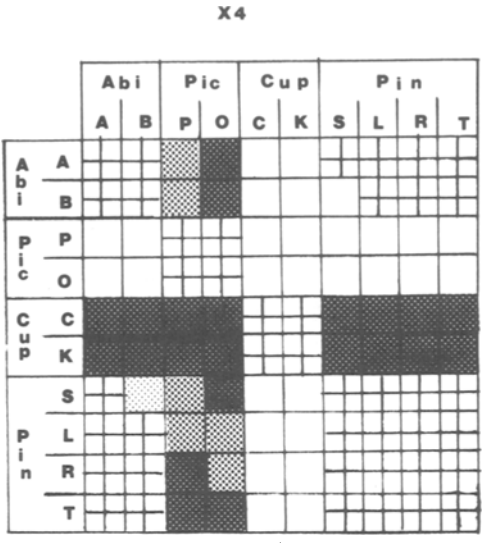

FIG. 3. Preference test results for *Dendroctonus micans.*

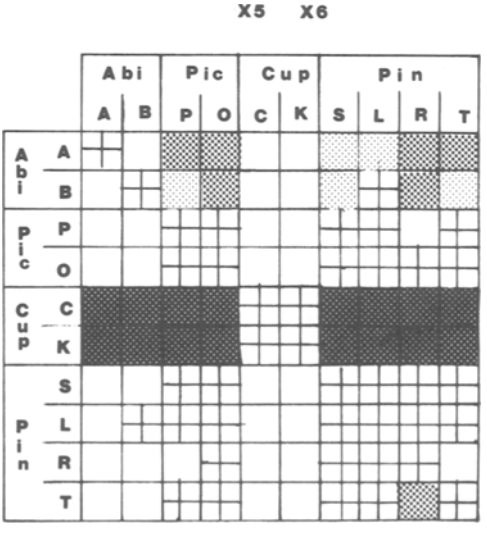

FIG. 4. Preference test results for *Ips sexdentatus* and *Ips typographus.*

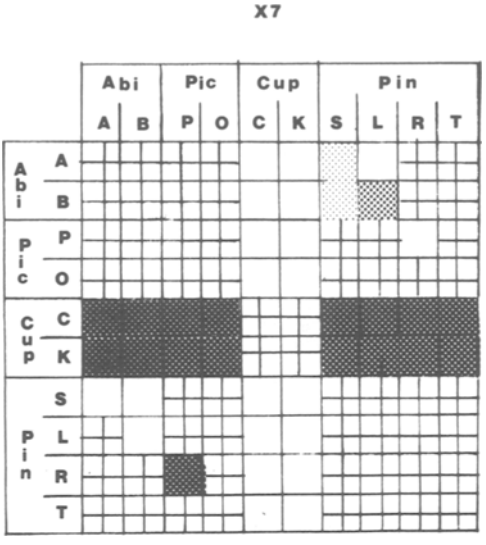

FIG. 5. Preference test results for *Orthotomicus erosus.*

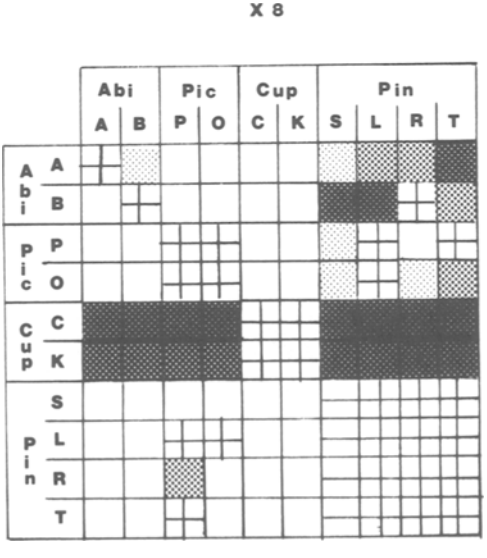

FIG. 6. Preference test results for *Tomicus piniperda.*

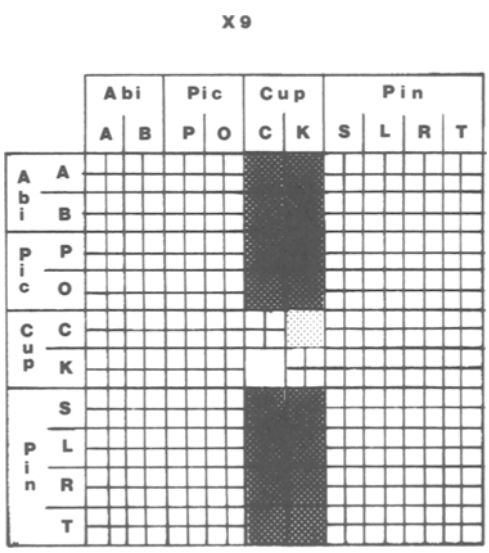

FIG. 7. Preference test results for *Phloeosinus bicolor.*

(level of confidence 0.05) for *Abies nordmanniana* (B) compared to *P. sylvestris* (S).

However, *D. micans* does not distinguish between the two *Abies* species: that difference is represented by a cross which is necessarily also found in the principal diagonal of each figure.

One can immediately see in Figure 7 the very great specificity of *Phloeosinus bicolor* for *Cupressus* as compared to all other species, with a very slight preference for *Cupressus sempervirens.*

It clearly emerges from our results that, as a general rule, within a conifer genus, the Scolytidae, except *P. bicolor*, do not exhibit a very marked preference for any one species. The clusterings by genus produced by the preceding statistical analysis are thus confirmed.

It should be noted that we were obliged to separate *I. sexdentatus* and *I. typographus* (Figure 4, X5 and X6) from *Orthotomicus erosus* (Figure 5, X7), whose differing behavior had not been demonstrated by correspondence analysis.

On the other hand, we found no clear differences in behavior for *Cryphalus piceae*, *Cryphalus abietis*, and *Pityokteines curvidens*, which are therefore grouped together in Figure 2, X1, X2, X3). That finer analysis of the olfaction tests completes Figure 1, by joining the insect groups to the conifer groups by means of arrows representing the specificity of the attraction: a single arrow (→) indicates preference at a level of confidence of 0.05, a double arrow (→→) 0.01; and a triple arrow (→→→) 0.001.

Thus *P. bicolor* (X9) is very exclusively attracted by *Cupressus*, whereas *O. erosus* (X7), repelled by *Cupressus*, seems to be indifferent to the other species, with the exception of the *Pinus*, to which it shows a slight preferential attraction.

All other Scolytidae studied are also repelled by *Cupressus*. *C. piceae* (X1), *C. abietis* (X2), and *P. curvidens* attack indiscriminately *Abies* or *Picea*, which they prefer to *Pinus*. *D. micans* (X4) attacks almost exclusively *Abies*. *I. sexdentatus* (X5) and *I. typographus* (X6) do not really distinguish between *Pinus* and *Picea*, which they clearly prefer, however, to *Abies*. Finally, *T. piniperda* chooses *Pinus*, for which it shows a clear preference.

As we have shown, the olfaction tests lead to a grouping of genus of the different conifer species. The examination of the odor spectra (Table 2) should normally lead to the same result. Actually, a rapid examination of the figures and tables does not reveal, in any simple way, what the two *Cupressus*, for example, have in common, or what distinguishes them from the other species.

We are therefore forced to admit that it must be possible to recognize a common pattern particular to the odor spectra of the *Cupressus*, although that pattern does not immediately appear.

Olfaction is a complex phenomenon resulting from the synergism of the different terpenic compounds present in the odor spectrum at particular concentrations. It is not possible in this study, where we only considered two *Cupressus* species, to recognize their spectral similarities; the number of results is insufficient to be able to legitimately apply a method of statistical analysis.

However, if all ten odor spectra are treated globally, the hierarchical classification, using a Euclidian distance, allows us to bring out a clustering of the *Pinus*, for which we already have four odor spectra. These results are presented in Figure 8.

DISCUSSION

From our research, we can thus conclude that certain insects show a strict specificity for a given species; this is the case for *P. bicolor* which, in all the statistical combinations and whatever the grouping of the species, unquestionably shows a strict preference for *C. sempervirens*, although it might occasionally settle on C. atlantica, without becoming permanently established. Under natural conditions, this insect shows a preference for *Cupressus*, and it may occasionally attack certain *Juniperus* and certain *Thuya*.

P. bicolor is attracted to *C. atlantica* only when in the experimental enclosure with other species, such as *Abies cephalonica*, *Abies nordmanniana*, or various *Pinus*.

Aside from that observed for *P. bicolor*, the other Scolytidae do not show

TABLE 2. TERPENOID COMPOSITION OF TREE SPECIES STUDIED[a]

	Peak number																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Abies caphalonica	100	5.8	24.5	35.0	–	–	9.1	2.9	3.3	0.3	–	1.6	–	–	6.6	8.3	–	15.8	11.2	8.3	12.9	6.2	–	–
Abies nordmanniana	100	3.3	13.3	–	–	10.8	15.0	6.6	–	1.4	–	–	–	–	–	17.5	–	5	–	2.5	6.6	3.3	–	–
Cupressus atlantica	0.6	–	–	–	–	–	0.6	–	–	–	–	1.8	2.4	–	100	–	3.7	10.8	12.0	1.6	12.6	–	–	–
Cupressus sempervirens	100	3.5	36.6	13.4	–	31.2	11.4	–	12.6	–	18.2	–	–	8.5	–	18.2	3.1	6.2	1.9	8.6	4.6	3.2	–	38.7
Picea abies	100	5.3	60.0	2.6	–	11.3	6.3	5.3	0.3	–	–	0.6	–	–	12.5	4.1	–	1.8	–	0.6	1.5	1.2	–	–
Picea orientalis	40.6	4.5	33.9	–	–	82.4	100	7.2	5.5	–	–	16.3	0.6	–	–	7.8	–	0.9	–	0.9	0.6	–	–	–
Pinus brutia	100	14.0	21.3	–	–	40.9	9.7	–	3.7	–	–	21.3	–	–	8.3	2.7	1.3	–	–	–	–	–	–	–
Pinus pinaster	100	10.0	62.2	–	15.7	31.1	9.1	1.8	2.8	–	–	4.7	0.8	–	–	11.0	1.7	2.9	–	1.5	2.7	–	–	–
Pinus laricio	100	5.9	4.2	1.5	–	0.7	9.6	1.2	3.8	–	–	1.9	0.7	–	1.7	–	–	1.3	–	–	1.5	0.6	–	–
Pinus sylvestris	100	3.3	12.0	–	3.3	–	14.3	6.6	0.6	–	–	4.7	–	6.2	–	8.6	0.7	8.1	–	–	7.1	–	3.8	–

[a]1. α-pinene; 2. camphene; 3. β-pinene; 4. myrcene; 5. unknown; 6. 3-carene; 7. limonene; 8. β-phellandrene; 9. terpinolene; 10. α-cubebene; 11. unknown; 12. copaene; 13. isobornyl acetate; 14. unknown; 15. longifolene; 16. caryophyllene; 17. unknown; 18. γ-muurolene + α-humulene; 19. unknown; 20. α-muurolene + germacrene D; 21. δ-cadiene; 22. γ-cadinene; 23. unknown; 24. cedrol.

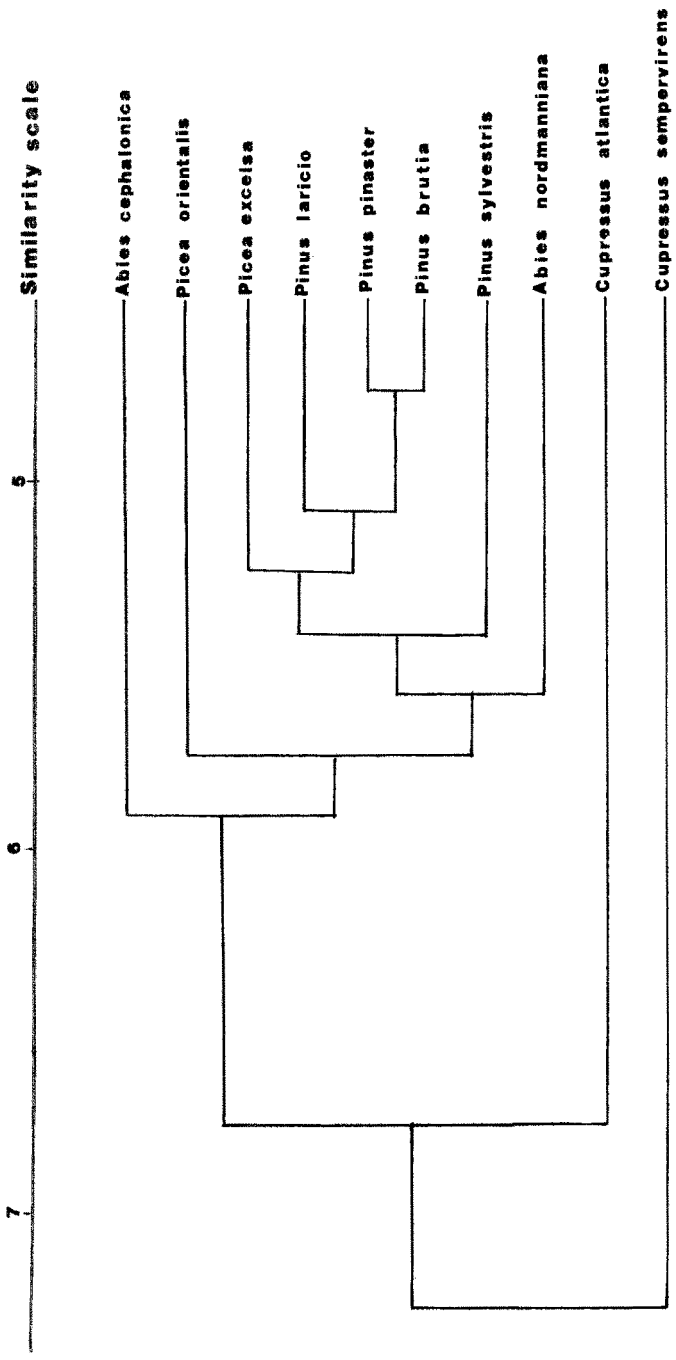

FIG. 8. Hierarchical classification of the conifers studied.

a strict specificity, as *D. micans* is attracted by *Picea orientalis*, as well as by *Picea abies*. In fact, under certain natural conditions, when the species are found mixed (*Pinus*, *Picea*, *Abies*), the *Picea* exert the strongest attractive power.

I. sexdentatus and *I. typographus* do not show a strict specificity towards one species, as they attack *Pinus* and *Picea* indiscriminately. On the other hand, *Cryphalus* and *P. curvidens* show a stricter specificity for *Picea* and *Abies*.

O. erosus does not show very strict specificity, as it turns indiscriminately towards various *Abies*, *Picea*, and *Pinus* with a slight preference for *Pinus*.

It can thus be seen that, aside from *Phloeosinus*, all the Scolytidae studied here are attracted by conifers whose odor spectra vary noticeably from one species to another. In fact, the permanent establishment of a scolytid does not depend on a given compound; the attracting power results from the synergism of different terpenes. Definitive establishment thus depends on sensory adaptation to volatile substances, an adaptation which is narrow for certain Scolytidae like *Phloeosinus bicolor*, or wider for the other Scolytidae studied here.

Under natural conditions, when there are several cut logs of different species present, a particular odor environment is created around each log which contributes towards the orientation of the adults during their emergence. Some authors have said that orientation is random at the time of emergence and that establishment is influenced by many factors, not only by the odor spectrum, but also by visual factors. Nevertheless, our research has shown that, in the presence of several species with identical bark colour, the Scolytidae distinguish among these species perfectly; this follows from olfactory stimuli that intervene at the time of their establishment on the host tree.

It must also be noted that a grouping of tree species by genus is observed, *Pinus* in particular. In fact, under natural conditions, we were able to observe that the Scolytidae may develop on different *Pinus*, and the disappearance of a species or genus does not prevent the establishment and development of this bark beetle on another *Pinus*. A typical example was given by Chararas (1962), who observed in Turkey that after the disappearance, caused by a forest fire, of 50 hectares of *Pinus brutia*, preferred species for *T. piniperda*, this insect heavily attacked a planting of *Pinus pinea* 8 km away, although the odor spectrum of the latter is completely different from that of *P. brutia*. In *P. brutia* α-pinene is the predominant monoterpene, while limonene is the major component of *P. pinea*. A similar example may be given for Tunisia, where American species have been introduced, particularly *Pinus radiata* (= P. insignis), *Pinus caribea*, and *Pinus taeda*, species which had never constituted a natural biotope for *T. piniperda* and *O. erosus*, insects which do not exist in the native site of the conifers we have just mentioned. These two Scolytidae,

which are usually encountered in Tunisia on *P. pinaster* and *P. halepensis*, have adapted themselves, from an olfactory and nutritional point of view, to the introduced species. All the Scolytidae are, in fact, capable of recognizing several compounds and of finding their bearings according to the odor spectrum of the appropriate species.

On the other hand, there are some Scolytidae which, while being attracted by a specific given compound of one species, such as atlantone in *Cedrus atlantica*, tolerate other compounds, such as α-pinene, which exert a repulsive stimulus at higher concentrations. This is the case for *Phloeosinus cedri* (Chararas, 1980).

Moreover, as a general rule, all terpenic compounds are repulsive at high concentrations, as olfaction tests have shown. But they can become attractive at low concentrations. This phenomenon has been studied by special devices in the laboratory as well as in the field (Chararas, 1958, 1980; Perttunen et al., 1970; Rudinsky, 1966). It must be said that in order for a species to exert an attraction on Scolytidae, the effects of one or more attractive substances must prevail over the effects of the repulsive substances. This results in a certain "pattern" of odor spectrum recognized by the bark beetle.

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IMPROVED PREPARATION OF (*E*)-β-FARNESENE AND ITS ACTIVITY WITH ECONOMICALLY IMPORTANT APHIDS

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Abstract—(*E*)-β-Farnesene, the major component of the alarm pheromone of many aphids, is prepared in 65% yield by passing commercial nerolidol through an evacuated column of alumina, treated with pyridine, at 200°C. The product is stored without air and applied to aphids as vapor at low speed (ca. 230 mm/sec) in air or nitrogen. The response of eight economically important aphid species was assessed in the laboratory; over 90% of *Myzus persicae* responded in a large scale trial.

Key Words—Aphids, *Myzus persicae*, Homoptera, Aphidae, pheromone, alarm pheromone, behavior, (*E*)-β-farnesene, nerolidol, dehydration.

INTRODUCTION

(*E*)-β-Farnesene (Figure 1) is the main component of the alarm pheromone of many aphids (Bowers et al., 1972; Edwards et al., 1973; Wientjens et al., 1973; Pickett and Griffiths, 1980). Crude (*E*)-β-farnesene [16% active ingredient (a.i.)] obtained from commercial nerolidol (Figure 1) by treatment with $POCl_3$/pyridine is as active on *Myzus persicae* as natural pheromone (Pickett and Griffiths, 1980) and in the laboratory increases effectiveness of the contact insecticide permethrin, presumably by stimulating aphid mobility (Griffiths and Pickett, 1980). The objective of this study was to develop a convenient route to (*E*)-β-farnesene on a large scale from easily obtained materials and to establish activity of this product with several species of economically important aphids. This would facilitate field investigation of the use of synthetic pheromone in conjunction with contact pesticides and the preparation of derivatives that might prevent settling and virus transmission of aphids (Dawson et al., 1982).

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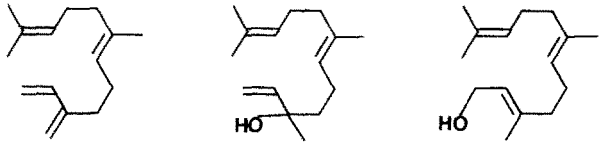

FIG 1. Structures of (*E*)-β-farnesene and precursors.

METHODS AND MATERIALS

Reaction products and aphid cornicle secretions were analyzed by gas chromatography (GC) (Ucon 5100, 50 m × 0.25 mm glass capillary, 40-110° at 40°C/min) and structures confirmed by nuclear magnetic resonance spectroscopy or GC coupled mass spectrometry (MS) as described previously (Pickett and Griffiths, 1980), the GC elution order being (*Z*)-β-, (*E*)-β-, (*Z,Z*)-α-, (*E,Z*)-α-, (*Z,E*)-α-, and (*E,E*)-α-farnesene. Air samples (8μl), saturated with *E*)-β-farnesene, taken with a 10 μl GC syringe with the plunger sealed with pentane (ca. 0.2 μl) were analyzed by GC-MS (single ion monitoring, *m/z* 69).

TABLE 1. PREPARATION OF (*E*)-β-FARNESENE

Method	Yield of β-farnesenes (%, theoretical)	(*E*)-β-farnesene in product (%)
Batch processes		
1. Nerolidol + $POCl_3$/pyridine, 20°C, 48 hr (Anet, 1970)	8	16
2. Nerolidol + DMSO reflux, 3 hr	10	13
3. Nerolidol + Al_2O_3 (neutral)/pyridine, oil bath 220°C, 6 hr (von Rudloff, 1961)	8	7
4. Farnesol + KOH, under vacuum, 200°C, <5 min (Bhati, 1963)	30	40
Column processes (under vacuum)		
5. Nerolidol, Al_2O_3 (neutral), 200°C	2	1.5
6. Nerolidol, Al_2O_3 (basic), 200°C	40	40
7. Nerolidol, Al_2O_3 (neutral)/pyrrolidine, 200°C	55	40
8. Nerolidol, Al_2O_3 (neutral)/triethylamine, 200°C	65	45
9. Nerolidol, Al_2O_3 (neutral)/pyridine, 200°C	65	47

Preparation of (*E*)-β-*Farnesene.* Table 1 gives reaction conditions and references. The general column procedure is illustrated by method 9: neutral alumina 100-200# (50 g) in a glass column (see Figure 2) was heated at 200°C under vacuum (0.1–0.2 torr, rotary pump). First pyridine (4 g) from the flask was allowed to evaporate and pass through the column. Nerolidol (80 g) was then allowed to drip into the column (4.5 hr). The product, collected as a light brown liquid (69.5 g) in the air-cooled trap below the column, was eluted from Florisil (200 g) with hexane which was removed under vacuum to give a straw-colored liquid (68.7 g, 65% β-farnesenes, 47% (*E*)-β-farnesene by gas chromatography). The final produce (10 mg and 1 g batches) was sealed under nitrogen in glass ampoules.

Aphids. Except for *Phorodon humuli*, which was collected outdoors, all aphids were from laboratory cultures maintained at 16 hr day length and 20 ± 5°C. The *Myzus persicae* used were obtained by confining adults on plants by means of clip cages for 2–3 days, then removing the adults and allowing the young to develop for 2–3 days before the tests, so they were all of similar age.

Bioassay Method. In the bioassay, a modification of the syringe test (Pickett and Griffiths, 1980), a detached piece of plant material bearing 10–30 aphids was placed on the stage of a low-power binocular microscope and the

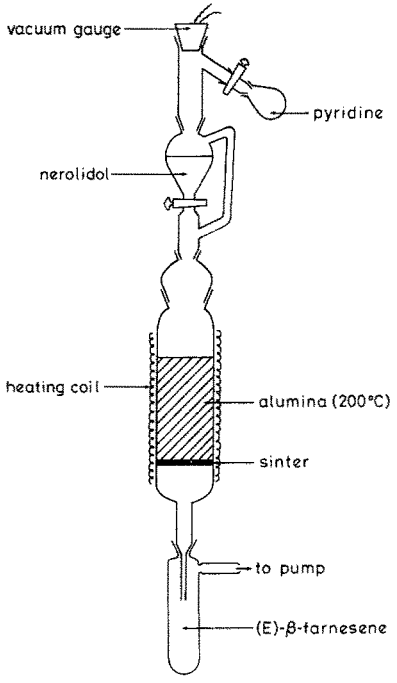

FIG. 2. Large-scale preparation (*E*)-β-farnesene.

settled individuals were counted. A glass ampoule containing 10 μl of the pheromone was crushed in the barrel of a 20 ml syringe. The saturated air from the syringe was discharged during 10 sec via a stainless-steel needle whose orifice was 10 mm above the plant surface. Settled aphids were counted at 1 min after pheromone discharge had ceased. Each test was repeated 4–10 times. To examine the effects of different conditions, the speed of delivery of pheromone was varied in some tests, and in others pheromone was applied in a current of air moving at 2.5 m/sec.

Large-Scale Test. Nitrogen (3 liter) was passed through a vessel containing a freshly broken ampoule of the new (*E*)-β-farnesene (1 g) absorbed onto filter paper and blown from a polypropylene pipe (4 mm ID) at 230 mm/sec sequentially over 16 large potted plants, *Brassica pekinensis*, infested with *Myzus persicae*, in two rows of eight in an area of 4 × 1 m in the laboratory at 18°C in daylight.

RESULTS AND DISCUSSION

Table 1 gives yields of (*E*)-β-farnesene under various conditions. Nerolidol and farnesol available commercially as mixtures of isomers: (*E*)-nerolidol with (*Z*)-nerolidol, ca. 1:1, and (*E,E*)-farnesol, (*Z,Z*)-farnesol, (*E,Z*)-farnesol with (*Z,E*)-farnesol, ca. 3:1:2:1 (Fig. 1), were used without further purification which is expensive and difficult on a large scale. Yields of (*E*)-β-farnesene were based on amounts of appropriate isomers, (*E*)-nerolidol or (*E,E*)-farnesol and (*Z,E*)-farnesol in the starting material. Of the batch methods, KOH dehydration of farnesol (method 4) gave best results. However, although a column procedure is desirable for larger-scale work (>20 g), to heat a column of KOH was not feasible, so modifications of method 3 using a column of alumina were examined. Surprisingly, results were better, particularly with method 9. Up to 200 g of nerolidol could be converted using a single 50 g column of alumina with consistent product quality.

In addition to (*E*)-β-farnesene, various isomeric nonfarnesene hydrocarbons (M 204) were obtained in the product from method 9 but (*Z*)-β-farnesene (23%) from the (*Z*)-nerolidol was the major contaminant. Nerolidol can dehydrate in three different ways to give β-farnesenes (terminal methylene) or α-farnesenes (*E*- or *Z*-trisubstituted double bonds). However, under the above conditions, the reaction gave almost exclusively the terminal methylene, the α-farnesenes formed extensively by $POCl_3$ dehydration being absent (<0.2% by GC). The nonfarnesene isomers may arise from heat cyclization since they predominate at higher temperatures which is likely to favor cyclization. Method 8 gave more nonfarnesene isomers than 9, although the yield of (*E*)-β-farnesene was similar. Cyclization and pyrolysis of the product, presumably responsible for lower yields by the method of von

Rudloff (1961), are substantially diminished by the low residence time (ca. 1 min) on the alumina in the column methods.

Method 9 for preparation of crude (*E*)-β-farnesene seemed suitable for large-scale production. It was therefore necessary to test the product with *M. persicae* under various conditions and in comparison with the $POCl_3$ product (method 1). Results (Table 2) show that the new product is as active as the original material (Pickett and Griffiths, 1980). At the higher levels air would be saturated with (*E*)-β-farnesene, 60 ppb (1.2 μg in 20 ml) from headspace analysis by GC-MS or 100 ppb calculated from the published boiling point (Bhati, 1963). Control treatments, e.g., 1-pentadecene [a compound chemically similar to (*E*)-β-farnesene] in experiment II, gave such low responses that they were not included in subsequent tests. In experiment III, contact with air lowered activity of the (*E*)-β-farnesene. This effect was less for the $POCl_3$ product presumably because the α-farnesene impurities protected the active isomer from aerial oxidation as observed previously with pure (*E*)-β-farnesene stored in air (Griffiths and Pickett, 1980). As GC analysis showed that saturation with (*E*)-β-farnesene was still achieved after contact with air, it was concluded that the activity was lowered by an inhibiting effect from the oxidation product. Experiment IV demonstrated that the low air speed (ca. 230 mm/sec) for applying(*E*)-β-farnesene gave better results than a speed over 10 times higher. This suggested that the treatment would be

TABLE 2. RESPONSE OF *Myzus persicae* TO (E)-β-FARNESENE (EBF) FROM NEW METHOD

Exp.	Test material and difference from standard bioassay conditions	Response (% ± standard error)	Difference
I	EBF (new) (7 mg a.i.[a])	90 ± 3.6	NS
	EBF ($POCl_3$) (7 mg a.i.)	92 ± 3.2	
	EBF (new) (20 ng a.i.)	77 ± 7.9	NS
	EBF ($POCl_3$) (20 ng a.i.)	75 ± 3.7	
II	EBF (new)	92 ± 4.7	Significant
	1-pentadecene	2 ± 1.3	($P = 0.001$)
III	EBF (new)	94 ± 2.0	Significant
	EBF (new) after exposure to air for 1 hr	74 ± 5.0	($P = 0.05$)
	EBF ($POCl_3$)	94 ± 6.3	NS
	EBF ($POCl_3$) after exposure to air for 1 hr	94 ± 3.0	
IV	EBF (new)	84 ± 4.7	Significant
	EBF (new) air speed *ca* 2.5m/sec	63 ± 8.0	($P = 0.05$)
V	EBF (new)	93 ± 2.4	NS
	EBF (new) in air draught 2.5m/sec	98 ± 1.5	

[a]a.i. = active ingredient.

TABLE 3. RESPONSE OF ECONOMICALLY IMPORTANT APHIDS TO (*E*)-β-FARNESENE FROM NEW METHOD

Aphid	Response (% ± standard error)
Myzus persicae	99 ± 0.6
Aphis fabae	71 ± 5.8
Phorodon humuli	78 ± 10.2
Sitobion avenae (green)	31 ± 11.7
Rhopalosiphum padi	47 ± 4.8
Nasonovia ribis-nigri	88 ± 5.9
Metopolophium dirhodum	61 ± 8.3
Brevicoryne brassicae	0

ineffective under windy field conditions. However, good response was obtained by applying the pheromone at low air speed to plants in an ambient draught with a high air speed.

Thus the new product can achieve maximum activity, if air is excluded during storage and it is applied as vapor at low air speed. Table 3 shows that the new product is also active with a number of other economically important aphids. *Brevicoryne brassicae* did not respond, although sufficient (*E*)-β-farnesene was detected in the cornicle secretions (<0.01 ng per insect by GC-MS) for *M. persicae* to respond. The response by *A. fabae* was substantially lowered if traces of solvent (e.g., hexane, diethyl ether) contaminated the (*E*)-β-farnesene.

Finally, (*E*)-β-farnesene from the new method was tested on a larger scale with *M. persicae* on mature chinese cabbage (*Brassica pekinensis*) plants arranged in rows on the laboratory bench. The amount of (*E*)-β-farnesene used to treat the 16 infested plants was calculated to be 180 μg, and over 90% of aphids responded by moving from feeding sites. This result indicates that (*E*)-β-farnesene from the large-scale method has potential value for use in the field.

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TRAIL PHEROMONE OF THE LEAF-CUTTING ANT, *Acromyrmex octospinosus* (REICH), (FORMICIDAE: MYRMICINAE)

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Abstract—The most active component of the trail pheromone of the leaf-cutting ant, *Acromyrmex octospinosus*, is methyl 4-methylpyrrole-2-carboxylate (I). Two pyrazine isomers (II) and (III) are present but inactive.

Key Words—Trial pheromone, leaf-cutting ant, *Acromyrmex octospinosus*, Hymenoptera, Formicidae, Myrmicinae, methyl 4-methylpyrrole-2-carboxylate, 3-ethyl-2,5-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, ant.

INTRODUCTION

Leaf-cutting ants of the genera *Atta* and *Acromyrmex* use trail-marking pheromones while foraging. The most active components of the pheromones of three species were identified as methyl 4-methylpyrrole-2-carboxylate (I) for *Atta texana* (Tumlinson et al., 1971) and *A. cephalotes* (Riley et al., 1974), and 3-ethyl-2,5-dimethylpyrazine (II) for *A. sexdens* (Cross et al., 1979), which also contained the pyrrole (I). The source of these compounds is the poison gland reservoir.

In the present study, we report that the most active component of the trail pheromone of *Acromyrmex octospinosus* (Reich) is the pyrrole (I); also present are the pyrazine (II) and its isomer, 2-ethyl-3,5-dimethylpyrazine (III).

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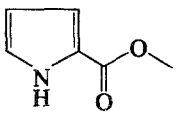

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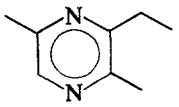

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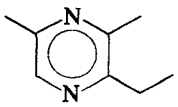

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METHODS AND MATERIALS

Acromyrmex octospinosus worker ants were obtained from nests in and near citrus and cacao plantations in Trinidad, West Indies, in August 1975. The abdomens of 120 g of ants (approximately 8000 ants) were removed and macerated in methylene chloride (CH_2Cl_2); the filtered solution was concentrated by distillation of the CH_2Cl_2 through a short packed column at atmospheric pressure, and the concentrated extract was subjected to short-path distillation (96°, 25 mm) to give a distillate that elicited a strong trail-following response in the laboratory bioassay (Cross et al., 1979).

Trail-marking deposits were accumulated from laboratory colonies of *A. octospinosus* at the University College of North Wales by allowing the ants to pass through an enclosed Plexiglas maze between the nest and the foraging area. Each nest had a fungus garden volume of 500–600 cc. A polythene tube, 2.5 cm in external diameter, led from the nest box into one end of the maze, which was a Plexiglas box 24 × 24 × 3.5 cm deep fitted with a series of internal partitions 2 cm apart running alternately from one side to within 3 cm of the other side. To traverse the maze, the ants had to take a zigzag path 2 m long. At the other end of the maze, a polythene tube led to a feeding box where privet leaves were provided as forage. The lid of the maze was removed, and 10.0 g of purified Porapak Q (Byrne et al., 1975) were poured onto the central section of the floor of the maze, to give a trail over Porapak 1.0 m long. After 50 days, the Porapak Q from two such mazes connected to two nests was extracted with 250 ml pentane in a Soxhlet apparatus for 24 hr, and the solvent was distilled through a short, packed column to leave 700 μl of a biologically active solution.

The distillate and the concentrated Porapak extract were fractionated by GC on the following glass columns: column A, 2.44 m × 4 mm ID, 5% SE-30 on Chromosorb G 50–80 mesh; column B, 6.10 m × 4 mm ID, 4% Carbowax 20 M on Chromosorb G, 50–80 mesh; column C, 1.53 m × 4 mm ID, 5% FFAP on Varaport 30, 80–100 mesh; column D, 6.10m × 4mm ID, 4% NPGA on Chromosorb G, 80–100 mesh; column F, 6.10 m × 4 mm ID, 5% Apiezon L on Chromosorb G, 80–100 mesh; column G, 6.10 m × 4 mm ID, 5% FFAP on Varaport 30, 80–100 mesh.

Mass spectra (electron impact) were obtained on a Hitachi RMU-6 and on a Finnegan GC-MS 3000. Fractions from the Varian 2700 chromatograph were collected in glass capillary tubes (30 cm × 2 mm OD) in a thermal-

gradient splitter/collector (Brownlee and Silverstein, 1968). Methyl 4-methylpyrrole-2-carboxylate (I) was obtained from Dr. P.E. Sonnet. A sample containing a 1:1 mixture of 3-ethyl-2,5-dimethylpyrazine (II) and 2-ethyl-3,5-dimethylpyrazine (III) was obtained as a gift from Pyrazine Specialties, Atlanta, Georgia; the isomers were separated on column F.

Laboratory bioassays were carried out as described by Robinson et al. (1974) and Cross et al. (1979).

RESULTS

Isolation and Identification. Fractionation of the concentrate of the Porapak extract on column A (60 ml He/min. 75° for 12 min, programed at 2°/min to 200°) gave a very active fraction (2) collected at 33–58 min. Slight activity was found in the other fractions. Fraction 2 was fractionated on column B (60 ml He/min, 155° for 24 min, programed at 4°/min to 190°). The most active fraction (2-8, 36–43 min) was fractionated on column C (60 ml He/min, 130°), and an active compound (2-8-4) eluting as a single symmetrical peak at 53 min was collected and identified as methyl 4-methylpyrrole-2-carboxylate (I) by congruence of the mass spectrum with that of an authentic sample (Tumlinson et al., 1972) and by coinjection with the authentic sample on columns B and C. In this way, approximately 20 μg of I was isolated from the deposits of ants foraging for 50 days from two laboratory nests.

The pyrazine that was found in *Atta sexdens* (Cross et al., 1979) was sought through the following fractionation sequence: Fraction 2-1 (0–6 min) from column B (60 ml He/min, 155° for 24 min, programed at 4°/min to 190°) gave fraction 2-1-4 (22–29 min) on column C (60 ml He/min, 90° for 6 min, programed at 2°/min to 150°), which gave fraction 2-1-4-2 (29–34 min) on column D (60 ml He/min, 115°), which in turn gave two peaks [55.5 min (II) and 60.5 min (III) on column G (60 ml He/min, 80°)]. These compounds were identified as II and III by coinjection with authentic samples on column G, and by congruence of the mass spectra with those of authentic samples [II and III, M = 136 (70%), M − 1 = 135 (100%)]. The ratio of II to III was 1:3 (54 ng–158 ng).

The short-path distillate from approximately 8000 ant abdomens yielded approximately 5 μg of the pyrrole (I) following the above fractionation procedure, but the presence of the pyrazines (II and III) could not be confirmed because of small amounts.

Bioassays. Acromyrmex octospinosus responded to the synthetic pyrrole (I) over a range of 4.0 pg/μl to 4.0 ng/μl in a laboratory bioassay; maximum response was at about 0.4 ng/μl (Robinson et al., 1974). In the present study, the GC fraction containing I gave a positive response over the three concentrations tested from 6 ng/μl to 0.06 ng/μl, with the highest response at

the highest dilution; presumably at higher concentrations, the fraction had other effects on the ants that interfered with the bioassay. Responses to the pyrazines II and III, alone or in combination, and to the fraction containing them were negative.

DISCUSSION

It is an oversimplification to ascribe the complex social behavior of trail following solely to the compound identified as the most active, satisfying though it may be to elicit this response in the laboratory and in the field with a defined chemical. In a recent study, Robertson et al. (1980) described the complex interactions of attractants, activators, synergists, and antitrailants in the trail following of the Argentine ant, and also noted that visual clues and physical contact with fellow trailers are involved.

In the present study, the role of methyl 4-methylpyrrole-2-carboxylate (I) in *Acromyrmex octospinosus* is clear, and its presence explains the earlier empirical observation by Robinson et al. (1974) that this ant would follow a trail of synthetic I. The role of the pyrazines II and III is less clear; The fractions containing II and III did not show significant activity, and we could not confirm our reported result that a 1:1 mixture (synthetic sample) elicited a response from *A. octospinosus* at a very high concentration (Cross et al., 1979). At any rate, such high concentrations have no biological significance. It is interesting to note the presence of II as the most active component of the pheromone of *Atta sexdens* together with the absence of III and the presence of I as an inactive compound in that ant. Certainly, sympatric species of many insects discriminate between species by recognizing specific blends of the same compounds that are shared and function both as pheromones and allelochemics (for example, Tamaki, 1977; Silverstein, 1977, Blum, 1979; Birch et al., 1980), and it has been shown that ants can distinguish their own trail from those of other species despite the commonality of components (Robinson et al., 1974; Cross et al., 1979).

Use of an absorbent such as Porapak Q, to accumulate trail pheromones from a limited number of ants, appears to be a useful procedure given a laboratory bioassay to track active compounds. We accumulated more of the compounds of interest from two nests than from the approximately 8000 ants laboriously harvested in the field and dissected.

We had hoped that a practical method of ant control might result from a pheromone-enhanced toxic bait. However, Robinson and Cherrett (1978) and Robinson et al. (1982) have shown that, although pick-up of filter paper disks in the laboratory was enhanced by the addition of synthetic I, adding it to citrus pulp bait did not produce worthwhile additional pick-up in the field. They suggested that this was due to a lower sensitivity to given concentrations of pheromone on baits in the field than in the laboratory, to the pheromone's

role being restricted to that of an attractant so that other chemicals are needed to induce pick-up of the bait, and to competition from naturally occurring "food odors" in the bait particles, which act as attractants in their own right. They concluded that the pheromone would not be a cost-effective addition to current baits, although it might be a worthwhile addition to the synthetic baits now being developed (Jutsum and Cherrett, 1981).

Acknowledgments—Grants from NSF, Rockefeller Foundation, and the British Overseas Development Administration are gratefully acknowledged. Ant colonies in the laboratory were maintained under MAFF license No. HH 10899A/67 issued under the Destructive Pests and Diseases of Plants Order 1965. We thank Mrs. H. Jennison and Dr. T. Wachs for mass spectra, Dr. P.E. Sonnet for the synthetic pyrrole (I) sample, Pyrazine Specialties for the synthetic pyrazines (II and III), and Mrs. O.M. Silverstein for assistance in gathering the ants. Dr. John J. McKelvey, grant administrator for Rockefeller Foundation, extended many professional and personal courtesies.

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EVIDENCE OF AN OVIPOSITION STIMULANT FOR PEACHTREE BORER[1,2]

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Abstract—Peachtree borer (PTB) cocoons, peach tree bark, and a mixture of frass and gum obtained arount PTB wounds were extracted with organic solvents of increasing polarity. Female PTB moths, *Synanthedon exitiosa* (Say), laid the greatest number of eggs on filter paper substrates treated with acetone extracts of PTB cocoons, ethyl acetate extracts of peach tree bark and acetone extracts of frass and gum mixtures. The extracts of cocoons or frass and gum mixture were separated by liquid chromatography and thin layer chromatography.

Key Words—Peachtree borer, *Synanthedon exitiosa* (Say), Lepidoptera, Sessiidae, oviposition stimulant, peach, *Prunus persica*.

INTRODUCTION

The peachtree borer (PTB), *Synanthedon exitiosa* (Say), is one of the most serious insect pests of peach [*Prunus persica* (L.) Batsch] in most peach production areas of the U.S.; every year, directly or indirectly, it causes the death of many trees (Snapp and Thomson, 1943; Pearson and Wieres, 1978). Larvae of this pest bore into the tree trunk near ground level and feed in the inner bark. Insecticides are commercially used for control, but researchers are looking for a nonchemical means of control. For example, the female sex

[1]*Synanthedon exitiosa* (Say).
[2]Mention of a commercial or proprietory product does not constitute an endorsement of this product by the USDA.

pheromone of the PTB, identified by Tumlinson et al. (1974) as (*Z*,*Z*)-3,13-octadecadien-1-ol actate, was used by McLaughlin et al. (1976) to disrupt the mating communication of the PTB by permeating the atmosphere with the synthetic pheromone. Gentry (1980) reported that mass-trapping PTB males with the synthetic pheromone resulted in greater control of PTB populations in peach orchards than in orchards treated with conventional insecticides. A variety of bait substances for attracting both male and female Sesiidae moths to a trap have also been tested. Frankenhuyzen (1979) reported that a solution of 10% molasses and 4% yeast with a drop of terpenyl acetate was used to trap the apple clearwing moth, *Synanthedon myopaeformis*, (Barkhausen). Frankenhuyzen and Wijen (1979) also found that a mixture of 85% water, 10% red wine, and 5% soft brown sugar was highly efficacious. About 25–30% of the moths attracted to these chemicals were females.

The attraction of female PTB to previously damaged host trees was noticed by Eli B. Blakeslee (unpublished manuscript, 1914), who stated that "clumping or aggregation of borer cocoons may occur because larvae are more likely to become established in previously infested trees." Later Snapp and Thomson (1943) reported that PTB females deposit most of their eggs on or close to the tree from which they emerge. There are several reports that indicate oviposition behavior in some cases may be elicited by pheromones. Norris (1963) demonstrated that the strong tendency for females of the desert locust, *Schistocerca gregaria* Forskal to lay their pods next to one another is in part pheromone mediated. A chemical factor associated with developing stages of *Culex tarsalis* Coquillett was reported by Hudson and McLintock (1967) to influence the choice of oviposition sites by females of that species.

Our research was designed to determine if substances that stimulate ovipositing of female PTB could be extracted from natural sources such as the bark of peach trees or cocoons from which adult PTB had emerged or frass and gum mixtures from PTB wounds.

METHODS AND MATERIALS

Source Materials. Bark, down to the xylem, was obtained from the trunk of 4-year-old "Dixiland" peach trees in healthy condition situated at the Southeastern Fruit and Tree Nut Research Laboratory, Byron, Georgia. Cocoons of the PTB were collected from commercial peach orchards in Peach County, Georgia, during August and September 1980. The frass and gum mixtures were collected in August 1981 from the base of PTB-infested 5-year-old Dixiland peach trees at Byron, GA.

Solvent Extractions. Twenty grams of cocoons or 36 g (fresh wt) of chipped bark were homogenized for 1 min in a Waring blender in 100 or 150 ml, respectively, in each of the following solvents (only one solvent per

extraction): hexane, petroleum ether, diethyl ether, chloroform, ethyl acetate, acetone, methanol or water. Homogenates, other than the one from water, were filtered through sodium sulfate, and then directly transferred to filter paper wicks under a stream of dry nitrogen to aid evaporation. A larger quantity, 250 g, of cocoons was extracted in 2 liters acetone and concentrated in a flash evaporator at 56° C under reduced pressure for liquid chromatographic fractionation. The frass and gum mixture was extracted with acetone (1:2 w/v), then concentrated 16-fold with a rotary evaporator at 30° and reduced pressure.

Bioassays. Biological activity of solvent extracts and of chromatographic fractions was monitored by placing gravid PTB females in a cage made of 0.64-cm thick Plexiglas (Figure 1). The cage was designed for assembly and disassembly in three sections (two tunnels, one center section). This arrangement allowed (1) easy handling and transport; (2) interchange of tunnels from one side of the center section to the other to eliminate any positional advantage; and (3) quick and efficient cleaning. Dimensions of each tunnel were 182.9 × 30.5 × 30.5 cm, while those of the center section were 30.5 × 30.5 × 30.5 cm. At the outer end of each of the tunnels and also on the open sides of the center section were 10-cm-wide frames of plexiglas mounted with 0.64-cm machine bolts on the outside of the three sections which protruded 5.1 cm out from the sections and also for two end panels. An opening 15.2 × 15.2 cm, with a hinged door on one side of the center section, allowed introduction of the gravid females into the cage. Located on top of the center section was a small 7.6-cm, 1/20-hp squirrel cage fan which drew ca. 50 cfm air through two 1.27-cm-diam. holes covered with 16 × 18 mesh screen wire in the end panels. The end panels could be removed to permit insertion or removal of the impregnated filter paper wicks or the females. Two lines made of 20-gauge piano wire were attached in each tunnel about 2.54 cm below the corners where the top joined the sides. The fiter paper wicks, No. 40, 150 cm, were suspended from the wire vertically at equal distances apart. In each test, 8–10 gravid females were released into the cage at ca. 1600 hr and exposed to the impregnated filter paper wicks as well as the control wicks not impregnated for 24 hr. Observations for oviposition on treated and untreated filter paper were made at the end of each test period.

Fractionation and Thin-Layer Chromatography. Acetone extracts of PTB cocoons were fractionated by liquid chromatography on a 4 × 45-cm glass column packed with silica gel (60/70 mesh) and eluted with 3 liters of a gradient that varied from 100% chloroform–0% methanol to 0% chloroform–100% methanol. Fractions of 10 ml were collected, and the last 4 of each 20 tubes were combined, concentrated, transferred to filter paper wicks, and bioassayed for activity. Bioassayed fractions were also subjected to thin-layer chromatography on silica gel using toluene–ethyl acetate–formic acid (TEF)

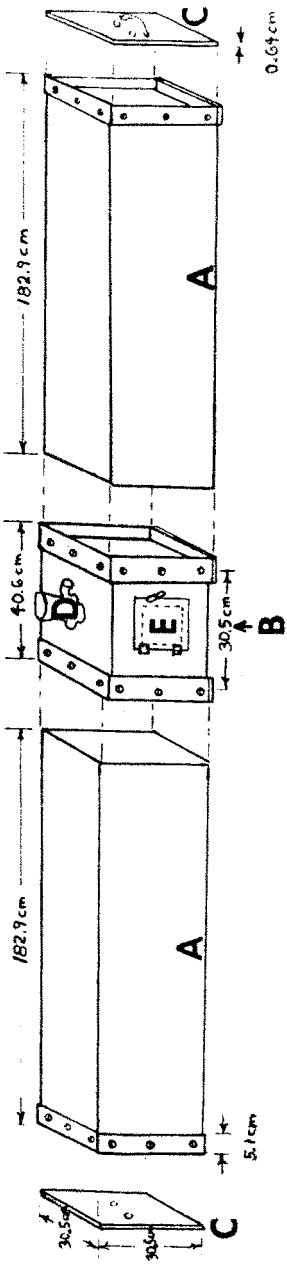

FIG. 1. Oviposition bioassay cage. A = tunnel, B = center section, C = end panel, D = squirrel cage fan, E = entrance door.

(5:4:1 v/v/v) for development. Plates were then sprayed with 1% ethanolic H_2SO_4 and charred at 150°C for 5 min to visualize the bands.

The concentrated acetone extract of the gum and frass mixture was subjected to column chromatography on a 2.5 × 10-cm column packing of LiChroprep RP-18 (MC/B Manufacturing Chemists, Inc., 2909 Highland Ave., Cincinnati, Ohio 45212).

Concentrate, 5 ml, was mixed with 25 ml of water and passed through the column followed by successive elutions of 50 ml water, 50 ml 30% acetonitrile, 50 ml 100% acetonitrile, and 50 ml acetone. Fractions were concentrated tenfold and used for bioassay or TLC using the TEF solvent system.

RESULTS AND DISCUSSION

The ethyl acetate extract of bark from peach trees contained a substance(s) that induced female PTB to oviposit (Table 1). Biological activity, in terms of increased egg deposits when compared with solvent checks, was also detected in the acetone extract.

Peachtree borer cocoons also contained a substance(s) that induced females to oviposit; it was found in diethyl ether, acetone, and methanol extracts (Table 2). Thin-layer chromatography of bark and cocoon extracts

TABLE 1. NUMBER OF PEACHTREE BORER (PTB) EGGS DEPOSITED ON FILTER PAPER WICKS IMPREGNATED WITH SOLVENT EXTRACTS OF PEACH TREE BARK AND WITH RESIDUES OF SOLVENT CHECKS

Solvent[a]	Number of eggs[b]	
	Bark extract[c]	Solvent check
Filter paper–untreated		12
Hexane	12	4
Petroleum ether	32	2
Chloroform	23	15
Ethyl acetate	240	9
Acetone	84	9
Methanol	51	17
Water	15	2

[a] Presented in order of increasing polarity.
[b] Number of eggs deposited by 10 gravid female PTB during a 24-hr test period.
[c] Based on 36 g of bark homogenized in 150 ml solvent, filtered, and evaporated on filter paper wick.

TABLE 2. NUMBER OF PEACHTREE BORER (PTB) EGGS DEPOSITED ON FILTER PAPER WICKS IMPREGNATED WITH SOLVENT EXTRACTS OF PTB COCOONS AND WITH RESIDUES OF SOLVENT CHECKS

Solvent[a]	Number of eggs[b]			
	Test 1		Test 2	
	Cocoon extract[c]	Solvent check	Cocoon extract	Solvent check
Filter paper–untreated		15		12
Hexane	14	3	19	55
Petroleum ether	22	0	30	38
Diethyl ether	53	5	8	0
Chloroform	16	7	40	31
Ethyl acetate	6	0	14	14
Acetone	189	3	231	15
Methanol	26	18	125	18
Water	0	10	4	7

[a] Presented in order of increasing polarity.
[b] Number of eggs deposited by 10 gravid female PTB during 24-hr test periods (Test 1, Oct. 23; Test 2, Oct. 30).
[c] Based on 20 g of PTB cocoons extracted in 100 ml solvent, filtered, and evaporated on filter paper wick.

indicated that the substances had similar physical properties despite the differences in solvent polarity.

The oviposition stimulant in cocoons appeared to elute in the liquid chromatography with the roughly 50:50 chloroform–methanol blend (Table 3). The reduction of total numbers of eggs layered was attributed to dilution of the attractant substance due to chromatography. Examination of the active fraction by TLC indicated the presence of three coral bands, which migrated closely (R_f = 0.03, 0.07, and 0.13) and were unique to that fraction. The same coral bands were present in biologically active bark extracts and gum and frass mixtures.

The 30% acetonitrile fraction of the acetone extract of the gum and frass mixture had the greatest biological activity of the separated fractions (Table 4). Active fractions from bark, cocoons, and gum and frass mixtures appear to have a common substance(s), as demonstrated by bioassay and TLC, which stimulates ovipositing of female PTB.

Efforts are now underway to purify and characterize the compound or compounds associated with the observed oviposition stimulant of PTB. The isolation and identification of the chemical or combination of chemicals in peach bark, cocoons, or from gum and frass mixtures could provide useful

TABLE 3. BIOLOGICAL ACTIVITY AND THIN-LAYER CHROMATOGRAPHY (TLC) PROPERTIES OF ACETONE EXTRACTS OF PEACHTREE BORER (PTB) COCOONS FRACTIONATED BY LIQUID CHROMATOGRAPHY WITH CHLOROFORM-METHANOL GRADIENT

Gradient characteristics		Properties of PTB cocoon fractions	
Fraction number[a]	Methanol (%)	Activity (no. eggs)[b]	TLC separation bands: R_f value and color[c]
0-15	0	0	0
15-19	5	0	0
35-39	12	0	0
55-59	20	2	0
75-80	27	2	0
95-99	34	3	0
115-119	41	3	0.30 sepia, 0.38 grey, 0.67 brick
135-139	48	3	0.13 gray, 0.21 peach, 0.37 saffron, 0.68 pink
155-159	55	17	0.03 coral, 0.07 coral, 0.13 coral, 0.37 saffron
175-179	62	1	0.03 coral[d]
195-199	70	1	0
215-219	77	1	0
235-239	84	0	0
255-259	91	1	0
275-280	100	0	0

[a] Ten-ml aliquots.
[b] Based on number of eggs deposited by 10 gravid female PTB on filter paper wicks impregnated with specified fractions during a 24-hr period.
[c] Developing system composed of toluene-ethyl acetate-formic acid (5:4:1 v/v/v). Developed plates sprayed with ethanolic H_2SO_4 and charred at 150°C.
[d] Trace.

TABLE 4. NUMBER OF PEACHTREE BORER (PTB) EGGS DEPOSTED ON FILTER PAPER WICKS IMPREGNATED WITH SOLVENT EXTRACTS OF FRACTIONS OF ACETONE EXTRACT OF GUM AND FRASS MIXTURES

Fraction[a]	Number of eggs[b]
Water	36
30% acetonitrile	70
100% acetonitrile	48
Acetone wash	18
Acetone extract of cocoons	14
Check	11

[a] Fractions obtained from crude acetone extract of gum and frass as described in Methods and Materials.
[b] Number of eggs deposited by 8 gravid female PTB during 24-hr test period.

tools for monitoring and perhaps aid peach breeders in developing cultivars resistant to the PTB.

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SCENT MARKS AS SOCIAL SIGNALS IN *Galago crassicaudatus* I. SEX AND REPRODUCTIVE STATUS AS FACTORS IN SIGNALS AND RESPONSES

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Abstract—In simultaneous presentations of the scent marks of male and female conspecifics, thick-tailed galagos (*Galago crassicaudatus*) of both sexes responded most strongly to the female scent. Males differentiated between the scents most clearly in their sniffing, females in their scent marking in response. The scent of estrus females elicited increased licking of the scent by both sexes. This greater response to female scent and the contrasts in response patterns between the sexes are discussed and related to galago social and spatial relationships in the wild.

Key Words—Primates, *Galago crassicaudatus*, galago, prosimian, scent marking, social organization, chemical communication.

INTRODUCTION

Galagos, a family of small, nocturnal African prosimians, share with many other mammals areas of cutaneous gland development and a variety of scent-marking behaviors. This paper reports the results of a laboratory study of the information on gender and reproductive condition contained in the scent marks of *Galago crassicaudatus* and the effects of these factors on galagos' responses to scent.

The importance of olfactory signals in the reproductive and social behavior of mammals has been extensively reviewed (e.g., Cheal and Sprott, 1971; Eisenberg and Kleiman, 1972; Epple, 1976; Johnson, 1973; Mykytowycz, 1972; Ralls, 1971; Thiessen and Rice, 1976). Scent marking has critical communicatory functions not only among nocturnal species, but in many visually oriented mammals such as marmosets (Epple, 1970, 1972, 1974)

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and pronghorn antelope (Müller-Schwarze and Müller-Schwarze, 1972). Mammals with multiple sources of scent are particularly interesting because potentially they produce distinct scents chemically specialized for different messages, as demonstrated in the dwarf mongoose (Rasa, 1973) and black-tailed deer (Müller-Schwarze, 1971).

Galago crassicaudatus, the thick-tailed galago, is the largest member of the family Galagidae (Prosimii) and has the greatest elaboration of scent sources of any galago species. These sources include chest glands, scrotal glands (or circumlabial) glands, facial glands, and urine. Galagos in both captivity and the wild deposit secretions from these sources using recognizable and consistent patterns of behavior. (For complete descriptions, see Bearder and Doyle, 1974; Clark, 1975.) Histological description of some of the glandular areas is given in Montagna and Yun (1962) and Dixson (1976). The volatile components of the chest gland secretion have recently been identified (Wheeler et al., 1977; Crewe et al., 1979). These components elicit sniffing when presented to galagos as a mixture, but not as individual compounds (Katsir and Crewe, 1980). Even more salient for this study, relative proportions of the compounds differed in a male and a female sample, and Crewe et al. (1979) suggest that a consistent sexual dimorphism might exist. A summary of the sources, their secretions, and associated marking behavior is presented in Table 1.

Field study indicates that, contrary to earlier descriptions, this arboreal prosimian is neither strictly territorial nor solitary. Rather, individuals associate on a nightly basis with other individuals which share all or part of their home ranges (Bearder, 1975; Bearder and Doyle, 1974; Clark, 1978a, 1980). Compared with other galago species (Charles-Dominique, 1971, 1974, 1977, Bearder, 1969; Bearder and Doyle, 1974), *G. crassicaudatus* appears to interact with conspecifics more frequently and to spend more time in contact with them (Harcourt, 1980).

The function or utility of scent marking in the coordination of this social system can be appreciated only when the signalling patterns, the effects of the signals on the receivers, and the information on which those effects are based are known. In this study, I investigated gender and reproductive condition as information present in scent marks and as factors in the response to scent marks. Results of a study of individual characteristics of scent and possible specialization of scent sources are presented elsewhere (Clark, 1975, 1982).

METHODS AND MATERIALS

Subjects. Eighteen *Galago crassicaudatus argentatus* (as identified by Buettner-Janusch, 1964), of the prosimian collection at Duke University's Center for the Study of Primate Biology and History, were the subjects of this study. Table 2 presents pertinent data on the individuals. It should be noted

TABLE 1. SCENT MARKING AND SCENT SOURCES OF *Galago crassicaudatus*

Marking behavior	Scent source	Histological/chemical characteristics[b]	Substrate[c]	Description of behavior
Urine washing (UW)	Urine	Variable composition; UV fluorescent	All surfaces and angles of branches	Urine deposited on palm head under urogenital opening then rubbed on sole of foot; often repeated on other side
Chest rubbing (CR)	Chest gland over clavo-sternal area	Sebaceous and apocrine, yellow, UV fluorescent; main volatile components: benzyl cyanide; 2-(*p*-hydroxyphenyl)ethanol; *p*-hydroxybenzyl cyanide	Usually 45° - vertical branches	Chest lowered to substrate, gland pressed down forward in "glide"
Anogenital rubbing (AR)	Scrotal glands, circumlabial glands	Sebaceous and apocrine; oily, yellow, UV fluorescent	All glands of branches	Hindquarters lowered to substrate and dragged forward using forelegs
Face rubbing (FR)	Glands on face, muzzle	Apocrine; *not* UV fluorescent	Small branches, especially the ends of points of objects	Side of face and area around lips rubbed forward against the object
Hind foot rubbing[a] (HFR)	Patch of cornified epithelium along tibial margin of plantar surface of foot	Aglandular; ??-dried, adherent urine	All surfaces	One foot rotated laterally bringing patch into contact with substrate; foot rubbed rapidly back and forth. On rough surface, makes scraping sound. Often repeated with opposite foot

[a] Provisionally classified as "scent-marking" since it occurs with UW and CR frequently; may have an auditory signal component (Clark, 1975).
[b] Clark, 1975; Crewe et al., 1979; Dixson, 1976; Montagna and Yun, 1962; Wheeler et al., 1977.
[c] Based on Clark, 1975, and unpublished observations.

TABLE 2. GALAGOS USED IN STUDY

Name[a]	Age[b]	Relation to/among donor galagos
Male test galagos		
Minos (150)	⩾ 5 yr.[c]	father-Phaedra
Hector II (780)	4 yr. 10 mos.	sib-Niobe
Orestes (113)	5 yr. 8 mos.	half-sib - Niobe
P.K. (728)	2 yr. 5 mos.	unrelated
Isis (736)	1 yr. 7 mos.	unrelated
Female test galagos		
Electra (114)	5 yr. 8 mos.	half-sib - Niobe
Cassandra II (721)	4 yr. 10 mos.	sib-Niobe
Iphegenia (111)	> 8 yr.[c]	unrelated
Usiku (715)	> 7 yr.[c]	unrelated
Zola (731)	11 mos.	daughter-Niobe
Female donor pairs		
Nova (708)	> 8 yr.[c]	mother-Charybdis, Bossa, Yeats
Niobe (718)	5 yr. 8 mos.	half-sib - Moja
Phaedra (152)	3 yr. 9 mos.	paternal half-sib - Yeats
Cressida (109)	7 yr. 9 mos.	unrelated
Male donor pairs		
Moja (732)	1 yr. 8 mos.	half-sib - Niobe, half-sib - Bossa
Charybdis (730)	1 yr. 11 mos.	son - Nova
Bossa (725)	3 yr.	son - Nova, ?half-sib - Moja, full?-sib - Charybdis
Yeats	7 mos.	son - Nova, half-sib - Bossa, Charybdis

[a] Official Duke University CSPBH names and numbers.
[b] As of beginning of study.
[c] Wild-caught.

that this large black or silver-grey subspecies and all wild populations thus far studied belong to the larger and coarser of the two subspecies groups or types (personal observation; T. Olson, 1979, personal communication; G. Contrafatti, Personal communication on karyology). The other, smaller type is distinct in morphology, reproductive characteristics, and karyology (De Boer, 1973; Clark, 1975; Patzor, 1976; Patzor and Van Horn, 1977) and probably warrants separate specific status (Dixson and Van Horn, 1977; Eaglen and Simons, 1980), although the nomenclature is still under dispute (e.g., Olson, 1980). Descriptions of social organization and the specific behavioral results, e.g., scent-marking frequencies reported here, should be considered characteristic only of the large type.

Five male and five female galagos served as scent recipients or test galagos. The other eight galagos, four of each sex, were scent donors. Lack of

enough suitable adults made it necessary to include three subadult individuals (less than 20 months old at the beginning of the experiments—see Table 2). One test galago was pregnant throughout her tests.

Donors were housed in same-sex pairs (see below) in a room separate from the test animals. Prior to the experiments, donors and test animals had been in the same room and with a variety of cagemates. With one exception (Cassandra II and P.K.), no donors and test animals had been cagemates immediately before the experiments. Test animals were kept in individual cages (approximately 66 × 66 × 88 cm) all in one room. Rooms were lit on a natural light cycle adjusted biweekly to local sunrise and sunset.

Design of Experiments. For each 15-min trial, one male-scent-marked plastic perch and one female-scent-marked perch were presented simultaneously, as a pair, to a test galago. Its responses to each perch in the 15-min period following presentation were recorded on an Esterline-Angus Event Recorder. In addition, trials using two male-marked perches or two female-marked perches were carried out as controls.

Responses recorded were: the frequency and duration of sniffing on each perch; the frequency of licking on each; frequency of all scent-marking behaviors (urine washing, chest rubbing, face rubbing, anogenital rubbing, hind foot rubbing) occurring during or immediately after contact with each perch.

A sniff was defined as the period of time during which a galago's nose was on or almost on the perch and inspirations could be heard and/or seen (nostril movement). (Because biting perches could be a form of olfactory sampling, "biting" was included in "sniffing.") A lick was recorded each time the galago's tongue was observed to contact the perch.

The eight donors were divided into four same-sex pairs (Table 2). Pairs rather than single individuals were used to decrease any possible effect of preferences for individual's scents. Although it was impossible to know which of a pair marked most on a given perch, both members of all pairs were observed to mark. Perches were arranged in donor cages so that donors had to walk over them. At the very least, urine adhering to their feet from urine washing was transferred to the perches. Thus, a male-marked perch carried the scent marks of two males, and a female-marked perch the marks of two females. Throughout this paper, "male perch" and "female perch" will refer to perches carrying the combined scent of two animals.

All but one of the test galagos received ten test trials with each of three male perch versus female perch combinations (Phaedra/Cressida vs. Moja/Charybdis; Nova/Niobe vs. Moja/Charybdis; Nova/Niobe vs. Bossa/Yeats; see Table 2) and five trials with each of the two control combinations (male perch versus male perch, female perch versus female perch) for a total of 40 trials. The pregnant female gave birth during the study and was removed after completing only 18 test trials. To minimize any response variation among test galagos due to changes in the reproductive condition of female

donors, each set of ten trials with a given donor combination was completed in two nights. All males were tested the first night, all females the second. The control trial sets were randomly interspersed among test trial sets. Every fifth night, no trials were run in order to reduce boredom and habituation effects on the test animals.

Throughout the trials, reproductive condition of female donor and test galagos was monitored (see below). Each female was characterized as to her stage in the estrous cycle for all trials.

Scent Collection. All scents presented to test animals consisted of freely laid-down scent marks in whatever combination scent donors happened to use. Plastic golf tubes (88.0 cm long, approximately 3.7 cm diameter; made of virgin, lineal polyethylene) were used as scented perches. Each tube was inserted into a donor's cage through the front wire to rest on the shelf at the back. Perches were handled only by myself or one other person, and only by the ring end of the tube which was never inside either donor or test animals' cages. The perches were inserted at an angle of about 30° from horizontal to provide a substrate for all kinds of marking (see Table 1). All types of marking were observed during the experiments.

Perches to be marked were left in donor cages a minimum of one-half hour during the galagos' active period and a maximum of 48 hr. Perches were removed minutes before they were used. Thus, most perches were freshly marked, but older accumulated scent marks were often present as well. Obviously, the amount of scent could influence the response. All urine and glandular secretions fluoresced bright yellow–white under UV light, chest and anogenital secretions usually more intensely than urine. Therefore, as an indication of the presence and relative amounts of scent on the perches (Clark, 1975), each perch was examined under long-wave UV light with a Blak-Ray lamp (UVL-S6, Ultraviolet Products, San Gabriel, California). The fluorescent marks were described as to intensity, distribution (how much of perch covered) and probable source (urine vs. glands). The more intense the fluorescence, the more scent was assumed to be present. Urine typically appeared as droplets or footprints, chest and anogenital secretions as "smudges." Marking on the two perches to be presented together was compared using subjective categories "equal," "greater than," "much greater than." If a perch was not obviously marked, it was returned to the donor cage and another perch was used.

The perches were new at the beginning of the experiments. They were reused after: (1) thorough cleaning with very hot water and then a detergent or an iodine-base surgical scrub, (2) thorough rinsing, and (3) air drying. Perches were not reused for at least a week. They were wiped with alcohol just before reuse. Such cleaned perches elicited no more interest than a new perch.

Scent Presentation and Test Protocol. Experimental trials were done under infrared light, after the galagos' nightly activity had begun. With rare exception, all testing ended before midnight when galagos often became less

active for several hours. Testing, like scent collection, was done in the home cages. This minimized disturbance and handling. It also provided a situation comparable to that in the wild in which an animal encounters scent marks in its own range. During a trial, the cage was placed between a wall and screen to further minimize disturbance. The observer inserted the perches into the front of the cage in full view of the galago. The perches were positioned as they had been in the donor cage. Then the observer stepped around the screen to watch through a small window. After the initial tests, galagos paid little if any attention to the observer and usually went directly to the perches. To control for any effect of which perch was closer to the observer, however, the two perches' positions were randomized.

Reproductive Condition Determination. Changes in vaginal epithelium resulting from the cyclical hormonal shifts during the female estrous cycle were followed throughout the experiments using vaginal smears, which serve as estimators for levels of estrogen and progesterone.

Approximately every four days, all females—donors and scent recipients—were hand caught and a swab from the vaginal walls taken with a sterile cotton-tipped applicator. The applicator was then rolled onto a clean glass slide. [Eaton et al. (1973) report a mean cycle length of 44 days including: vaginal estrus, 4.7 days; behavioral/vaginal estrus, 5.8 days; and diestrus, 24 days. In *G. c. argentatus*, unlike most other subspecies for which data are available, the vaginal orifice did not become imperforate during the cycle and smears were obtained during all cycle phases.] The smears were allowed to air-dry overnight, fixed in methyl alcohol, and stained with a Giemsa B stain. Each was then classified as to stage in the estrous cycle using criteria given in Eaton et al. (1973).

Analysis of Results. Eighteen trials in which one or both perches were never contacted were discarded. The remaining 282 test trials (male vs. female) were analyzed for differences in cumulative time per trial spent sniffing the male vs. female perches. The effects of donor sex vs.: (1) individual variability of test galagos' responses; (2) sex of test galago; (3) relative amount of scent on perches; (4) perch position; and (5) reproductive condition of donor and test females on the time spent sniffing, and on the frequencies of licking and all marking responses were analyzed by two-way analysis of variance and/or t tests.

The control trials were analyzed similarly for consistent response differences to specific male or female pairs which, if present, could affect the results of the test trial analysis.

Because of the small number of galagos and the intertrial variability, results were only accepted as significant at 0.025 or less.

In describing the results, I have used "preference" to mean that galagos sniffed more (marked more often, etc.) in response to one of two scents. This does not imply that the greater response was affiliative; aggressive or territorial responses could be involved as well.

RESULTS

Effects of Scent Donor's Sex. Test galagos taken as a group spent more time sniffing the female than the male perches ($\bar{X}$ = 151 sec vs. 87 sec, respectively; $P < 0.001$, 2-tail paired t test). Between-trial and between-animal variability in time spent investigating perches was high and independent of how heavily they were marked.

As individuals, all but one galago spent significantly more time sniffing female perches. For all males, this result was highly significant ($P < 0.001$, t test), while the significance level of preferences for four females ranged from $P < 0.02$ to $P < 0.001$. One female, Electra, sniffed the male perch longer in 12 of 29 trials and, thus, showed no overall preference.

Licking, urine washing, anogenital rubbing, and chest rubbing all occurred more frequently on the female perches ($P < 0.001$, $DF = 1, 539$).

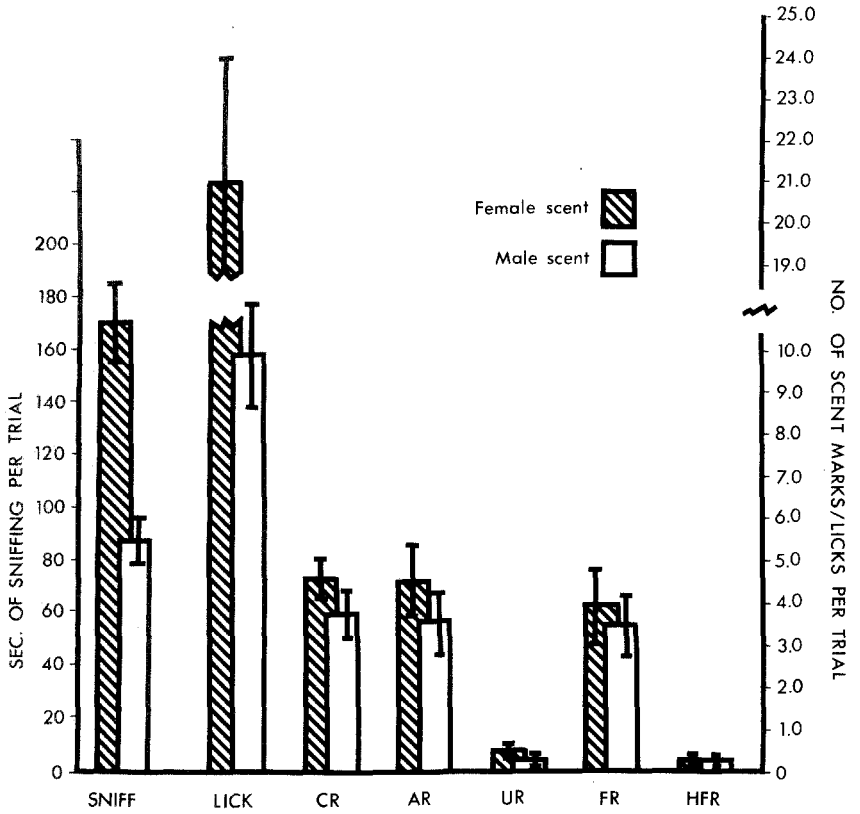

FIG. 1. Responses of male test galagos to male versus female perches ($\bar{X} \pm$ SEM; $N = 282$). CR, chest rub; AR anogenital rub; UR, urine wash; FR, face rub; HFR, hind foot rub.

There was little evidence for differential distribution of face rubbing and hind foot rubbing ($P < 0.05$, $DF = 1, 539$ and $P < 0.1$, $DF = 1, 538$, respectively).

Effect of Test Galago's Sex. Males' vs. females' responses are summarized in Figures 1 and 2, respectively. Males spent more time sniffing female perches more than did females ($\bar{X} = 130$ sec vs. 170 sec; $P = 0.001$, t test). The sexes did not differ in their mean sniffing times on male perches ($\bar{X} = 87$ sec vs. 85 sec). Females were more variable than males in the degree of their sniffing preference.

Females anogenital rubbed, hind foot rubbed, and face rubbed more frequently than did males during the trials ($P < 0.001$, $P < 0.025$, $P < 0.001$, $DF = 1, 555$ respectively). Differences in the overall frequencies of licking, urine washing, and chest rubbing by male vs. female test galagos were not significant. Females did, however, show a statistically stronger bias toward female perches in their urine washing ($P < 0.001$ vs. 0.01, paired t test) and

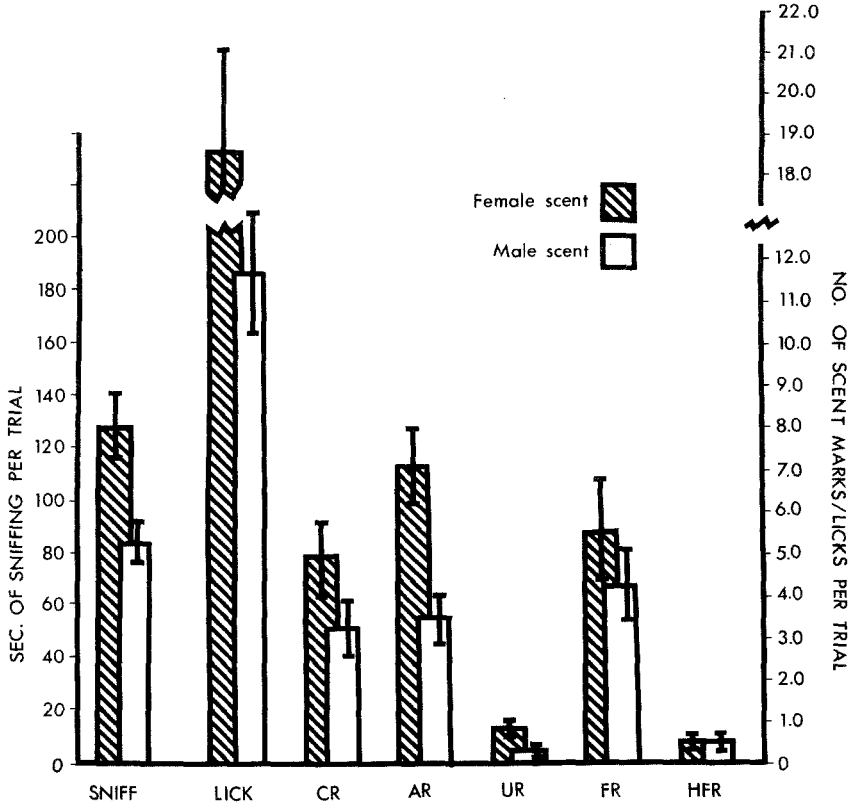

FIG. 2. Responses of female test galagos to male versus female perches ($\bar{X} \pm$ SEM; $N = 282$). Abbreviations as in Figure 1.

anogenital rubbing ($P < 0.001$ vs. 0.05, paired t test) than did males. While females also chest rubbed more frequently on female perches ($P < 0.001$, paired t test), males as a group did not. One male, Minos, actually marked in all ways more frequently on male perches.

Effect of Reproductive Condition of Donor Females. The female scent presented in any given trial was designated by the combination of estrous cycle stages (estrus, diestrus, mid-diestrus, proestrus) of the two donors at the time. Thus, if one member of the donor pair was in estrus, and the other in proestrus, the scent was estrus/proestrus (E/P). The responses of male and female test galagos to each combination of stages was compared using a two-way analysis of variance. Differences between the length of the stages in the cycle result in an inadequate spread of trials across all stages and small samples. Some results, however, are quite suggestive.

Licking, rather than sniffing, differed most clearly between scents ($P < 0.001$, $DF = 4, 250$). Test animals of both sexes licked the scent of estrus/diestrus donors more than those of either proestrus or diestrus donors (Figure 3). The contrasts between sniffing and licking (Figures 3 and 4) is

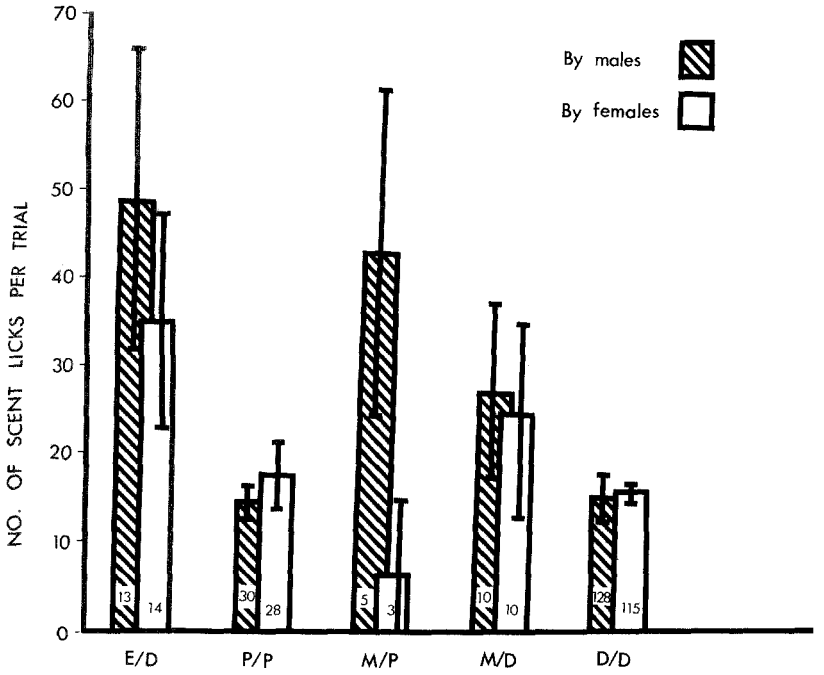

FIG. 3. Mean licking responses per trial to female scent when donors were in different stages of the estrus cycle. Sample sizes are shown on each bar. E/D, P/P, etc., indicate cycle stages of two females contributing scent. E, estrus; P, proestrus; D, diestrus; M, mid-diestrus.

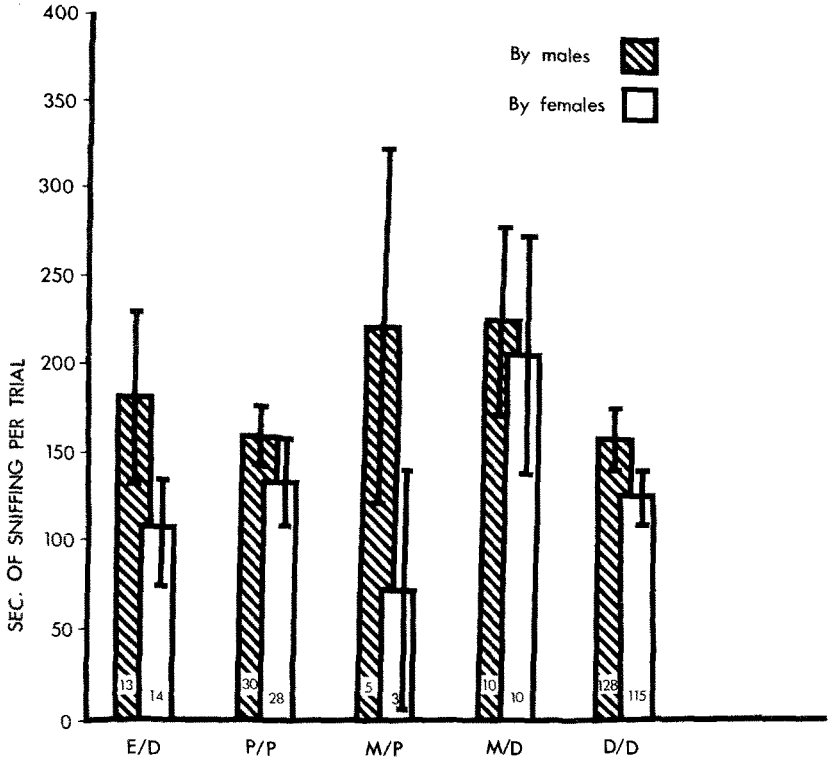

FIG. 4. Mean sniffing per trial in response to female scent when donors were in different stages of the estrus cycle. Sample sizes are shown on each bar. (See abbreviations in figure 3.)

notable since sniffing and licking responses varied in parallel in all other comparisons in this study.

The results for marking were equivocal, with no differences in hind foot rubbing or anogenital rubbing and only marginal differences in chest rubbing ($P < 0.05$, $DF = 4, 250$) between scents. Urine washing and face rubbing varied significantly among different stages ($P < 0.025$, and $P < 0.01$, $DF = 4, 250$) with urine washing highest and face rubbing lowest on estrus/diestrus-scented perches.

Effect of Reproductive Condition of Test Females. Females' trials were grouped by the cycle stage of the female test galago. The response to male and female perches for each stage was explored using a two-way analysis of variance. Sniffing and chest rubbing differed significantly between stages ($P < 0.01$; $P < 0.025$, $DF = 3, 224$). Urine washing, anogenital rubbing, and face rubbing did not. Pairwise comparisons of the four stages with respect of sniffing, licking, and chest rubbing showed only one significant difference: licking by diestrus females on both male and female perches was more

frequent than licking by proestrus females ($P < 0.025$, t test).

Three of the five females were tested during all four stages of their cycle. No differences in their sniffing scores at each stage were found nor was variation in degree or direction of perference with the cycle suggested. Chest rubbing did differ among stages ($P < 0.01$, $DF = 3, 74$) but the small number of trials in estrus, proestrus, or mid-diestrus precluded further statistical analysis. Qualitatively, two of the three galagos chest rubbed distinctly more often when in estrus, and the third not quite as often in estrus as in diestrus. All three chest rubbed least during proestrus.

Throughout the study, the pregnant female had the lowest mean sniffing scores of all test galagos for male, female, and all perches taken together.

Effect of Perch Position. The sniffing scores for each test animal were grouped according to the position of the perch (closer or farther from the observer). There was no significant difference in sniffing between the positions in either the set of control trials or in test trials. Responses to a perch were therefore not influenced by its location.

Effect of Relative Amount of Scent. Trials were grouped according to my estimate of the relative amount of marking on each of a simultaneously presented pair of perches (see Methods and Materials). The duration of sniffing on the female perch compared to the male perch did not differ between trials in which the male perch was marked less and those in which the male perch was marked more than the female perch for any test galago.

Effect of Specific Donor Pairs. It was possible that donors differed in some unspecified quality of their scents and that particular donor pairs were more or less attractive than others. Presenting the least attractive male pair with the most attractive female pair or vice versa could seriously alter at least the degree of differential response to the two sexes. Furthermore, strong individual preferences among test galagos for particular donor pairs might contribute to the individual variation in responses. Two-way analysis of variance of differences in the mean sniffing of the two female donor pairs, and of the two male donor pairs, for the group of test galagos was performed.

When sniffing of female perches during both control and test trials was considered, no preference for particular donors was detected. In control trials alone, female donors Nova/Niobe may have elicited more sniffing than female donors Phaedra/Cressida ($P < 0.025$, $DF = 1, 76$), but the highly significant interindividual differences preclude this generalization. In both the total and control trial analysis of male donor pairs, test galagos sniffed perches of Moja/Charybdis more than those of Bossa/Yeats ($P < 0.005$, $DF = 1, 358$; $P < 0.001$, $DF = 1, 76$). Individual variation was also significant, but there were no exceptions among the test galagos in direction of preference.

These results suggest that in test trials pairing P/C (the less-preferred female pair) with M/C (the more-preferred male pair), the difference between the sniffing durations on the perches might be smaller than when N/N was

paired with M/C. No such difference was found, however. Similarly, no difference was found between trials pairing B/Y with N/N and those pairing M/C with N/N. There is therefore no evidence that preferences for individual donor pairs overrode or even influenced the direction and magnitude of the observed preference for female over male scent.

DISCUSSION

Experimental Conditions and Basis for Interpretation of Results. In these experiments, scent marks of unfamiliar individuals were introduced into the home cage of the test galago. The alternative procedure of presenting strange scent marks in a "neutral" situation confounds the interpretation of responses, since it is difficult to imagine an analogous situation for wild galagos. Without arguing that the kind and degree of response in caged animals is precisely that of wild animals, I feel the situation provided some approximation to conditions under which scent marks are used meaningfully in the wild. Consistent patterns in laboratory experiments can thus indicate how scent marks may affect behavior in the field. Field observations on male and female ranging patterns and relationships within several galago populations in South Africa, made both before and subsequent to these experiments (Bearder, 1975; Bearder and Doyle, 1974; Clark, 1978a,b, 1980, 1982) have provided a basis for inference, and are briefly summarized here.

Wild *Galago crassicaudatus* live in systems of overlapping home ranges, none of which is exclusive of galagos of the same sex. Adult males' ranges are large, overlapping those of adult females and younger individuals of both sexes. In comparison, ranges of adult females are smaller and appear more stable. They are likely to be shared in part with mothers, daughters, or sisters (Clark, 1978b). Particular females may exclude each other territorially on an individual basis.

All types of scent marking observed during these experiments were also regularly observed during the field studies. The branch angles preferred by wild animals conform to those given in Table 1 (personal observation). Both males and females scent mark in the wild as in the lab, with males more frequently chest rubbing than females (Bearder, 1975; Clark, 1980). Prior to marking, which, again excepting urine washing, occurs repeatedly in particular trees, galagos sniff actively.

Where population density is very low, or there are physical barriers separating female ranges, apparently stable associations between an adult male and female over periods of two years have been observed (Bearder, 1974; Clark, 1978a, 1982). Where a large number of animals use an area, however, females associate and mate with more than one male, and males mate with several females. Breeding is reported to be seasonal throughout this species' distribution (Bearder, 1975; Haddow and Ellice, 1964; Kingdon, 1971).

Patterns of Response to Scent Marks in Galago. The consistently and

significantly longer investigation of female-marked perches indicates that galagos easily discriminate the scent marks of females from those of males. Discrimination occurs even when donors are equally unfamiliar and donor females are not in estrus. Males and females do, however, respond differently to the scent marks, probably relecting a dichotomy in the import of male vs. female scent.

Preferential sniffing of female perches was more pronounced in males than in females. One functional explanation for their interest may be reproductive synchronization of potential mates through olfactory cues, as has been shown or suggested for many other seasonally breeding mammals (see Doty, 1976, for pertinent articles) including primates (Epple, 1976; Harrington, 1975; Jolly, 1967; Schilling, 1979).

Wild female galagos as well as females in stable, captive groups decreased their scent marking when in estrus (Clark, unpublished data; 1975), just as captive lion tamarins (*Leontopithecus rosalia*) are reported to do (Kleiman, 1978). Similarly, male black-tailed deer (*Odocoileus hemiones*) show flehmen to a female more frequently before than during her estrus (Müller-Schwarze, 1979). Thus, in galagos and some other mammals, pertinent olfactory cues from females are released before estrus, as might be expected of stimulatory signals, and males would be expected to show great interest in the scents of nonestrous females.

It should be noted here that the increased licking, as opposed to sniffing, that distinguished the response to estrous donor scent strongly suggests the preferential use of vomeronasal receptors for sampling reproductive information. As in other prosimians, New World monkeys, and many other mammals, *G. crassicaudatus* has a functional vomeronasal organ (Stephan, 1965). It opens into the nasopalatine canal. Chemicals carried on the tongue would be likely to reach the organ. Estes (1972) argued and reviewed the evidence for an important role of vomeronasal reception in detection of reproductive information among mammals. Since then, experimental results with rodents have given increasing support to this hypothesis of specialized function (Powers and Winans, 1975; Winans and Powers, 1977; Fleming et al., 1979). A prosimian's probable use of the vomeronasal organ has been demonstrated for *Lemur catta* (Bailey, 1978).

Competition for resources may be the strongest reason for females' strong response to female odor. The size and shape of female ranges seem to be based on the distribution of limited resources necessary for successful rearing of young (Clark, 1978b). Strange females could be detrimental competitors for food, should they remain. Indeed, aggression between certain females can be intense, especially between older females and unrelated subadults or young adults. Males, in contrast, forage over wider areas than females. They seek out females rather than the reverse. Females might thus be expected to pay more attention to strange female scent than male scent at any time of year.

I have argued that female competition for space, and males' maintaining

associations and physiological synchrony with reproductive females in the range are possible factors making the female scent most critically informative. I now consider the marking responses of each sex.

Of the five types of marking given in response to test perches, three—urine washing, chest rubbing, and anogenital rubbing—were differentially distributed on male and female perches. Hind foot rubbing was connected with general excitement and alerting to strange noises during the trials; face rubbing could serve to collect scent as easily as deposit it. Neither will be discussed further. Urine washing occurs in a variety of nonsocial and social contexts. In other experiments, little interest was shown in urine alone (Clark, 1975; 1982). In the field, it is distributed according to where galagos spend time (Clark, 1980) and may have been so in these experiments.

Females' chest rubbing and anogenital rubbing in response to female scent supports the inference that they were identifying potential competitors. In both captive groups (Clark, 1975; Sauer, 1974) and wild animals (Bearder, 1975; personal observation) the more dominant adults, and the aggressors in agonistic situations, chest rub more frequently than subordinates or "losers."

Males, on the other hand, distributed their marks between male- and female-marked perches more equally than did females. This could reflect both a male's animosity to strange males and, as has been suggested for the marking over, "endorsement," of female scent by male sifaka *Propithecus verreauxi* (Richard, 1974) and golden pottos *Arctocebus calabarensis* (Manley, 1974), the establishment of his presence and association with a female in his home range.

Large individual differences in all the above responses are to be expected of animals which are capable of modifying their behavior on the basis of past experiences. As mentioned, galago sociality appears to be based largely on individual relationships. Idiosyncratic responses by captive animals may result both from age and from experiences which I could neither define nor separate.

Mammalian Scent Marking in a Social Context. Elicitation of a greater response by female scent from galagos contrasts with other mammalian species in which, if one sex were preferred overall, it was the male scent. Reindeer *Rangifer t. tarandus* (interdigital gland scent, Müller-Schwarze et al., 1978), brown bears *Ursus arctos* (Tschanz et al., 1979) and probably collared peccaries *Dicotyles tajacu* (Sowls, 1974) respond most strongly to odors of their same sex conspecifics. Similarly, introduction of strange animals into groups of duikers (*Lephalophus maxwelli*) or common marmosets (*Callithrix jacchus*) elicited increased marking from the same-sex dominant members of the group (Ralls, 1974; Epple, 1970). Male brown lemurs *Lemur fulvus* investigate male scent more than female, at least outside the breeding season (Harrington, 1977). Females were not tested. Female mule deer *Odocoileus hemiones* (Müller-Schwarze, 1971) licked male tarsal scent more than did males. Finally, Epple's extensive studies of scent marking

in saddle-backed tamarins *Saguinus fuscicollis* have shown that both males and females investigate and scent mark on male scent more than on female, but males to a lesser degree and less consistently than females.

I suggest that thick-tailed galagos differ because their social and spatial relations rest heavily on the spatial organization of the small, stable female home ranges, as opposed to spacing of exclusive individual or group territories. The interest in and marking of female scent will tend to produce areas in the ranges of adult females where, due to those females' regular scent deposition, many other galagos will also regularly visit and mark. Centers of olfactory information exchange could develop. Indeed, such centers, consisting of a few, large contiguous trees, were noted during the field study (Clark, 1980). These also served as regular meeting and socializing points for all galagos whose ranges included the center. Presumably any transient individual would be able to determine the concentration of males and females using the scent marks present, even when the residents were elsewhere.

In conclusion, this study demonstrates that both male and female galagos can discriminate the sex of conspecifics by their scent marks. Both males and females investigate female scent in preference to male, but degrees of preference and patterns of marking in response vary with the sex of the responder. The results, when placed in the context of field observations and compared with similar data for other mammals, suggest that interspecific contrasts in male and female responses to scent marks may reflect interspecific differences in the bases for social and spatial organization.

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SCENT MARKS AS SOCIAL SIGNALS IN *Galago crassicaudatus* II. DISCRIMINATION BETWEEN INDIVIDUALS BY SCENT

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Abstract—Thick-tailed galagos, *Galago crassicaudatus argentatus*, which had been habituated to the scent marks of a conspecific through repeated presentations, increased their sniffing when presented with scent marks from a second conspecific of the same sex. Thus, they discriminated between the scents of individual conspecifics. This result was obtained using naturally scent-marked perches and perches carrying only chest gland scent—the latter even two weeks after the marking. When urine, the prevalent scent signal among most other galago species, was tested, it elicited little interest and discriminations were not clearly made. It is suggested that the use of more specialized scents is related to the social characteristics of *Galago crassicaudatus*.

Key Words—Chemical communication, galago, *Galago crassicaudatus*, individual discrimination, primates, prosimians, scent glands, social organization.

INTRODUCTION

Individual recognition and its effects of behavior may be the single major basis for structuring mammalian and avian social relations. Its importance is easily appreciated in dominance relations, mother–offspring recognition, kin-directed behavior, and mate recognition, as familiar examples. The ability to discriminate between the scents of conspecifics has been demonstrated in a wide variety of mammals including flying phalangers *Petaurus breviceps* (Schultze-Westrum, 1965, 1969), brown lemurs *Lemur fulvus* (Harrington, 1974, 1976), ring-tailed lemurs *Lemur catta* (Mertl, 1975), tamarins *Saguinus fuscicollis* (Epple, 1973, 1978), black-tailed deer *Odocoileus hemionus*

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(Müller-Schwarze, 1971), dwarf mongooses *Helogale undulatus* (Rasa, 1973), common mongooses *Helogale auropunctatus* (Gorman, 1976, 1979) and possibly, to a limited degree, even man (Russell, 1976).

The thick-tailed galago (*Galago crassicaudatus*), like many other prosimian primates (see review by Schilling, 1980), has a number of distinctive scent-marking behaviors by which it deposits urine and secretions of cutaneous glands of the chest, scrotal or circumlabial, and possibly muzzle areas. The characteristics of the scent sources and marking behaviors are described elsewhere (Andrew, 1964; Bearder and Doyle, 1974; Clark, 1975, and summarized in Clark, 1982). In this study, individual discrimination on the basis of scent marks by the thick-tailed galago (*G. c. argentatus*) was investigated experimentally.

The social life of this nocturnal, arboreal African prosimian is structured around the highly overlapping home ranges of adults (Bearder, 1975; Bearder and Doyle, 1974; Clark, 1978, 1980) within which the scent marks, except for urine, are concentrated at places of frequent social interaction (Clark, 1980). Thus, scent marks may play an important role in organizing the fluid social relations of this galago. A knowledge of the information that the scent marks carry is essential to understanding their role. I report elsewhere (Clark, 1975, 1982) that galagos can distinguish between and respond differently to the scents of males versus females, and of females in estrus versus females in other cycle stages. The experiments reported here were designed to answer two questions: do thick-tailed galagos detect individual characteristics in a conspecific's scent marks; and is there evidence that individual distinctiveness is not equally present in all the galagos' scent sources?

METHODS AND MATERIALS

Subjects

Sixteen galagos, part of the Duke University Center for the Study of Primate Biology and History collection, were used in these experiments. All belonged to the subspecies *Galago crassicaudatus argentatus*, as used at the Primate Center. This East African subspecies and the South African *G.c. umbrosus* on which all field information is based, are very similar in morphology and reproductive characteristics (Olson, 1979; personal observation), karyotype (G. Contrafatti, personal communication), and choice of habitat (Olson, personal communication). They contrast in these respects with a group of smaller-sized subspecies (see Clark, 1975; Dixson and Van Horn, 1977; Eaglen and Simons, 1980; Olson, 1979; Patzor, 1976; Patzor and Van Horn, 1976, for discussions of important subspecific differences and taxonomic problems). All but one subject (a 7-year-old captive-born female, used

as a test galago) had also been used in the gender discrimination tests (Clark, 1982). Pertinent data on the other individuals is given in Clark (1982, Table 2).

Experimental Design

A modification of the habituation technique used by Schulze-Westrum (1965) for *Petaurus breviceps* and Harrington (1976) for *Lemur fulvus* was used. A test galago in its home cage was presented with a series of perches scent-marked by one galago (donor I), one after another, each for one minute. During the minute, the time spent sniffing the perch, the number of sniffs, licks, and all types of scent marks were recorded. (A sniff was recorded as that period of time when an animal's nose did not move from a few millimeters of that perch and inspiration movements could be seen.) The time between presentations was 30 sec or less. Presentations of donor I scent continued until, for four consecutive perches, the galago's time spent sniffing per presentation was less than a predetermined, low number of seconds. The exact number of seconds, the same for all test animals, was chosen during pretrial runs such that, in all cases, obvious behavioral signs of waning interest closely preceded this level of sniffing. This level was considered to indicate "habituation."

When the habituation criterion has been met, a perch scent-marked by a second galago of the same sex (donor II) was inserted as the next in the series of presentations. Responses were recorded as before. After the donor II perch, a donor I perch was again presented and responses recorded.

If a galago recognized the donor II scent as being of a different individual from donor I, it was expected to show a higher level of interest and an increase in sniffing time relative to donor I habituation perches. Upon immediate representation of donor I scent, interest and sniffing time should again decrease if the galago not only discriminated the scents, but specifically recognized the familiar donor I scent. Thus, the galago could demonstrate both its ability to discriminate two donors' scents and the ability to recall a known scent, at least over a short period of time.

The first two of the four experiments were designed to show that galagos could discriminate the scents of individual conspecifics when those scents were naturally deposited composites of glandular secretions and urine. The two subsequent experiments tested for discrimination on the basis of urine alone and chest gland scent alone. Anogenital gland scent was not tested, because a procedure for collecting it free of urine contamination could not be devised. The composite scent and single-source scent experiments are described separately below.

Composite Scent Tests. Scent was collected in exactly the same way as has been described for the gender discrimination experiments (Clark, 1975, 1982): perches (plastic golf tubes) were placed in donor home cages for at least

half an hour of the active period, then removed, checked for the presence of the fluorescent marks (Clark, 1975) under UV light, and used within the hour following removal from donor cages. All perches used in a given test had been in the donors' cages for the same length of time and were collected at the same time. Except for five trials, all marks had thus been made within an hour or two of each other. Perches were left in 24 hr and 48 hr in three and two tests, respectively.

During the first experiment, ten galagos (five males and five females) were tested for discrimination between two males (once each between Moja and Charybdis, and between Bossa and Yeats) for a total of twenty tests. In the second experiment, one female and two males of the original ten galagos (Cassandra II, Hector II, and Orestes—see Table 2 of Clark, 1982) were chosen as being calm in the test situation and consistently willing to contact the perches. These three were each tested five more times (once or twice with each of the two male vs. male comparisons and one female vs. female comparison) for a total of 15 additional trials. For both experiments, a habituation criterion of 15 sec was used. The results were analyzed using a Wilcoxon matched-pairs signed-rank test for differences in sniffing time, average length of sniffs, and frequencies of marking between (1) the last habituation perch of donor I and the donor II perch; and (2) the donor II perch and the final donor I perch.

Single Scent Tests. Collection of urine and chest gland scent free of other scents proved difficult. In the case of urine, catheterization of females yielded enough urine for only one test. Males' urine was used in all other tests. Male galagos tended to urinate freely when caught. Large amounts of "clean catch" urine were collected by holding the animal at an angle so the urine fell away from the body and into a clean jar. The urine was immediately stored in closed jars in a refrigerator and used the same evening. Cotton swabs were used to apply it to the ends of the perches, after which the perches were examined with UV light to assure a similar area covered and a similar intensity of fluorescence on all.

During the 10–20 days over which their chest gland secretion was collected, the donor galagos wore jackets of nylon web lined with gauze which covered the glandular area and kept it free of urine and all other scents. All but one donor adapted well and wore the jackets without apparent disturbance. For these donors, the glandular area was cleaned with alcohol prior to putting on the jacket. Scent was then collected by removing the jacket and rubbing the ends of the perches on the now-oily chest area while the animal was restrained. In the case of one male galago (Charybdis) who always managed to rip off his jacket overnight, scent was collected by cleaning the glandular area with alcohol, then waiting several minutes for new secretion to appear as tiny droplets over the surface. (Galagos when upset and/or restrained readily produced the yellowish oily secretion.) The ends of the perches were then

rubbed on this fresh secretion. All perches were checked under UV light for the presence of comparable fluorescence. The order of donor I "scent marks," as collected, was then randomized for presentation.

Perches marked with chest gland scent were normally used within 4–6 hr. In five tests, the "scent marks" were 24 hr old and once, 2-week-old perches which had been stored in a closed, air-conditioned room were tested.

For both urine and chest gland trials, galagos' discriminations between two males (Moja vs. Charybdis) and two females (Nova vs. Niobe) were tested. Each of eight galagos (five male, three female—see Table 1) received at least one trial with the chest gland secretions of each pair. Only one (the first) test for each was used in the analysis so as to weight the responses of all test galagos equally. In trials using urine, seven galagos were tested with the urine of the male donor pair. One of the seven was then tested with the female pair on the one occasion when urine was obtained from both females. Analysis was similar to that of composite scent results.

RESULTS

Composite Scent

In the first experiment, galagos sniffed longer at the donor II scent than at the last habituation perch of donor I (19 of 20 trials, $T = 2$, $P < 0.005$, Wilcoxon one-tailed test for all comparisons unless specified). Their sniffing decreased markedly upon re-presentation of donor I scent (16 of 20 trials, $T = 13$, $P < 0.005$). Mean responses and a typical trial is shown in Figure 1.

TABLE 1. DURATION OF SNIFFING (SECONDS: $\bar{X} \pm$ SE) ON PERCHES CARRYING SCENT OF DIFFERENT AGE

Donors	Perch	Age of scent		
		⩽ 6 hr	24–48 hr	2 wk
Females	1st D.I	(6)[b] 28.9 ± 11.0	(5) 22.8 ± 5.1	(1) 39.0
($N = 12$)[a]	Last habit.	(5) 3.4 ± 0.2	(6) 3.9 ± 1.5	(1) 3.2
	D.II	(6) 17.5 ± 3.5	(5) 16.8 ± 3.4	(1) 14.8
	D.I reintro.	(5) 0.6 ± 0.2	(6) 4.3 ± 2.0	(1) 12.0
Males	1st D.I	(7) 24.6 ± 1.8	(2) 14.5 ± 5.5	
($N = 9$)[a]	Last habit.	(7) 2.3 ± 1.3	(2) 1.9 ± 1.9	
	D.II	(7) 7.7 ± 2.9	(2) 8.7 ± 3.4	
	D.I reintro.	(7) 5.0 ± 2.3	(2) 3.5 ± 1.5	

[a]Includes 5 trials not included in Figure 2 which is based on the first trial only for each galago–see text.
[b]Number of trials using perches in each category.

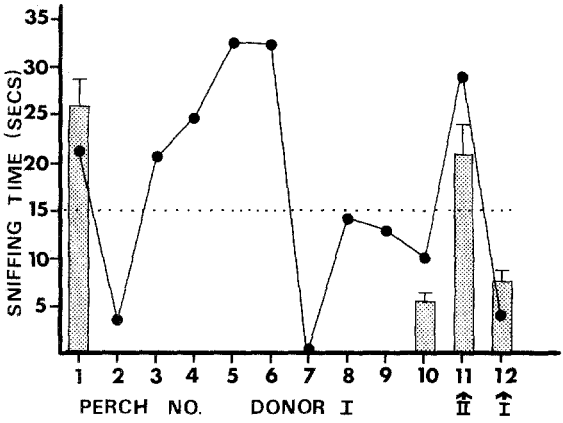

FIG. 1. A typical trial using a naturally marked perch (composite scent) shown against the mean (±SE) responses of 10 test galagos in 20 trials of composite scent. Because the number of perches presented until habituation was reached varied, only the means for perches comparable over all trials are shown. The single trial graphed [Cassandra II responding to donors Charybdis (I) versus Moja (II)] was chosen so that trials of one galago responding to chest gland scent and urine of the same donors could be shown in Figure 2 for comparison (. . . = habituation criterion).

Time spent sniffing did not show a simple decrease with repeated presentations. The first donor I perch was not the perch sniffed most frequently or longest in 23 of the total 42 trials. In four trials, the maximum actually occurred on the donor II perch. Thus, a consistent immediate drop in sniffing upon re-presentation of a donor I perch appears to be explained best by familiarity with the scent rather than by a general tendency to decrease sniffing over successive presentations. The final donor I perch was, however, usually sniffed longer than the last donor I perch of the habituation series ($T = 37$, $0.025 < P < 0.05$, see Figure 1). A new scent appeared to have restimulated interest in sniffing perches.

The length of individual sniffs is a good indicator of interest in or concentration on the scent. The average length of sniffs was calculated as the total time spent sniffing divided by the total number of sniffs. Sniff length increased significantly upon presentation of donor II scent ($T = 14$, $P < 0.005$). As with total sniffing, sniff length on the final donor I perch was greater than that on the last donor I habituation perch ($T = 37$, $P < 0.005$).

Marking activity was low throughout and, in contrast with sniffing, showed no significant change from the last donor I habituation perch to the donor II perch, or from the donor II perch to the final donor I perch.

The additional sets of trials for each of three galagos were entirely in agreement with the above results. As testing continued, those galagos began to

habituate in five to seven presentations rather than the 12–15 required at first, and apparently became tired of the test situation. Nevertheless, all three sniffed longer at donor II scent than at the last donor I habituation perch ($T = 0$, $P = 0.05$ for each individual; $T = 0$, $P < 0.005$ for all 15 trials together). In all but two trials, sniffing time decreased during re-presentation of donor I scent ($T = 4$, $P < 0.005$, for all 15 trials; $T = 0$, $P = 0.05$ for the female and $T = 1$ and 2, NS for the two males). The two exceptional trials are interesting in that they suggest that more than "familiarity" is influencing relative interest in scents of conspecifics. In both these cases, donor II (Yeats) was a much younger male than the donor I male (Bossa) and the test galagos were males (see Table 1). The one other male tested with this donor combination during the 20-trial series also responded with an increase in sniffing upon re-presentation of donor I. The three females tested with this donor combination during the 20-trial series and the one female tested in the 15-trial series all responded with the typical decrease in sniffing upon re-presentation.

I further investigated the apparent age effect by making pairwise comparisons of the magnitude of the increase in sniffing upon presenting donor II when donor II was the older male of this pair (Bossa/Yeats) with the magnitude of the increase when donor II was one of an age-matched pair (Moja/Charybdis) for six galagos tested with both pairs. The magnitude of the increase was significantly greater for Bossa/Yeats ($T = 0$, $P = 0.025$, one-tail).

Single Scents

Chest Gland Scent. Quite different results were obtained for the male and female donor scents. In tests of female scent, sniffing times increased upon presentation of donor II and decreased upon representation of donor I without exception ($T = 0$, $P < 0.005$, one-tail). (For mean responses and an example, see Figure 2a). Marking did not vary in any consistent way.

In tests of male scent, five of the eight galagos increased and decreased their sniffing time as for the female scent. But the other three (all males: Minos, P.K., and Isis) not only showed no discrimination on the basis of sniffing, but also showed no detectable behavioral signs of recognizing a new scent. Their sniffs were perfunctory; their ears did not move forward; attention shifted easily to other events, noises, etc. In all three cases, donor I was Moja and donor II, Charybdis. Statistically, the increase upon presentation of donor II and decrease upon re-presenting donor I was significant for male donors ($T = 4, P < 0.025$ and $T = 1, P < 0.01$, respectively—see Figure 2a).

Responses to and discriminability of individual's scents did not vary with the age of the marks (see Table 1). The male scent marks which were

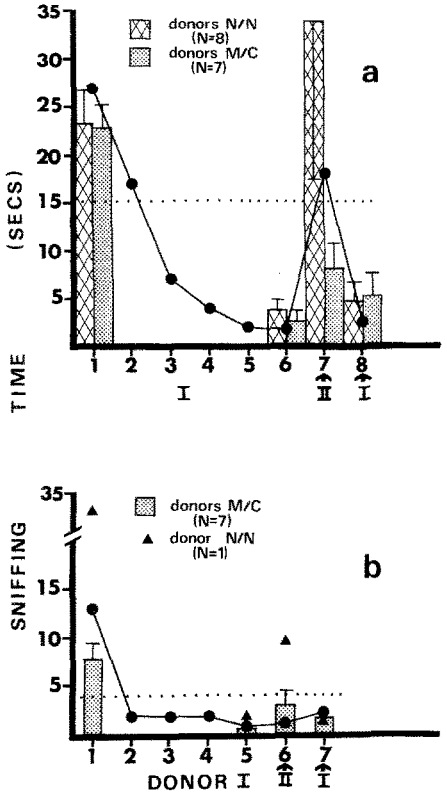

FIG. 2. Typical trials of (a) chest gland scent; (b) urine shown against the mean (±SE) responses of all 8 test galagos. Note that responses to male and female donors are shown separately. Test galago for single trials, Cassandra II; donors, Charybdis (I) versus Moja (II). Compare levels of sniffing with Figure 1 (. . . = habituation criterion).

apparently not differentiated (see above) were 4–6 hr old. In the two tests of male chest gland scent after 48 hr, the increased sniffing of donor II scent was observed. Finally, in the one test using female chest gland scent two weeks old, discrimination was as clear as for other tests and the exact spot of the marks, known to the observer, was "zeroed in on" by the test galago. UV fluorescence was still present.

Urine. Urine excited the least interest of all scents presented, and the habituation criterion had to be lowered accordingly, to 4 sec (compare Figure 2b with Figure 1 and Figure 2a). There were no statistically significant increases or decreases in sniffing times during urine presentations. There was suggestive evidence of individual discrimination: in six of the eight tests, the test galago increased its sniffing time for donor II scent. However, the

increases were small (mean of 4.7 sec) and in only two cases was I convinced by the behavior of the animal that it recognized the scent as different. One of these cases was the only female urine test, and the small amount of blood in the donor II urine could easily have enhanced interest. The low level of interest in urine made addition of another set of tests impossible.

DISCUSSION

The experimental results demonstrate that the combination of scents left by galagos when scent marking were sufficiently distinctive for a conspecific to discriminate between two individuals' odors. In the wild, a galago's scent mark would often exist as a mixture from several sources. Urine adheres to galagos' feet and is probably present to some degree near all scent marks. Chest gland and anogenital secretions are often placed near or on top of each other (personal observation; Clark, 1980). Such mixed scent marks might then inform a galago of the presence of one versus several conspecifics. There are, however, several possible levels of discrimination, any one of which would explain the observed increase in sniffing of donor II scent:

Level A. Galagos comparing two scents presented close together can usually tell they are not from the same individual. All pairs of scent marks may not differ in the same factors nor do any two galagos' scents necessarily contrast in the same ways at all times.

Level B. Galagos recognize only a few general characteristics, e.g., sex and age, by which many scent pairs may be discriminated if presented close together.

Level C. Galagos detect scent characteristics beyond this small set, characteristics which allow them to distinguish and respond appropriately to the odors of a number of frequently encountered conspecifics.

These levels are not, of course, mutually exclusive. A hierarchy of differences, such as age–sex–social status, may serve to identify a particular individual within a given local population. But only if level C is the case can galagos be expected to learn to identify many individuals by their scents and relate scent mark information to other signals, such as calls.

These experiments clearly demonstrate discrimination at least at level A. Since two of the three pairs were closely matched for age, as well as sex, B alone is improbable. The decrease in sniffing upon representation of donor I scent is consistent with the galagos' identifying it as the previously presented scent over at least a short time. The exceptional increases in sniffing upon representation of donor I scent obtained in the case of the donor pair not matched for age suggest that age or maturity is indeed detectable. Since males and females responded differently to this apsect, with males alone responding

more to the older male's scent, I suggest that age probably influences the response, but is not the actual basis for individual discrimination.

The decreasing interest and quicker habituation to donor I perches over the span of the experiments could have been due to the animals' growing boredom with the test situation. It is, however, also consistent with their recognizing the donor scents from previous trials. Thus, all available evidence suggests that galagos can detect and respond to some consistent information in conspecific scent marks beyond age and sex. If and exactly how they use this information in the wild remains a matter of conjecture.

Results of single-scent tests demonstrated that at least some individuals of the same sex are discriminable by chest gland scent alone. Urine either does not provide this or other interesting information, or else it is only informative after some bacterial action while on the feet.

The chemical basis of chest gland discrimination remains to be determined. Subsequent to these experiments, Crewe et al. (1979) identified the three major volatile components [benzyl cyanide; 2(*p*-hydroxyphenyl) ethanol, first reported by Wheeler et al. (1977); and *p*-hydroxybenzyl cyanide] in all chest gland secretions of *G. c. umbrosus*, which they collected from the glandular area without covering pads or jackets. At least two components had to be present on a perch for a wild galago to respond with the sniffing elicited by a naturally marked perch (Katsir and Crewe, 1980). The authors suggest, on the basis of waning responsiveness of wild galagos to artificial marks, that differential volatility of the components (the latter two of which last over 48 hr) allows animals to determine the age of the scent mark. They also suggest that other less volatile components might vary between individuals and contain more individual identity information. Certainly the discriminations made on the basis of 2-week-old perches support this. The authors, however, have not yet analyzed and compared secretions of different individuals, so consistent differences even in proportions of the volatile compounds are uninvestigated, and the chemical basis for individuality remains unknown. In this study, the three instances of apparent inability to discriminate between two males, one of whose scent was collected as it was freshly secreted, suggests that postsecretion bacterial action might play a role in scent quality.

That by-products of bacterial decay can be important elements of mammalian olfactory communication has been demonstrated in the common mongoose (*Herpestes auropunctatus*) by Gorman (1976, 1979). Relative amounts of four carboxylic acids produced by bacteria in the anal pockets are the basis of individual recognition. Similar data for red fox and some other carnivores is presented by Albone et al. (1978), who point out that the relatively anaerobic anal sacs are excellent fermentative chambers. The contribution of cutaneous surface bacteria to animal communication remains to be demonstrated.

Galago crassicaudatus differs from its congeners in its lack of response to

urine. For most other galago species, e.g., *G. demidovii* (Charles-Dominique, 1972; Vincent, 1969) and *G. alleni* (Charles-Dominique, 1977a), as well as lorisids and many Malagasy prosimians, urine washing appears to be the most frequent and important marking behavior (Charles-Dominique, 1977b; Schilling, 1979, 1980). Schilling (1980) reports interindividual discrimination of urine by *Microcebus coquereli.* Charles-Dominique (1977a), on the basis of his ingenious radiotelemetry study of urine washing in *G. alleni*, suggests that urine marks function in territorial boundary demarcation. In contrast, *G. crassicaudatus* did not urine wash more frequently at the borders of their ranges nor at social meeting points. Urine washing was done near nest sites, along frequently used travel routes, and in feeding or resting spots in approximate proportion to the time they spent in those areas (Clark, 1980, 1982). Chest rubbing, however, did occur primarily in those areas of home-range overlap where galagos gathered. From the field reports on other galagos (Bearder and Doyle, 1974; Charles-Dominique, 1972, 1977a,b; Harcourt, 1980), *G. crassicaudatus* may be the least strictly territorial. Even compared with *G. senegalensis*, which appears to have a similar spatial structure (Martin and Bearder, 1980), wild *G. crassicaudatus* more frequently interact with a relatively large number of conspecifics (Harcourt, 1980). A recent field study by Harcourt (1981) indicates that *G. senegalensis* may use urine washing for grip rather than territorial boundary marking.

Results strikingly similar to those of the urine tests reported here have been obtained for the tamarin *Saguinus fuscicollis* (Epple, 1978). Urine, which occurs with skin gland secretions in circumgenital marks, excites little interest by itself and does not signal individual identity as does glandular scent. Female tamarins can discriminate between urine of the sexes, but it is interesting to note that, for this family-group-living primate, gender without individual identity may be of limited importance or use.

Urine, ubiquitous in mammalian olfactory signal repertoires, is also, as Schilling (1980) points out, an extremely complex mixture of chemicals. Some aspects of these will vary with environmental factors like diet (Beauchamp, 1976). Many urinary odors are contributed by gonads, adrenals, and the skin rather than the kidneys (Adams, 1980). All in all, it may be information-packed, but is unavoidably "noisy." Selection of odor production as intraspecific signaling is likely to result in formalization (sensu Smith, 1977) and increased control of its chemical as well as spatial and contextual characteristics (see Adams, 1980, for a discussion of endocrine and neural control mechanisms). I suggest that a set of glandular sources may offer more control over these characteristics and clearer messages than would urine alone. Within the Galagidae, a shift from urine to several glandular sources may reflect an increase in complexity or specificity of olfactory messages with an increase in the number of frequent associates and the complexity of its social life.

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NEROL: AN ALARM SUBSTANCE OF THE STINGLESS BEE, *Trigona fulviventris* (HYMENOPTERA: APIDAE)

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Abstract—Bees of the genus *Trigona* and subgenus *Trigona* possess volatile materials in their mandibular glands, used as alarm substances and as marking pheromones. Heads of workers of *Trigona fulviventris* were analyzed by gas chromatography–mass spectrometry. The two major volatile components were nerol (~50%), and octyl caproate (~20%). Relative to other substances tested at a Costa Rican nest, treatments containing 20 μg of nerol attracted *T. fulviventris*, depressed numbers of bees leaving the nest by about 50%, and elicited wing vibration and biting. The responses were similar to those obtained with the contents of one worker head. Attraction and biting were also seen in response to captures of colony members by assassin bugs (*Apiomerus pictipes*) outside a nest entrance; one bee responded in about 15% of the captures. This alarm behavior, although weak, is of interest since it was thought that *T. fulviventris* was unusual for its subgenus in its lack of nest defense behaviors.

Key Words—Alarm substance, nest defense, nerol, mandibular gland, Hymenoptera, Apidae, Meliponinae, stingless bees, *Trigona fulviventris*, *Apiomerus pictipes*, Hemiptera, Reduviidae.

INTRODUCTION

Most species of eusocial Hymenoptera actively defend their nest by biting or stinging (Baroni Urbani, 1979); additional attacking workers may be recruited to the site of invasion by means of alarm pheromones released from the mandibular, Dufour's, sting, or anal gland (Wilson, 1971). It might be expected that *Trigona fulviventris fulviventris* Guérin (Apidae: Meliponinae) would possess an active, pheromone-mediated nest defense, for the other members of its subgenus, *Trigona*, are well known for their mass biting

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attacks (Rau, 1933; Kerr and Lello, 1962; Wille, 1965). Yet the absence of attack by *T. fulviventris* even when its nest is breached by a large animal has been widely reported (Michener, 1946, 1974; Schwarz, 1948; Wille, 1965; Wille and Michener, 1973; Johnson, 1974; Brian Bateson, personal communication). In addition, Weaver et al. (1975b), who presented a crushed colony member at a nest entrance of *T. fulviventris*, reported no evidence of an alarm response.

The purpose of this work was threefold: (1) to determine if *T. fulviventris* naturally exhibited an alarm response of any kind; (2) to identify chemical components of the mandibular gland, the site of alarm pheromones in other *Trigona* species (Blum et al., 1970; Blum and Brand, 1972; Luby et al., 1973); and (3) to bioassay identified mandibular gland components at the nest.

METHODS AND MATERIALS

Natural Observation of Alarm Behavior. Two unbroken 12-hr days (dawn to dusk, February 17 and March 4, 1973) were spent at a nest of *T. fulviventris* in and among the roots of a tree in Guanacaste Province, Costa Rica. Bees outside the nest were potential prey of more than two dozen assassin bugs (*Apiomerus pictipes* Herrich-Schäffer, Hemiptera: Reduviidae) that lived around the entrance. A bug would seize a hovering bee with its raptorial legs and inject a paralyzing saliva with its beak. All prey captures and other interactions between bees and the individually marked bugs were recorded, and the time of encounter and distance from the nest entrance were noted. An alarm reponse to the capture of a nestmate was defined as the close approach (0–1 cm) of a bee to the bug with prey during the minute after prey capture. The rate of approach under such circumstances was compared to the rate of approach to bugs without freshly killed prey.

Collection of Bees for Chemical Analysis. Trigona fulviventris workers were collected from a nest in Guanacaste Province, Costa Rica, in January and July 1980 and from a nest on Barro Colorado Island, Panama Canal Zone, in August 1981. Individual bees were trapped in a net as they left the nest, transferred to a plastic bag, and refrigerated. Once they became torpid, they were decapitated with a razor blade or dissecting scissors. The heads were immediately sealed into glass ampoules containing a small volume of solvent (methylene chloride or pentane). Replicate samples were taken during both collecting periods.

Sample Analysis. The sealed glass ampoules were cooled and opened, and the heads were crushed with a Teflon rod. After addition of an appropriate volume of solvent, the resulting solutions were analyzed by GC and GC-MS. Gas chromatographic analyses were conducted on either a Varian 3700 or a Hewlett-Packard 5840 gas chromatograph, each equipped

with capillary injection and data systems. GC-MS data were collected on a Hewlett-Packard 5985B GC-MS system, operated under standard Autotune conditions. All solvents were purified by distillation and checked for purity by GC and GC-MS analysis before use.

The samples from Costa Rica and Panama were analyzed separately. In both cases the two major components of the *T. fulviventris* extract together accounted for approximately 70% of the total volatile materials, as measured by GC-MS (integrated area table).

The peak of lesser retention time (~ 50% of the volatiles) was identified as a monoterpene alcohol by its fragmentation pattern; computerized comparison of its spectrum against the NBS Mass Spectral Data Base suggested that this compound was either nerol or geraniol. Comparisons with authentic samples allowed assignment of the nerol structure, molecular weight 154.

The peak of longer retention time (~ 20% of the volatiles) showed a weak molecular ion (0.3%) at m/z 228 in its electron impact spectrum, but the chemical ionization spectrum (methane reagent gas) showed a strong (28%) ion at m/z 229 ($M^+ + 1$). The assumption of a molecular weight of 228 for the compound allowed straightforward interpretation of the mass spectrum. A base peak of m/z 117 and significant ions at m/z 112 (37%) and 99 (57%) suggested an *n*-alkyl ester and appeared most consistent with octyl caproate. Comparison with an authentic sample (K & K Chemicals) allowed confirmation of this assignment.

Preparation of Material for Bioassay. GC and GC-MS analysis of a commercial nerol sample showed a purity of >99%, so this sample was used without further purification. Because the octyl caproate was contaminated with a second compound, apparently hexyl octanoate, it was purified by distillation through a spinning band column to >99.9% purity before use. Solutions for bioassay contained nerol (3.0 g/liter), octyl caproate (2.0 g/liter), or both (at the same concentrations), dissolved in ether. Also tested in the bioassay were ether alone, vanillin in ether (1.0 g/liter), and methyl butyrate in ethanol. Portions of stock solutions were sealed in glass ampoules for transportation to the assay sites and opened only for immediate use in the bioassays.

Bioassays. The bioassays were done in May and June 1981 at a *T. fulviventris* nest in Guanacaste Province, Costa Rica, and in July 1981 at a nest on Barro Colorado Island, Panama Canal Zone.

The first bioassay was concerned with the attractiveness of the test chemicals and their effect on nest traffic. In a test, a pin bearing a 2-cm square of filter paper was placed 2 cm to the side of the nest entrance. Plain filter paper was tested, or 20 μl of a test chemical were pipetted onto the paper. During the ensuing minute the numbers of bees entering, leaving, and landing on the paper were recorded. After the test the pin and paper were removed and

sealed in a Zip-loc plastic bag. Two minutes were allowed to elapse between each test.

The test substances were (a) nerol in ether, (b) octyl caproate in ether, (c) nerol and octyl caproate in ether in the relative concentrations in which they occurred in the collected samples, (d) ether, (e) methyl butyrate in ethanol, and (f) vanillin in ether. The methyl butyrate and the vanillin were chosen as fruity or floral controls to see if the bees would respond with alarm to the sudden introduction of any strange odor. Substances a, b, c, and e were tested in Costa Rica, and a, b, c, d, and f in Panama.

A test series began with a "background count" of the number of bees entering, leaving, and landing when just pin and paper were present. The background count was followed by tests of the substances and a control (just pin and paper) in systematically varied order, such that each substance occupied each position in the ordering exactly once, and was not preceded by or succeeded by any other test substance twice. Each test series then ended with a test of the response to the juice of one crushed head. Results in all tests of a series were compared with the numbers obtained in the background count. To prevent a possible build-up of irritation in the guard bees only one series was run per day, and the most exciting treatment, the head, was run last. The series was run between 1000 and 1400 hr, when transit activity of *T. fulviventris* reaches a plateau.

The second bioassay investigated the kinds of behaviors exhibited by bees attracted to a 2-cm filter paper square pinned beside the entrance. The paper received one of the treatments described previously, and was placed for 2 min 2 cm below the entrance. Five kinds of behavior were recognized in bees that touched the filter paper, and the number of occurrences of each type were counted in each half minute. The behaviors were "touch," in which a bee touched the paper as if it were going to land but did not land; "land," in which a bee alighted on the paper; "v-wing," in which a bee on the paper held its wings out from the body; "buzz," in which a bee on the paper vibrated its wings; and "bite," in which a bee closed its mandibles on the paper. The second bioassay was done only on the Costa Rican nest, on days when the first bioassay was not scheduled.

RESULTS

Natural Observation of Alarm Behavior. On February 17 the 26 active assassin bugs outside the *T. fulviventris* nest made 17 captures of bees, and on March 4 the 27 active bugs made 25 captures, all within 21 cm of the nest entrance. In three of these captures the events in the minute following capture could not be clearly seen (e.g., the capturing bug tumbled into the entrance shaft). In six of the remaining 39 captures, a bee flew to within 1 cm of her

captured sister. Four of these bees simply hovered in the vicinity of the bug with prey, and two of the bees bit the bug. Thus in slightly over 15% of the captures, a bee was attracted, that is, there were 0.154 alarm responses per capture in the minute after capture. This response rate can be contrasted with 0.0007, the background response rate to bugs without prey killed in the last min.[3] With such a background response rate, the probability of 6 or more spontaneous responses in 39 trials is less than 4×10^{-13} (binomial test). Therefore it appears that capture of a nestmate can elicit an alarm response in *T. fulviventris*.

Bioassay: Nest Traffic and Attraction. For the Costa Rican nest, an analysis of variance was performed using as the dependent variable the deviation of the number of entering bees, exiting bees, or bees landing on the filter paper from that observed in the background count. A Duncan's multiple-range test was then used to find groups of treatments that differed significantly from one another in their deviation from the background count. Table 1 shows that while the treatments did not differ significantly in their effect on incoming traffic, there was a depression in the number of outgoing bees, especially with the head and the nerol-containing treatments. The nerol treatment gave a mean of 25 fewer bees exiting per minute; nerol + octyl caproate, 28 fewer; and the head, 31 fewer. There was no significant difference between the nerol + octyl caproate treatment and the head.

The results were comparable for landings (Table 2), in that the head and the nerol-containing treatments elicited significantly more landings than the other treatments. The head in turn elicited significantly more landings than the other treatments.

The analysis was repeated on the data from the nest in Panama. The effects of the treatments on outgoing traffic were similar to those observed in Costa Rica (Table 3). With nerol, nerol + octyl caproate, and the head, 15–30 fewer bees went out per minute (mean deviation from background count). The effect of the head was significantly different, that of the head and nerol + octyl caproate significantly different, and that of the nerol and nerol + octyl caproate significantly different from the remaining treatments. In Panama a possible depression of incoming traffic was also noted, with the strongest reductions occurring with the head and the nerol-containing treatments (Table 3).

Somewhat different in Panama was the result that only the head stimulated a significantly higher number of landings. The number of landings observed during different treatments are compared for Costa Rica and Panama (Figure 1).

[3] Over the 2 days there were 7 hovering approaches in 9780 bug-minutes, including one in which a bee bit the foot of a bug without prey. Bugs were included in the calculation of bug-minutes only while they were in the open, where bees could readily perceive them.

TABLE 1. EFFECT OF SIX TREATMENTS ON TRANSIT ACTIVITY AT NEST ENTRANCE IN COSTA RICA

In				Out			
Duncan test[a]	Treatment	N	Mean deviation[b]	Duncan test[a]	Treatment	N	Mean deviation[b]
	Methyl butyrate	6	17.2		Control	6	−3.7
	N + O	6	14.5		Octyl caproate (O)	6	−8.1
	Octyl caproate (O)	6	8.8		Methyl butyrate	6	−16.8
	Control	6	6.7		Nerol (N)	6	−24.8
	Nerol (N)	6	3.7		N + O	6	−27.7
	Head	6	−1.5		Head	6	−31.0

[a]Bars indicate the range of nonsignificant differences at the $P = 0.05$ level, Duncan's multiple range test.
[b]Mean deviation from the number of bees entering (In) or leaving (Out) the nest per minute during the "background count" that began any given series of treatments.

TABLE 2. EFFECT OF SIX TREATMENTS ON ATTRACTION TO FILTER PAPER SQUARE

Duncan test[a]	Treatment	N	Mean deviation[b]
	Head	6	4.3
	N + O	6	2.3
	Nerol (N)	6	2.2
	Octyl caproate (O)	6	1.3
	Methyl butyrate	6	0.2
	Control	6	0.0

[a]Bars indicate the range of nonsignificant differences at the $P = 0.05$ level, Duncan's multiple-range test.
[b]Mean deviation from the number of bees landing on the paper square per minute during the "background count" that began any given series of treatments.

The relative effects of the treatments on transit activity are displayed in Figure 2, which combines the data from Costa Rica and Panama. In Figure 2 it is clear that the control is the treatment most like the background count in its effects on transit and that there is a noticeable depression in the number of outgoing bees by nerol, nerol + octyl caproate, and head, in that order.

We also tested for the consistency of treatment effects on nest traffic. We asked whether the number of bees entering or leaving during a treatment was greater than (+) or less than (−) the number during the background count (Table 4). If a treatment was without effect, and chance fluctuations in traffic held sway, one would expect an increase or decrease to occur with equal probability. Accordingly, the binomial probability of six effects in the same direction is $P = 0.03$. Significant by this criterion are decreases of incoming bees by nerol + octyl caproate in Panama and decreases of outgoing bees by the head and the nerol-containing substances in both Costa Rica and Panama. When data from both Costa Rica and Panama are combined, the head, nerol, and nerol + octyl caproate depressed outgoing traffic 12 out of 12 times. It is improbable ($P = 0.00048$) that 12 effects in the same direction are due to chance fluctuation, so we accept the alternate hypothesis that the depressing effect is due to an alarm substance, nerol.

Bioassay: Kinds of Alarm Behavior. An analysis of variance was performed using as the dependent variable the number of times during a treatment a behavior was performed by a bee in contact with the filter paper. A Duncan's multiple-range test was then applied to the mean relative number of occurrences.

The results indicated first that the half-minute out of 4 in which a behavior could occur was not a variable that predicted number of occur-

TABLE 3. EFFECT OF SEVEN TREATMENTS ON TRANSIT ACTIVITY AT NEST ENTRANCE IN PANAMA

In				Out			
Duncan test[a]	Treatment	N	Mean deviation[b]	Duncan test[a]	Treatment	N	Mean deviation[b]
	Vanillin	6	1.3		Ether	6	6.0
	Octyl caproate (O)	6	-1.0		Control	6	4.0
	Ether	6	-2.5		Vanillin	6	0.3
	Control	6	-11.0		Octyl caproate (O)	6	-4.7
	Nerol (N)	6	-11.3		Nerol (N)	6	-15.2
	Head	6	-18.0		N + O	6	-19.8
	N + O	6	-19.8		Head	6	-29.2

[a]Bars indicate the range of nonsignificant differences at the $P = 0.05$ level, Duncan's multiple-range test.
[b]Mean deviation from the number of bees entering (In) or leaving (Out) the nest per minute during the "background count" that began any given series of treatments.

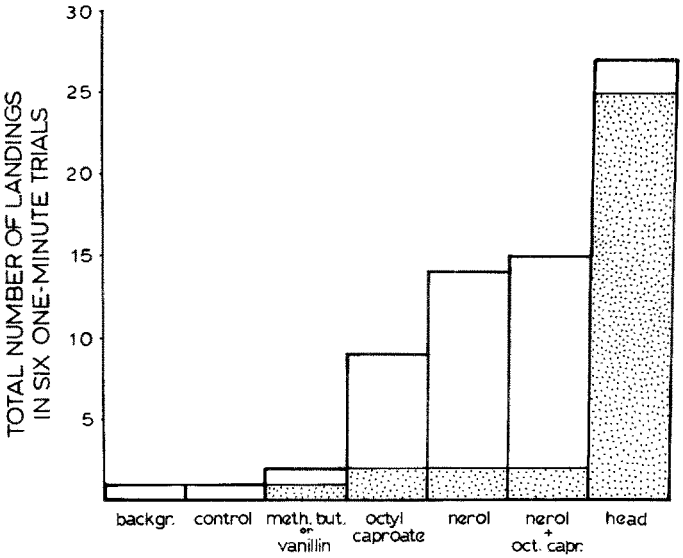

FIG. 1. Number of bees that landed on a 2-cm filter paper square totaled for six 1-min trials for each of six treatments and the "background count." Total height of bars gives the magnitude of the responses at the Costa Rican nest; height of the stippled bars gives the magnitude of the responses at the Panamanian nest. For ether, tested only in Panama, no landings were recorded in six trials.

rences. This means, for instance, there was not a multiplicative effect of bees building up on the paper, spurring each other to more and more acts of alarm. Instead it is probable that alarm behaviors were elicited directly by a fairly stable test stimulus.

Treatment, on the other hand, was a variable that did affect the occurrence of alarm behavior (Table 5). No alarm behavior occurred on the paper during the control. All categories of alarm behavior were elicited by the head, namely touch, land, v-wing, buzz, and bite; the other treatments elicited some of the behaviors. The only treatments to differ significantly along with head were the nerol-containing substances: nerol + octyl caproate and head elicited significantly more touches than the other treatments, and nerol and head elicited significantly more buzzes. Head, nerol, and nerol + octyl caproate, furthermore, were the only treatments that elicited bites, the most defensive of the behaviors.

DISCUSSION

While there is no evidence that *T. fulviventris* defends its well-protected subterranean nest against large animals, the species does possess an alarm

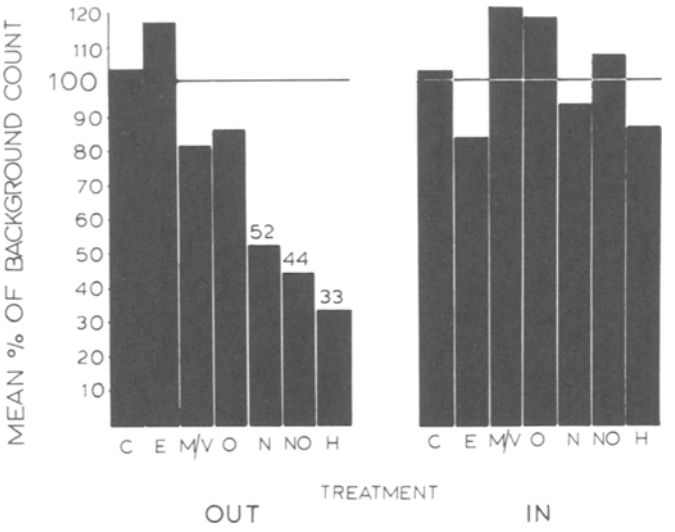

FIG. 2. Mean percentage of "background count," averaged over all trials of a given treatment. For ether $N = 6$; for all other treatment categories, $N = 12$. C = control, E = ether, M = methyl butyrate, V = vanillin, O = octyl caproate, N = nerol, H = head. The nerol treatments depressed outgoing traffic to 52% of that in the background count, and the nerol + octyl caproate treatments depressed outgoing traffic to 44%. Because of the considerable minute-to-minute variation in traffic, and because the traffic level in the background count differed from day to day, it was not worthwhile to compute confidence intervals for these data.

response to small arthropods. When the provocation is small, the response is relatively weak, even compared to that of a timid species like *T. dorsalis*.[4] Nonetheless, *T. fulviventris* is capable of mounting a large-scale defense. For example, Johnson (1982b) found 322 pairs of dead *T. fulviventris* and *Lestrimelitta limao* locked in combat beneath the opening to the nest that *L. limao* had taken from *T. fulviventris*. Hubbell and Johnson (1977) described two battles, in which 1768 bees died, between a group of *T. fulviventris* attempting to move into an empty nest cavity and a defending colony of *T. fulviventris* nesting on the opposite side of the tree.

This study shows that alarm behaviors can be elicited by nerol, the major volatile constituent of the mandibular gland. Nerol is closely related to other pheromones that have been identified in the family Apidae, including the

[4]Whereas *T. fulviventris* exhibited 0.154 responses per provocation by an assassin bug, *T. dorsalis* in 2 days in the same month in the same site at equivalent levels of assassin bug infestation exhibited 0.840 responses per capture in the minute after capture (Johnson, unpublished observations).

TABLE 4. DIRECTION OF CHANGE IN TRANSIT ACTIVITY[a] AT NESTS IN COSTA RICA (CR) AND PANAMA (P) DURING TREATMENTS

	Ether	Control	Methyl butyrate or Vanillin	Octyl caproate	Nerol	Nerol + octyl caproate	Head
In							
CR		−++−++	+−++++	++−+++	+−+−−+	+++−++	−+−+−+
P	+−+−+−	+−+−−−	−−+−++	−−−++−	−−+−−−	−−−−−−	−−−−+−
Out							
CR		−−−++−	−−−−+−	−−−+++	−−−−−−[b]	−−−−−−[b]	−−−−−−[b]
P	+++−+−	−+++−+	−−++−+	−−+−+−	−−−−−−	−−−−−−	−−−−−−

[a]The comparison in each trial was made to the "background count." Plus signs indicate trials in which the transit activity count was higher than the background count; minus signs indicate trials in which the transit activity count was lower than the background count.
[b]Consistent direction of effect on number of outgoing bees ($P = 0.00048$, two-tailed binomial test).

TABLE 5. MEAN NUMBER OF OCCURRENCES[a] OF FIVE BEHAVIORS IN RESPONSE TO TREATMENTS

	Touch	Land	V-wing	Buzz	Bite
Control	0.00	0.00	0.00	0.00	0.00
Methyl butyrate	0.06	0.13	0.00	0.13	0.00
Octyl caproate (O)	0.08	0.50	0.00	0.42	0.00
Nerol (N)	0.00	1.08	0.00	1.25[b]	0.17
N + O	0.50[b]	0.25	0.00	0.56	0.06
Head	0.25[b]	2.50[b]	0.80[b]	1.50[b]	0.38

[a]Mean number of occurrences per 2-min trial are given; however, the number of trials per treatment varied from 2 to 4.

[b]The treatments superscripted in this column differed significantly ($P < 0.05$) from other treatments in mean number of occurrences of the given behavior (Duncan's multiple-range test).

neral and geranial that are alarm and marking pheromones in *T. subterranea* (Blum et al., 1970), raiding allomones in *Lestrimelitta limao* (Blum, 1966), and components of the Nasanov secretion of honeybees (Shearer and Boch, 1966). In fact, small amounts of nerol itself have recently been detected in the Nasonov gland (Pickett et al., 1980). As a hymenopteran alarm pheromone nerol is typical, possessing volatility, a molecular weight between 100 and 200, and an oxygen atom (Wilson and Bossert, 1963; Blum, 1970).

Octyl caproate, the other major volatile isolated from heads of *T. fulviventris*, does not appear to be an alarm substance, nor does it consistently act synergistically with nerol, at least to a degree detectable by our bioassay. We suspect that octyl caproate is not a pheromone, but is instead a component of the cuticular wax. It is of interest, however, that the octyl caproate, methyl butyrate, and vanillin elicited a slightly greater response by some measures than did the ether treatments or the control. It is possible that guard bees fly up to strange odor sources around the nest as a way of locating or harassing potential invaders.

The behaviors elicited by nerol and by the fresh contents of a *T. fulviventris* head are also seen in the alarm responses of other bees in the Apidae, as well as in *T. fulviventris* in other contexts. "Touch" was exhibited by foraging *T. fulviventris* hesitant to land where a model of a competitor bee had been pinned (Johnson, 1982a). Attraction to small arthropods near the nest entrance, including close hovering and landing, has been seen in *T. dorsalis* in reponse to both assassin bugs and ants (Johnson, unpublished observations) and in *T. fulviventris* in Mexico as well (Weaver et al., 1975a). The "v-wing" display is given by *T. fulviventris* in intraspecific fights over resources (Hubbell and Johnson, 1978) and when signs of aggressive *Trigona* species are detected at resources (Johnson, 1980). We have not seen "buzz" in *T. fulviventris* before, but vibration of the wings has been observed in the

alarm responses of *Melipona* (Smith and Roubik, 1982), honeybees (Collins et al., 1980), and *T. remota* (Nogueira-Neto, 1970). In this case the vibration may be a form of fanning to disperse odor messages more widely, as appears to be the case for *T. postica* (Blum et al., 1970). "Bite" is the best-known active defense in stingless bees (cf. Darchen, 1966; Wille and Michener, 1973), but biting behavior also occurs in stinging members of the Apidae (Sakagami and Akahira, 1959; Koeniger, 1975; Collins et al., 1980). For its part, *T. fulviventris* not only bites small enemies around the nest, but also bites rivals in the course of intraspecific aggression for food resources (Hubbell and Johnson, 1978).

The depression of outgoing but not incoming traffic in response to nerol also has its parallels: Leuthold and Schlunegger (1973) and Moser et al. (1968) report that laden, returning ants were the least responsive to the presence of alarm pheromones at the nest. It might be hypothesized that outgoing and incoming foragers are responsive to different kinds of stimuli simply in order to orient effectively, as Hertz (1930, 1935) and Jacobs-Jessen (1959) showed for honeybees. An additional adaptive explanation might be that when danger threatens, the safest place to be is in the nest. Bees inside would be hesitant to leave, and bees outside would be stimulated to enter, or at least not be reluctant to do so.

Our study is not the first instance of presentation of nerol at a *T. fulviventris* nest. Weaver et al. (1975b) presented over 80 compounds or mixtures, including nerol, to a nest in Mexico. Although responses to nerol called "excite" and "investigate" were detected, the responses were not greater than they were for many of the other substances. Geographic differences in the behavior of the bees or in their pheromones may be involved, or perhaps their colony and ours were in different states of excitability.[5] Particular colonies of stingless bees have been known to fluctuate in their defensiveness (Weaver et al., 1975b), perhaps in response to fluctuations in value of the stored food.

Clearly, systematic studies of variation in colony responsiveness, geographic variation, and the effects of auxiliary stimuli need to be done. What we have established here is that *T. fulviventris* possesses an alarm pheromone, nerol, and a measurable alarm response.

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[5]Even in the absence of differences between the Mexican and Central American colonies, it is difficult to compare our results with those of Weaver et al. They used different and more inclusive categories of response, qualitative response measures rather than counts, unspecified quantities of chemicals, and long series of chemical presentations rather than short series separated by at least a day.

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CONTACT STIMULANTS FROM *Heliothis virescens* THAT INFLUENCE THE BEHAVIOR OF FEMALES OF THE TACHINID, *Eucelatoria bryani*[1,2]

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Abstract—Factors acting at contact or close range affected the behavior associated with host seeking of females, but not of males, of the tachinid, *Eucelatoria bryani* Sabrosky. Females were arrested by components of larvae of *Heliothis virescens* (F.) and by a dichloromethane extract of okra leaves. A hexane extract of *H. virescens* frass and a chloroform–methanol extract of *H. virescens* larvae were both active. In addition to chemicals, shape and size were involved because females were arrested by small inert objects the size of *H. virescens* larvae and frass.

Key Words—Kairomone, contact chemicals, arrestant, host seeking, *Eucelatoria bryani*, Diptera, Tachinidae, *Heliothis*, Lepidoptera, Noctuidae, parasitoid.

INTRODUCTION

Tachinids are important parasites of many important insect pests, and a better understanding of the role of chemicals in the location and acceptance of their hosts is essential for the optimal utilization of these parasites in pest-management programs. Chemicals clearly affect tachinid host-selection behavior. Sucrose, fructose, a protein, and several unidentified chemicals stimulate oviposition when certain species of tachinids contact hosts, host frass, or host habitat damaged by host feeding (Burks and Nettles, 1978; Hassel, 1968; Nettles and Burks, 1975; Roth et al., 1978). Unidentified volatile

[1]In cooperation with the Louisiana Agricultural Experiment Station and the Texas Agricultural Experiment Station.
[2]Mention of a proprietary product does not constitute an endorsement by the USDA.

chemicals emitted by the food plant of the insect host (Monteith, 1955; Nettles, 1979, 1980) and by the host (Mitchell and Mau, 1971; Monteith, 1955, 1958) are also attractive to certain species of tachinids.

Contact chemicals stimulate oviposition by the tachinid *Eucelatoria bryani* Sabrosky (Burks and Nettles, 1979), and a volatile chemical(s) is involved in host habitat seeking (Nettles, 1979, 1980). *E. bryani* females are attracted to the odor of the host's food plants, okra and cotton (Nettles, 1979, 1980), and to hosts [*Heliothis virescens* (F.)] fed okra leaves (Nettles, 1980), but they are not attracted to odor of hosts fed an artificial diet (Nettles, 1980). The volatile attractant probably is much more important for host habitat finding than for final host location because the plant odor is stronger than the plant-derived host odor (Nettles, 1980) and the mass of the plant is much greater than that of the host larva. Thus, another chemical(s) may be involved in host location and, in support of this hypothesis, I have frequently observed that the behavior of *E. bryani* adults is affected when flies come in contact with feces, vomit, and hemolymph from *Heliothis* larvae. Although the role of contact chemicals as stimulants of host-seeking behavior is well established for several hymenopteran parasites (Vinson, 1977), there has been no research with tachinids on the role of contact chemicals other than as ovipositional stimulants. This paper reports the effects of components of *H. virescens* larvae on the behavior of the tachinid, *E. bryani.*

METHODS AND MATERIALS

Insect Rearing. E. bryani and *H. virescens* were reared as described previously (Nettles, 1980). The *H. virescens* larvae were fed either a Nutrisoy® diet (Raulston and Lingren, 1972) or a lima bean–wheat germ diet (Shorey and Hale, 1965; Burton, 1969). The parent stocks and the flies being held before they were used in the bioassays were given water at 0800 and at 1600 hr each day by saturating a 9 × 9-cm square of Cellucotton® on the top of the cage.

Tests with H. virescens *inside a Box.* This test was performed on insects used to rear the *E. bryani* parent stock. Two hundred last-stage *H. virescens* larvae were placed inside a white plastic box (11 × 24 × 30 cm, Transco Plastics Corp., Cleveland, Ohio) containing five layers of hardware cloth, which served as spacers (1.2 cm between layers), and a layer of absorbent paper toweling on the bottom of the box. This box was placed inside a cage where the larvae were exposed to adult parasites. After 2 hr of such exposure, the box was covered and removed from the cage. The numbers of each sex of the flies inside the box and inside the cage (outside the box) were determined.

Bioassay Using Paired Papers. Flies (5–15 days old) were separated according to sex using nitrogen anesthesia and placed in cages so that each

contained about 125 flies. These cages (28 × 24 × 43 cm) were covered on the outside with Tubegauz® (Scholl, Inc.), and the bottom was lined with heavy-duty paper toweling. On the day that a group of flies were tested, water was provided one time after tests were completed. [There is a drastic reduction in fly activity when water is given to thirsty flies (Nettles, 1981).] Sugar cubes were removed from the cages while the tests were being performed. Naive and experienced flies were used in the bioassays because flies responded positively to *Heliothis* components regardless of whether they previously had been exposed to *Heliothis* larvae.

Two Whatman 3 M papers (15 × 15 cm) were placed in the bottom of the cage and arranged so they were spaced ca. 2 cm apart. One paper was treated with the test material and the other served as the control. The cages were held at ca. 25° C and, because the flies are photopositive, rotated on a turntable at a rate of 1.2–1.3 revolutions per minute in front of a large window that provided indirect light. Direct light came from overhead fluorescent lights. In addition, the test and control papers inside the cage were placed equidistant from the turntable's central axis and were alternated in case there was a tendency for the flies to prefer one end of a cage.

Numbers of flies on each paper were recorded once per minute for 15 min, and the data were eventually expressed as the percentage of exposed flies on each paper. The use of percentages overcame any possible variations in numbers that may have been caused by differences in fly activity during the day and by mortality. [From previous experience (Nettles, 1979), we know that the response of flies in olfactometers varies during the day.] A response was considered positive when there was a statistically significant difference (paired *t* test) in the percentage of flies on the test and control papers. The ratio of the test to control percentage is presented to give a rough measure of the relative activity of a test material.

Materials Used on Test Papers. Except for the tests with fresh frass, all materials (including papers removed from storage at −20° C) were air dried for at least 1 hr before they were tested. Drying was rapid because all tests were performed in the late fall and winter when the relative humidity in the laboratory was usually low.

Papers exposed to *H. virescens* larvae came from the bottom of the white plastic boxes used for exposing hosts to parasites for rearing. These papers (Whatman 3 M chromatography paper) replaced the paper towels ordinarily used to line the bottom of the boxes. For these tests, we held 200 *H. virescens* larvae in the box for 1 hr. Although hardware cloth spacers kept many of the larvae off the papers, the papers were well spotted with excreta and vomit of the hosts. The frass samples consisted of 1–2 larval equivalents of fecal pellets from last stage *H. virescens*) sprinkled on a test paper. In tests with hemolymph, 200-μl samples were taken from host larvae and spotted in such a way that the paper was covered as evenly as possible. For the vomit tests, a *H.*

virescens larva was agitated with forceps so that it regurgitated on the paper in several places.

Frass extracts were made by placing 20 g of fresh frass in 30 ml of either acetone, ethanol, or water in an Erlenmeyer flask covered with aluminum foil. Each flask stood for about 24 hr and was swirled periodically. The test liquid was obtained after centrifuging. A Virtis® homogenizer was used to prepare a chloroform–methanol (2:1, v/v) extract (200 ml) of last-stage *H. virescens* larvae (90 g), and a dichloromethane extract (800 ml) of okra leaves (370 g). These homogenates were filtered through a sintered glass filter, and the filtrate was used for bioassays. One-ml extracts of frass, larvae, and okra leaves were streaked so as to cover as much of each paper as possible, and the papers were air dried at 25° C for at least 1 hr.

RESULTS

Effect of H. virescens *Larvae on Distribution of* E. bryani. When boxes containing 200 *H. virescens* larvae were held inside a fly cage for 2 hr, 21 ± 3% of the males and 76 ± 2% of the female parasites moved inside the host box. There was a highly significant difference ($P < 0.01$, paired t test) between the distribution of sexes inside the box ($t = 10.80$). Quite obviously, the *H. virescens* larvae were more attractive to females than to males, and it seems likely that chemicals may be at least partially responsible for the difference.

Response of Female E. bryani *in Bioasays.* Using the criterion of percentage distribution of flies between treated and untreated test papers in cages used for bioassays, females responded either positively or not at all (Table 1); there were no negative responses. Females responded positively to papers bearing excreta and vomit of *H. virescens* larvae (tests 1, 2, and 5); frass from *H. virescens* larvae (tests 6, 8, and 10); hemolymph and vomit from *H. virescens* larvae (tests 15, 17, and 18); and pieces of solder (test 13) and wire (test 14). Dry frass from *H. virescens* larvae was more active than unused, dried *Heliothis* diet (test 12).

Papers excreted and vomited on by *H. virescens* larvae elicited a response by flies even after they had been stored for 6 days at −20° C (test 5); however, flies did not respond if the same papers were held 3 and 5 days at 25° C (tests 3 and 4). Although the females responded positively to fresh *H. virescens* hemolymph (test 15), hemolymph was inactive after it was held on the test paper at 25° C for one day (test 16). On the other hand, a mixture of *H. virescens* vomit and hemolymph was active after aging one day under similar conditions (test 18). Hexane extracts (test 19) of *H. virescens* frass affected female fly behavior, but acetone, ethanol, and water extracts of frass (tests 21, 22, and 23) were inactive. A chloroform–methanol extract of *H. virescens* larvae (test 25) and a dichloromethane extract of okra leaves (test 26) were active.

Responses of Male E. bryani *in Bioassays.* Males responded positively to fresh moist frass from *E. virescens* larvae (test 7) but did not respond to 1-day-or 1-week-old frass (tests 9 and 11); hexane and water extracts of *H. virescens* frass (tests 20 and 24); or to a dichloromethane extract of okra leaves (test 27). There were no negative responses.

DISCUSSION

Our tests utilizing both sexes of *E. bryani* demonstrated that factors acting at contact or at short range, and associated with *H. virescens* larvae, were responsible for the greater than 3:1 ratio of females to males inside the box. A volatile attractant probably was not responsible because our earlier studies indicated that *H. virescens* larvae fed artificial diet were not attractive to *E. bryani* females in olfactometer tests (Nettles, 1980). The hosts probably had less effect on the males than the data indicate because it is likely that the females provided some attraction to the males seeking mates.

In bioassays based on a comparison of the distribution of flies on paired papers on the bottom of cages, females spent more time on papers containing components of *H. virescens* than they did on control papers. Except for tests in which there was a loss of activity with time, all tests with *H. virescens* components arrested or positively affected female behavior. Positive responses to different types of extracts strongly indicate chemical involvement. Additional evidence for chemical mediation of these responses is the retention of activity by samples stored at −20° C (test 5) and the loss of activity by samples held at 25° C. The loss of activity by aged host components would increase parasite efficiency by tending to limit searches to areas where hosts are more likely to be present.

The retention of activity by the vomit–hemolymph combination after 1 day at 25° C and the loss of activity by hemolymph alone under the same conditions suggest either that vomit is a richer source of arrestant than hemolomyph or that vomit contains something that protects the arrestant from decomposition. Vinson et al. (1975) reported that the mandibular gland of *H. virescens* is the source of several methyl-branched saturated C_{32}, C_{33}, and C_{34} hydrocarbons which stimulate searching by a hymenopteran parasite.

Factors other than chemicals also affected fly behavior at a short range. Males probably responded only to moisture because fresh moist frass was active but 1-day-old frass was inactive. Females were arrested by both fresh moist frass and 1-day- and 1-week-old dry frass. The response to older dry frass suggests that either shape or texture or fly vision may be involved in host location–acceptance. This apparent physical effect was confirmed by the response of *E. bryani* females to pieces of solder about the size of last-stage *H. virescens* larvae and to pieces of wire about the size of dried frass. Texture

TABLE 1. RESPONSE OF *E. bryani* ADULTS TO DIFFERENT STIMULI

Test	Number of tests	Sex of flies	% Distribution of flies on each test paper: Test	% Distribution of flies on each test paper: Control	Ratio	*t* value (probability)
Paper exposed to *H. virescens* larvae						
1. ca. 3 hr old[a]	44	♀	65	35[b]	1.9	8.89 (<0.01)
2. 1 day old[a]	15	♀	66	34[b]	1.9	4.85 (<0.01)
3. 3 days old[a]	9	♀	49	51[b]		−0.29 (n.s.)
4. 5 days old[a]	4	♀	44	56[b]		−1.25 (n.s.)
5. 6 days old[c]	18	♀	76	24[b]	3.2	22.08 (<0.01)
Frass from *H. virescens* larvae						
6. Fresh, moist frass	22	♀	66	34[b]	1.9	6.10 (<0.01)
7. Fresh, moist frass	9	♂	60	40[b]	1.5	3.83 (<0.01)
8. 1-day-old dry frass[a]	8	♀	65	35[b]	1.9	3.18 (<0.05)
9. 1-day-old dry frass[a]	4	♂	52	48[b]		0.99 (n.s.)
10. 1-week-old dry frass[a]	5	♀	61	39[b]	1.6	3.07 (<0.05)
11. 1-week-old dry frass[a]	13	♂	52	48[b]		0.30 (n.s.)
Dry frass, host diet, solder, and wire						
12. 1-day-old dry *H. virescens* frass[a]	6	♀	68	32[d]	2.1	5.59 (<0.01)
13. Solder[e]	20	♀	57	43[b]	1.3	4.79 (<0.01)
14. Wire[f]	14	♀	61	39[b]	1.6	4.28 (<0.01)
Hemolymph and vomit from *H. virescens* larvae						
15. Fresh hemolymph (200 μl)[a]	6	♀	62	38[g]	1.6	4.13 (<0.01)
16. 1-day-old hemolymph (200 μl)[a]	18	♀	51	49[g]		0.26 (n.s.)
17. Fresh vomit	18	♀	59	41[b]	1.4	4.49 (<0.01)
18. Vomit and hemolymph, 1 day old[a]	8	♀	66	34[b]	1.9	8.69 (<0.01)
Extracts of *H. virescens* frass						
19. Hexane	14	♀	58	42[h]	1.4	2.93 (<0.05)
20. Hexane	6	♂	47	53[h]		−2.33 (n.s.)

21. Acetone	4	♀	54	46[h]		0.86 (n.s.)
22. Ethanol	5	♀	51	49[h]		0.21 (n.s.)
23. Water	10	♀	51	49[h]		0.25 (n.s.)
24. Water	5	♂	48	52[h]		0.74 (n.s.)
2:1 Chloroform–methanol extract						
25. *H. virescens* larvae	30	♀	57	43[h]	1.3	5.26 (<0.01)
26. Okra leaves	13	♀	60	40[h]	1.5	2.69 (<0.05)
27. Okra leaves	7	♂	52	48[h]		0.50 (n.s.)

[a]Held at 25°C.
[b]Untreated paper.
[c]Held at −20°C.
[d]Several air-dried pieces of *Heliothis* bean–wheat germ diet never exposed to *H. virescens*, size and number similar to pieces of frass.
[e]10 pieces of solder, size comparable to last stage *H. virescens* larvae.
[f]10 pieces of wire insulated with black cloth, diameter = 0.15 cm, length = 0.70 cm.
[g]200 μl of water.
[h]Equal volume of the appropriate solvent.

probably is not very important because the texture of solder is somewhat different from that of *Heliothis* larvae. The positive response to pieces of wire may suggest that the female response to frass is limited to visual or tactile stimuli. However, in a direct comparison, the greater response of females to dry 1-day-old pieces of *Heliothis* frass than to pieces of dry *Heliothis* diet untouched by *Heliothis* larvae again demonstrated the importance of a chemical.

The well-known fact that flies generally have good vision and the use of proboscis by females to probe the darkened spots left when hosts walked on papers led me to suspect initially that vision was responsible for some of the response to papers. However, unless a part of the spectrum outside that detected by humans was involved, vision was not a factor because the papers lost their activity but not their spots with time (after 3 and 5 days, tests 3 and 4). This does not rule out vision when a female *E. bryani* is near a host. Host motion may be very important to the parasite because Monteith (1956) observed that female tachinids (*Drino bohemica* Mesnil) responded to host movement when host odor was present and motion enhanced the acceptability of dead gypsy moth larvae to the tachinid *Compsilura concinnata* (Meigen) (Weseloh, 1980).

Although okra emits volatile chemicals attractive to *E. bryani* from a distance (Nettles, 1979, 1980), the experimental design, certain behavioral responses, and especially the use of the proboscis when females came in contact with the okra extract on paper indicated that okra was acting mainly as a contact stimulant in the current tests. In view of the fact that okra and the *Heliothis* components affected the females and not the males, it is likely that contact chemicals associated with the host's food plant and the host are involved in the female host-seeking response.

This report clearly demonstrates that contact stimulants other than ovipositional stimulants affect the host-seeking behavior of a tachinid. Because active *Heliothis* and okra components held flies on the treated papers only briefly, chemicals other than those already described for *E. bryani* in this and other reports probably are involved.

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TRAIL-FOLLOWING IN TERMITES: EVIDENCE FOR A MULTICOMPONENT SYSTEM

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Abstract—Several African termite species from different subfamilies and different habitats are sensitive to trail-active extracts or to naturally laid trails from other species. Using single-extract bioassays, it is shown that the response threshold for trail-following is nearly identical for all tested species (except for *Hodotermes mossambicus*). However, when termite workers have a choice between trails from their own species and from other species, conspecific trail-following is exclusively observed. This phenomenon can be counteracted by dilution (1 : 10) of the conspecific trail-pheromone extract. Tests of the trail activity of various synthetic alcohols show that among these, the highest sensitivity of termite workers is to (*Z*)-3-dodecen-1-ol. Based on our experimental data, we postulate that, in addition to a generally active trail-pheromone constituent (an unsaturated primary C_{12} alcohol) or a pool of chemically closely related alcohols, other species-specific components are present in termite trails.

Key Words—Isoptera, termites, trail pheromones, non-species-specificity, species specificity, (*Z*)-3-dodecen-1-ol.

INTRODUCTION

The ability of termites to lay pheromone trails is well documented (Stuart, 1963, 1969; Moore, 1974). Pheromone trails are laid to recruit nestmates to significant locations inside and outside the nest structure, e.g., to food and water sources, building sites, or areas of disturbance. Due to the small quantity of pheromone present on the trail or in the termite's sternal gland, the

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behaviorally active substances have, to date, been difficult to identify chemically. As a result, only a few trail-active components have yet been identified and subsequently synthesized (see Priesner, 1973; Ritter, 1977). Most evidence points towards (*Z,Z,E*)-3,6,8-dodecatrien-1-ol as being a natural trail pheromone of *Reticulitermes virginicus* (Matsumura et al., 1968; Tai et al., 1969). The same applies to Neocembrene-A, a diterpene hydrocarbon, for several *Nasutitermes* species (Moore, 1966; Birch et al., 1972), and to hexanoic acid for *Zootermopsis nevadensis* (Hummel, 1968; Hummel and Karlson, 1968; Karlson et al., 1968).

(*Z,Z,E*)-3,6,8-Dodecatrien-1-ol elicits trail-following behavior in several species of various termite genera (Matsumura et al., 1972; Ritter, 1977) and thus possesses at least some interspecific activity. This compound is present in wood attacked by the fungus *Gloeophyllum trabeum* (Matsumura et al., 1969). Its synthesis, from precursors made by the fungus, has been partially analyzed (Matsumura et al., 1976).

Howard et al. (1976), however, have shown that four different species within the subfamily Rhinotermitidae, although following other species' trails, prefer conspecific trails. It remains to be seen whether the observed species specificity is based on chemical variation in a postulated "one-component" system or on a "multicomponent" system in which an unspecific common trail pheromone is modulated with species-specific constituents.

Preliminary field experiments with workers of *Trinervitermes geminatus* foraging at their natural sites, and trail-active extracts of *T. bettonianus* workers, indicate that artificial trails—in choice situations with naturally laid trails—were not followed even when made from highly concentrated active extracts.

In this report evidence obtained from several African termite species (from diverse habitats and belonging to various subfamilies) indicates that, under certain experimental conditions, trail-following substances are responded to in a non-species-specific way. Individual workers in a noncompetitive choice situation demonstrate equal trail-following activity for both conspecific and nonconspecific trails. However, when given a choice, workers of the species tested show a clear preference for the conspecific extracts as well as conspecific natural trails.

METHODS AND MATERIALS

Test Animals. Workers of 13 species from different genera and families were collected in different habitats. Except for *T. bettonianus*, which came from laboratory colonies, all tests were carried out with freshly collected material.

Nasutitermitinae	
Trinervitermes bettonianus	from Ruiru and Narok, Kenya
Trinervitermes gratiosus	from Kibwezi and Voi, Kenya
Trinervitermes trinervius	from Mokwa, Nigeria
Trinervitermes togoensis	from Mokwa, Nigeria
Trinervitermes geminatus	from Mokwa, Nigeria
Trinervitermes occidentalis	from Mokwa, Nigeria
Nasutitermes kempae	from Kwale, Kenya
Termitinae	
Amitermes messinae	from Voi, Kenya
Amitermes unidentatus	from Kajiado and Kwale, Kenya
Macrotermitinae	
Macrotermes michaelseni	from Kajiado, Kenya
Rhinotermitinae	
Schedorhinotermes lamanianus	from Kibwezi and Kwale, Kenya
Reticulitermes lucifugus var.	from France
Hodotermitinae	
Hodotermes mossambicus	from Kajiado, Kenya

Pheromone Extracts. A known but variable number of termite workers were immersed in a calibrated volume of *n*-hexane and extracted for 24 hr at −16 to −18° C. The relative trail-following activity released by the extracts was measured in "trail units" (TU) according to Leuthold and Lüscher (1974). Prior to each bioassay, the extract activity (in TU) was determined using conspecific workers and workers of *T. bettonianus* and/or *S. lamanianus.*

Statistical Methods. The results of all tests followed a binomial distribution, and the *t* test was applied. The significance level was set at $P < 0.01$, except when otherwise stated. All tests were performed with at least 10 termites (with two decisions in each run) and were repeated at least three times.

Laboratory Bioassay. To avoid reinforcement of the trail, each trail was tested with one termite only.

Two types of bioassay were employed: In the single-extract bioassay the routine "figure-eight" test measured the trail activity as described by Leuthold and Lüscher (1974). It was used for tests with all species except *H. mossambicus.* Equivalent tests with *H. mossambicus* were made with the trail bioassay arrangement (triple V-shaped maze) described by Leuthold et al. (1976).

The dual-extract bioassay was used to determine the preference for different extracts of known trail-following activity. It consists of two channeled runways (5 cm long) joining together and forming a V-shaped maze (Figure 1A). Two extract trails (t_1 and t_2) applied on paper, one on each arm of

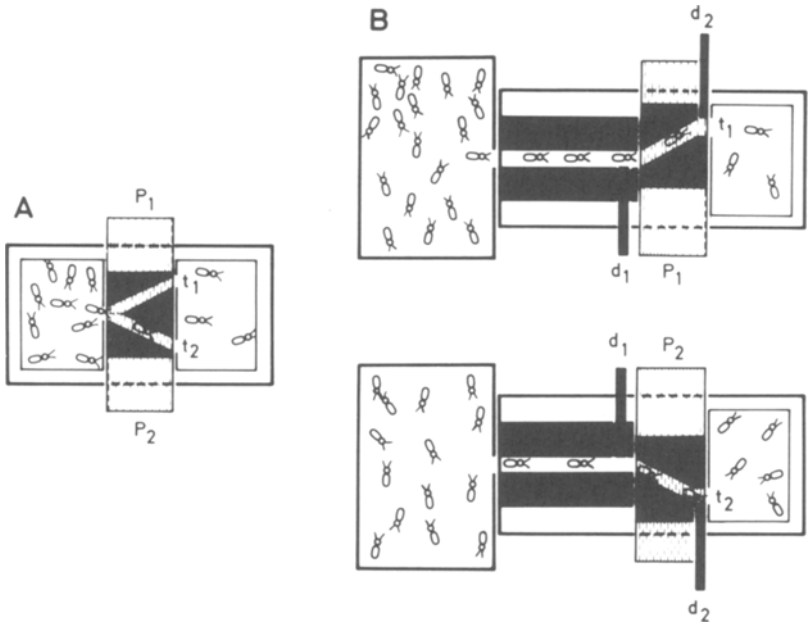

FIG. 1. (A) V-shaped maze bioassay arrangement for competitive trail bioassays. (B) Arrangement for simultaneous establishment of naturally laid trails. For further explanations see text.

the V-maze, made a choice available to the termites. The application of extracts and the scoring system were identical to those referred to above.

Naturally laid trails of *T. bettonianus*, *T. gratiosus*, and *S. lamanianus* were examined in a similar choice bioassay; this bioassay also consisted of a V-shaped maze (Figure 1B). Workers of different nests kept in the laboratory were lured through a tunnel. They laid a natural trail on the underlying paper strips P_1 and P_2 (Figure 1B). By operating the sliding doors, d_1 and d_2, respectively, equal numbers of workers from both species tested were allowed to pass over the paper strips. The two paper strips were then removed and placed one on each arm of the V-shaped bioassay arrangement. The amount of time required for the simultaneous establishment of the natural trails of both species did not differ by more than 1 min.

Field Experiments. Choice tests with extract trails applied in the field were carried out on *T. geminatus* foraging sites in Mokwa, Nigeria. Trail-active hexane extracts of *T. bettonianus* of known activity (TU) were mixed homogeneously with 5 cm^3 fine quartz sand together with 3 μl of liquid paraffin oil (Merck No. 7161). After evaporation of the hexane, the inpregnated quartz sand was poured through a small funnel to form a trail of 5 cm^3 of sand/m. Those trails containing between 20 and 2000 TU/cm yielded a

good following response with *T. geminatus* workers isolated from their foraging area, as they were followed without difficulty over a distance of 1 m. Identical trails were laid on a natural foraging site during foraging, either as a choice—branching off from a natural trail—or as a substitute for an artifically removed section of a foraging trail.

RESULTS

Single-Extract Bioassay. The amount of extractable trail pheromone(s) varies from 2000–6000 TU/gland (workers) in *S. lamanianus* to 1–10 TU in *M. michaelseni* (minor workers). Prior to each interspecific trail test the species-specific activity to the extracts was tested on conspecific workers. Table 1 summarizes the results for the single-extract bioassays with various African termite species and demonstrates the extent to which interspecific trail-following is observed. If an extract releases the same behavioral activity in workers of a different species as it did conspecifically, and vice versa, we conclude that workers of both species respond with equal sensitivity to both extracts.

Apart from *H. mossambicus*, all species tested show the same (or a similar) range of sensitivity in trail-following behavior to the extracts. Extracts from *H. mossambicus* major and minor workers repel workers of *T. bettonianus* and *S. lamanianus.* This repellent effect diminishes when the extracts from *H. mossambicus* are applied in a diluted form. In *S. lamanianus* the dilution required is at least 1 : 100.

Dual-Extract Bioassay. To determine the preference for different extracts, the dual-extract bioassay (V-shaped maze) was applied. Specificity can be demonstrated as shown in Table 2. If conspecific extracts are offered in competition with an extract of another species, conspecific extracts were always preferred to intraspecific extracts. When conspecific extracts are not offered, the termites favor extracts from a species of the same genus. This species-specific or genus-specific preference can be neutralized by diluting the conspecific, or the preferred, extract by 1 : 10 (Table 2).

Species-specific trail-following of natural trails laid by workers of *T. bettonianus*, *T. gratiosus*, and *S. lamanianus* is demonstrated in Table 3.

The trail activity of various alcohols was tested. Table 4 summarizes the results obtained. Tests with workers of *S. lamanianus*, using a homologous series of saturated primary alcohols, show that the highest trail-following activity is to an alcohol with a chain length of 12 carbon atoms. The position shift of the functional group demonstrates that the highest activity is present in a primary dodecanol. Dodecan-3-ol repels workers from trail-following. The repellent response of this compound declines to zero when the dilution exceeds $1 : 10^5$.

TABLE 1. INTERSPECIFIC TRAIL-FOLLOWING AMONG AFRICAN TERMITE SPECIES TESTED WITH THE SINGLE-EXTRACT TRAIL BIOASSAYS[a]

Test species		Extract from 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
T. bettonianus	1	●	●	●	●	●	●	●	●	●	○	○	●		–	–
T. gratiosus	2	●	●	●	●	●	●		●	●			●			
T. trinervius	3	●		●	●	●	●						●			
T. togoensis	4	●	●		●	●							●			
T. geminatus	5	●	●	●		●							●			
T. occidentalis	6	●					●						●			
N. kempae	7	●						●								
A. messinae	8	●	●						●	●			●			
A. unidentatus	9	●							●	●			●			
M. michaelseni																
Major worker	10	○	○								●		○			
Minor worker	11	0	0									●	0			
S. lamanianus	12	●	●	●	●	●	●	●	○	○	○	○	●	●	–	–
R. lucifugus[b]	13	●	●							●			●	●		
H. mossambicus																
Major worker	14	○											○		●	
Minor worker	15	0											0			0[c]

[a]Equal sensitivity (●) to non-species-specific extracts as compared with the species' own extract is demonstrated in most cases. For abbreviations see Methods and Materials. Sensitivity: ● = 1–10 TU; ○ = 10–30 TU; 0 = no trail-following; – = repellent function to workers; blanks = not tested.

[b]Combined results of two different test series.

[c]This result is due to the standardized extraction technique. Trail-following can be observed with longer extraction times.

TABLE 2. SPECIES-SPECIFIC TRAIL-FOLLOWING OF EXTRACTS FROM FOUR EAST AFRICAN TERMITE SPECIES IN DUAL-EXTRACT TRAIL BIOASSAY[a]

	Test species	Competing extracts					Diluted competing extracts									
		1 2 Y	1 3 Y	1 4 Y	2 4 Y	3 4 Y	$\frac{1}{10}$ 2 Y	$\frac{1}{10}$ 3 Y	$\frac{1}{10}$ 4 Y	$\frac{2}{10}$ 4 Y	$\frac{3}{10}$ 4 Y	1 $\frac{2}{10}$ Y	1 $\frac{3}{10}$ Y	1 $\frac{4}{10}$ Y	2 $\frac{4}{10}$ Y	3 $\frac{4}{10}$ Y
1	*T. bettonianus*	1	1	1	2	3	NP	NP	NP	2*	4	1	1	1	2	
2	*T. gratiosus*	2	1	1	2	3	2	3*	4	4	4*	NP	1	1	2	3
3	*S. lamanianus*	2	3	1	NP	3	2	3	4	4	NP	1	NP	1	2	
4	*A. unidentatus*	1	1	4	4	4	2	3	4		4	1	1	NP	NP	NP

[a]Extract numbers correspond with the species numbers they were obtained from. The dilution factor is marked underneath the extract numbers. The numbers in the table refer to the preferred extract. NP = no preference. * = significance level: $P \leq 0.05$. For further explanation see text.

TABLE 3. SPECIES-SPECIFIC TRAIL-FOLLOWING OF NATURALLY LAID TRAILS[a]

Test animals		Competing natural trails		
		1 2 Y	1 3 Y	2 3 Y
1	*T. bettonianus*	1	1	2
2	*T. gratiosus*	2	1	2
3	*S. lamanianus*	2*	3	3

[a]For further explanation see Table 2.

The introduction of one *Z* unsaturation of the C3 position of a dodecan-1-ol increases the activity approximately ten thousand times (Table 4).

The testing of trail-following activity elicited by (*Z*)-3-dodecen-1-ol with workers of *T. bettonianus*, *S. lamanianus*, and *R. lucifugus* demonstrates the same range of sensitivity in all three species (Table 5).

Microchemical Functional Group Analysis. Trail-active hexane extracts of *S. lamanianus* and *T. bettonianus* were tested as outlined by Beroza (1975). For both extracts (1000 TU each) saponification, alkaline extraction, or lithium aluminium reduction did not result in a loss of activity. After bromination and acetylation, a complete loss of the total activity is registered. These results indicate the presence of both unsaturation and a hydroxylic group in the trail-active substance(s).

Field Experiments. All attempts to deviate foraging workers from natural trails to extract trails failed. Nevertheless, in the field these extract

TABLE 4. THRESHOLD AMOUNTS OF VARIOUS ALCOHOLS ELICITING TRAIL-FOLLOWING BEHAVIOR IN *S. lamanianus*[a]

Alcohol	Amount (g/5 cm trail)	Alcohol	Amount (g/5 cm trail)
Hexan-1-ol	NR	Dodecan-1-ol	2×10^{-6}
Octan-1-ol	NR	Dodecan-2-ol	NR
Decan-1-ol	NR	Dodecan-3-ol	repellent ($\geqslant 10^{-5}$)
Undecan-1-ol	2×10^{-5}	Dodecan-4-ol	NR
Dodecan-1-ol	2×10^{-6}	Dodecan-5-ol	NR
Tridecan-1-ol	2×10^{-5}	(*Z*)-3-Dodecan-1-ol	3×10^{-10}
Tetradecan-1-ol	NR		

[a]The "figure-eight" trail bioassay is used for measuring the threshold amounts. The quantities listed correspond to 1 TU. NR = no response.

TABLE 5. THRESHOLD AMOUNTS OF (*Z*)-3-DODECEN-1-OL ELICITING TRAIL-FOLLOWING BEHAVIOR

Test species	Amount (g/5 cm trail)
T. bettonianus	10^{-10}
S. lamanianus	10^{-10}
R. lucifugus	3×10^{-11}
R. virginicus[a]	2×10^{-10}

[a]From Tai et al. (1971).

trails released a high trail-following response in isolated workers. Similar highly active artificial trails made from (*Z*)-3-dodecen-1-ol never successfully competed with naturally laid trails.

DISCUSSION

Unlike ants, which may use secretions from different glands in trail-laying for homing behavior and for the recruitment of nestmates (Wilson, 1971, 1974; Hölldobler, 1977), only the sternal gland serves this function in termites. Quennedey (1977) distinguishes morphologically different types of cells in the sternal glands, probably producing different components of the total gland's secretion. This may be linked with polyethism in termites since number and ratio of the cell types vary in larvae, workers, soldiers, and imagoes, and may explain some of the caste-specific responses. However, since all types of cells secrete directly or indirectly into the intersternal fold, different behavioral effects of, e.g., worker-generated trails are more likely to be quantitative (Tschinkel and Close, 1973; Traniello, 1981; Kaib and Leuthold) than qualitative ones.

When no choice is available, workers of several termite species respond with trail-following behavior to extracts from different species or genera at approximately the same threshold levels as to conspecific extracts (Table 1). This high degree of interspecific response suggests the existence of a common component or a pool of structurally very closely related components being present in the trail-active extracts tested (including the Nasutitermitinae).

Earlier chemical studies revealed that (*Z*, *Z*, *E*)-3,6,8-dodecatrien-1-ol is a trail active compound present in *R. virginicus* (Matsumura et al., 1969) and that it elicits trail-following behavior in several species of the genera *Reticulitermes* and *Coptotermes* (Matsumura et al., 1972). A shift of the double bond from the C-3 position to another position causes a considerable decrease in trail-following activity with respect to the alcohol (Tai et al., 1971; Akkerboom, 1974). A similar phenomenon has been reported for *Kalotermes*

flavicollis (Fabricius) and *Microcerotermes edentatus* (Wasmann), which respond to (*Z*)-3-hexen-1-ol but not to the (*Z*)-2 isomer (Verron and Barbier, 1962). Thus the (*Z*)-3 double bond appears to be important for the response of several termite species in non-species-specific trail-following behavior. However, contrary to various authors and to our own results (Table 4), Larue and and Barbier (1976) indicated that an unsaturated C_{14} alcohol and its corresponding ester are the trail-active substances in *S. lamanianus*.

Our results show that (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol (the trail pheromone of *R. virginicus*), or an alcohol related to (*Z*)-3-dodecen-1-ol, is one component of the trail-active extracts from the tested species (except for *H. mossambicus*).

The evidence is: (1) Microchemical tests suggest that the trail-active fractions of extracts from workers of *S. lamanianus* and *T. bettonianus* contain an unsaturated alcohol. (2) In trail-activity tests employing a series of homologous saturated aliphatic alcohols, workers of *S. lamanianus* show the highest sensitivity to dodecan-1-ol (Table 4). (3) (*Z*)-3-Dodecen-1-ol is followed by *S. lamanianus* with a sensitivity approximately 1000 times greater than dodecan-1-ol (Table 4), and it is also followed with approximately the same range of sensitivity by two other species (Table 5). In addition, the threshold concentration of (*Z*)-3-dodecen-1-ol eliciting trail-following behavior in *S. lamanianus* is 10^{-10} g/5 cm trail; this is nearly identical with that of *R. virginicus* (2×10^{-10} g/5 cm trail) (Tai et al., 1971). (4) Trails made from worker extracts of various East African termite species are followed by *R. lucifugus* with the same sensitivity level as the extract of its own species (Table 1).

Only one species, *H. mossambicus*, differs in many aspects from the rest of the termites studied. During foraging activity, workers use optical orientation (Leuthold et al., 1976) and follow pheromone trails by klinotactical orientation (Kaib and Leuthold, 1982) while workers of all the remaining species employ tropotactical trail orientation. Extracts from *H. mossambicus* workers' sternal glands repel *T. bettonianus* and *S. lamanianus* workers from trail-following. Furthermore, preliminary chemical investigations of extracts from *H. mossambicus* do not show the presence of an C_{12} alcohol (Schlunegger, personal communication).

When workers are simultaneously given the choice between equally active extracts from their own species and from a different species, they invariably follow conspecific extract trails (trail-following, Table 2). Furthermore, a genus-specific preference is indicated (Table 2). A species-specific component appears to bias the termite's response towards its own species' extract. This phenomenon is present, although the determined threshold concentration for trail-following behavior of extracts in the various species is the same as for conspecific extracts (based on the results of the single-extract bioassay).

This preference at the species-specific level can be counteracted by diluting the preferred extract of the tested species by a factor of 10. Presumably this effect is caused by the increasing concentration of the postulated common unsaturated C_{12} alcohol.

Moore (1974) has postulated that—based on experiments with extracts of *Nasutitermes* species—species specificity in artificially laid termite trails may be caused by the presence of components of greater volatility in addition to a non-species-specific basis pheromone. Our results with termite extracts confirm the presence of a multicomponent trail pheromone system in termites. However, to ensure that the species-specific substances are not artifacts from the extracts of whole bodies—such as colony odors, etc.—similar bioassays were carried out with naturally laid trails. These experiments confirm the species-specific trail-following response of termite workers to natural pheromone trails which is in good accord with the results obtained from termite extracts.

Preliminary field experiments completed under natural trail-following conditions demonstrate that artificially laid extract trails are refused even when these extracts are behaviorally highly active when tested in isolated situations. It is likely that these termites in isolated conditions—without having any other cues available—will follow most termite trails at the level of the species-specific threshold concentration. However, in the complex field situation, offering other stimuli as well or due to the motivation of the termites (e.g., foraging), the lack of all species-specific cues may become decisive for refusing the artificially laid trails.

There is no doubt that, in dense termite populations, often with overlapping foraging areas, efficient mechanisms exist to keep members of sympatric species or of different colonies of the same species apart. Our experiments demonstrate that the separation of sympatric species may occur at the level of species-specific pheromone trails. Based on our own results, we cannot answer whether or not colony-specific constituents are present in the pheromone trails. These constituents could be responsible for separating conspecific termites from neighboring colonies. However, other signals—such as mechanical contacts between individuals—may also be responsible for our observations.

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A REAPPRAISAL OF INSECT FLIGHT TOWARDS A DISTANT POINT SOURCE OF WIND-BORNE ODOR

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Abstract—This communication reappraises the behavioral evidence concerning insect flight toward a point source of wind-borne odor in the light of meteorological information not yet considered in this context. The horizontal tracks of puffs of smoke from a generator in the open air were videorecorded and found to continue along nearly straight lines from the source for at least 25 m, while the shifting wind direction caused the plume formed by the succession of puffs to "snake" to and fro. It is inferred from this and much previous work that within such a distance the wind will be aligned on the source of any wind-borne odor wherever the odor can be detected. This being so, a strategy of finding the odor source by flying roughly upwind on meeting the odor, but holding station against the wind with or without casting across it on losing the odor (odor-modulated anemotaxis), seems likely to be highly adaptive, whereas a strategy of flying along the plume ("odor-trail following") seems unlikely since it would often take the flier in "wrong" directions and would be more disrupted by turbulence.

Key Words—Insect attraction, orientation, anemotaxis, chemotaxis, odor plume, odor trail, pheromone, wind, turbulence.

INTRODUCTION

In a wind tunnel where the wind direction is constant, any plume of air-borne material from a stationary point source remains directly in line with the wind.

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Thus a male moth, for example, flying upwind towards a source of female sex pheromone is also flying up the odor plume. This coincidence of wind and plume direction in a wind tunnel lent color to the suggestion that chemotactic plume-following and anemotaxis may both be used by flying male moths in finding their way to a distant point source of wind-borne pheromone (Shorey, 1973, 1976; Farkas and Shorey, 1972, 1974). It also suggested an anemotactic mechanism of turning back into a lost plume (Kennedy, 1977). In the field, however, the wind direction is not constant but swings about, causing a plume to meander and "snake." How a male moth's approach path relates to this erratic field plume is unknown; authors concerned with the physical behavior of pheromone plumes in the field (see review by Cardé, 1979; also Aylor, 1976; Aylor et al., 1976; Murlis and Jones, 1981) have not yet dealt specifically with this problem of orientation in relation to a meandering plume (except for Legg and Strange, 1980). Nevertheless, it is already known that at any instant much of such a plume lies more or less across the wind, rendering upwind flight and plume-following incompatible. It has been established by atmospheric

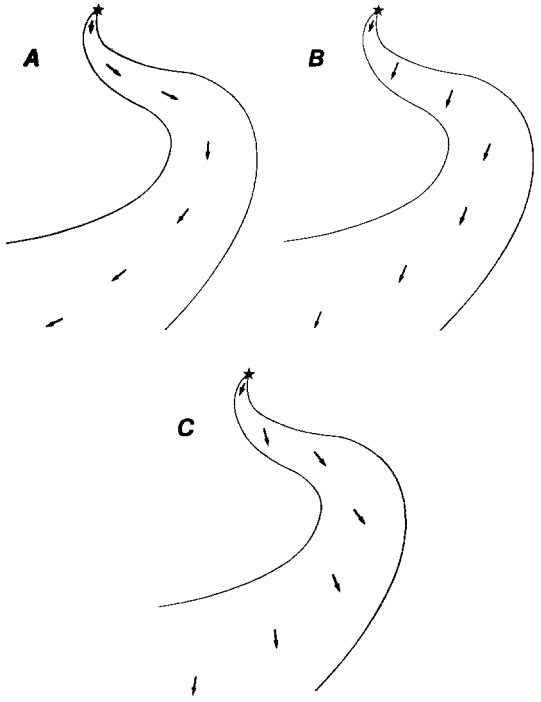

FIG. 1. Wind directions (arrows) within a plume of smoke or other wind-borne material (diagrammatic); stars, smoke sources; curved lines, plume outlines. A and B, naive preconceptions; C, observed.

physicists (Davidson and Halitsky, 1958; Pasquill, 1974) that over short grass in open country each single "parcel" of smoke (or other material) composing a plume is carried away from the source in a relatively straight line for a considerable distance while the plume as a whole may be snaking to and fro across wind. The snake is not the path of the wind: the plume snakes simply because the wind "swings," that is to say the air parcels that arrive in succession to carry material away in straight lines from its source, do so in divergent directions (Figure 1C).

The object of this communication is to illustrate this phenomenon, which may not be familiar to many biologists, and then to draw attention to the logical inference from it that flying along a plume to a distant odor source may be a misconception.

METHODS AND MATERIALS

The horizontal displacement of wind-borne smoke puffs, having an initial diameter of ca. 0.5 m and released at 0.5 m above ground level over short or tussocky grass, was recorded on videotape from towers at Silwood Park, Berkshire, on two occasions with light and variable winds (Table 1). On April 1, 1980, the smoke was emitted continuously from a pyrotechnic generator, and "puffs" were produced by the natural turbulence. For the three sets of recordings on June 30, 1981, regular puffs were produced by injecting diesel oil for 1 sec at 3- to 5-sec intervals into the exhaust pipe of a Swingfog insecticide sprayer. Conspicuous markers were set out over each area at 5-m intervals in a square grid of 25-m sides. Wherever the track of a smoke puff was sufficiently clear, it was first traced as seen in oblique view on the TV monitor screen and then corrected to a plan view for measurement. When a well-defined portion of the outline of a puff could be followed for at least 2 m, its horizontal track was traced for as long as it remained identifiable and close to the ground. The directions of these track segments were the unit measurements used in Figures 2–4 which include more than one segment from some of the puff tracks.

RESULTS AND DISCUSSION

Tracing the instantaneous outline of an entire snaking plume on the TV screen was not often possible because of breaks and diffusion, but when this could be done and the directions of segments of smoke-puff tracks within the plume had also been recorded, as in the examples in Figure 2, then these directions were seen neither to follow the changing direction of the plume axis as in Figure 1A, nor to run parallel with the wind direction seen at the emission point at the same time, as in Figure 1B. Instead, each segment of puff track

TABLE 1. CONDITIONS DURING SMOKE-PUFF TRACKING

Date time	Site topography	Nearest windbreaks	Camera height (m)	Surface	Wind		Air temp. at 1 m (°C)	Cloud cover
					From	Mean speed (m/sec ± SD)		
April 1, 1980 1200–1300 hr	Flat ground at top of a 1 : 10 slope to S and less steep to SE	Woods at ca. 150 m to W, N, and E; 5-m hedge running ESE from 40 m E (=downwind) of smoke emission	30	Grass tussocks	NW–S	1.6 ± 0.5	13	8/8
June 30, 1981 1100–1300 hr	1 : 17 slope rising to SE out of broad hollow, flanked by rises to NE and SW	Tall trees 40 m to NE and SW; building 100 m long 12 m high running NE–SW 75 m to SE (=downwind) of smoke emission	18	Mown grass	SW–N–NE	1) 1.2 ± 1.1 2) 1.5 ± 0.7 3) 2.0 ± 0.7	20	7/8

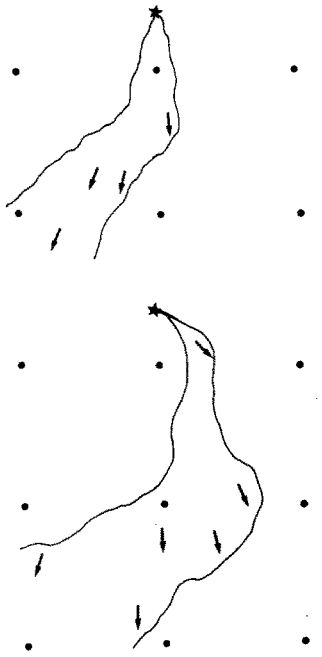

FIG. 2. Wind directions recorded simultaneously within smoke plumes.

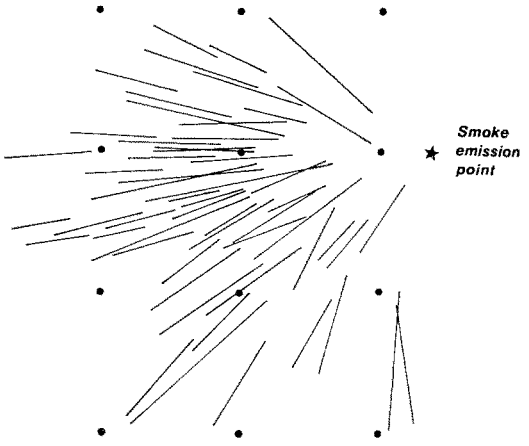

FIG. 3. Segments of tracks of smoke puffs recorded within a 10-min period. Grid markers 5 m apart.

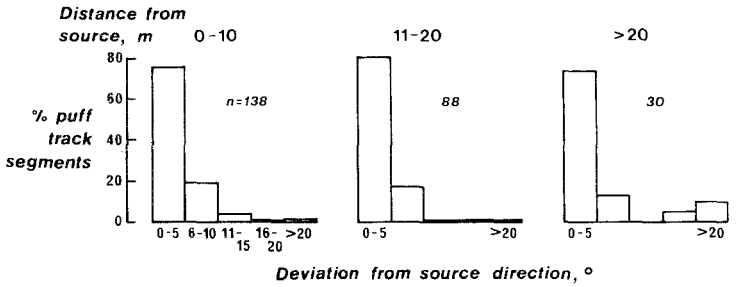

FIG. 4. Frequency distribution of the angle between a recorded segment of the track of a smoke puff and a straight line joining its midpoint to the smoke source; positive and negative deviations pooled.

was a straight line approximately coincident with a straight line extending from that segment to the emission point. Since different puff directions were recorded simultaneously, the wind plainly could not have changed direction over the whole area at once, as pictured in Figure 1B.

The same radiating pattern of puff tracks emerged clearly from each of four full sets of records, illustrated by one example in Figure 3. Some puffs could be traced by eye (on the ground or on the TV screen) traveling continuously in a straight line from the emission point for considerably further than the 25-m maximum range of the marker grid. The high frequency with which the puff track segments lay close to straight lines extending from them to the emission point is indicated by the mean absolute deviation ($4.42 \pm 0.27°$) and is shown for the four sets of records combined in Figure 4. The frequency distributions did not change significantly with distance from the emission point ($\chi^2_8 = 12.88$, $P > 0.05$), confirming the rarity of bends in the tracks despite the nearby windbreaks and slopes (Table 1).

These results are no more than what students of atmospheric diffusion would expect (F.B. Smith, personal communication; Cramer et al., 1959; Gifford, 1959, 1960; Pasquill, 1974). Given that the wind carries odor away from a source in straight lines but shifting directions so that the odor plume "snakes" to and fro across the mean wind direction (Figure 1C), then a flying insect that flew along this snaking plume would often be advancing neither upwind nor towards the odor source. Moving along a plume across wind could even entail travelling downwind with it, away from the source. Or again, if, having once picked up the scent, the insect flew persistently upwind, even during intervals when it had lost the scent temporarily, then too it would often not be flying towards the source. Only scent-laden air will have come from the source and would thus "point the way" to it by upwind flight. Unscented wind will point in other directions so that flying upwind would incur a high probability of not finding a lost plume again and of missing the source. Indeed,

the nearer the insect is to the source while in unscented air, the more serious the consequence of misdirected upwind flight is likely to be; for example, the insect is more likely to move into an area not reached by pheromone from that source.

Neither flying persistently along the plume nor flying persistently upwind therefore appear to be behavioral strategies that would make good use of the directional information available in the field. A much better strategy (Figure 5) would seem to be (1) to fly upwind on encountering the odor, since whenever it is encountered the wind will be coming straight from and thus pointing to the source, and (2) to cease advancing into wind but hold station against it with or without casting across it as soon as the odor disappears from the wind. The use of this pheromone-modulated anemotactic strategy, rather than flying along a plume, is supported by evidence from wind-tunnel experiments (Traynier, 1968; Marsh et al., 1978, 1981; Kennedy et al., 1980, 1981) and field data (Wall and Perry, 1978), especially observations of moths approaching a pheromone source in straight lines, without casting, from widely divergent directions in a fluctuating wind. In the field turbulence is likely to disrupt this strategy of approach to an odor source much less than a strategy that depends on flying along the plume.

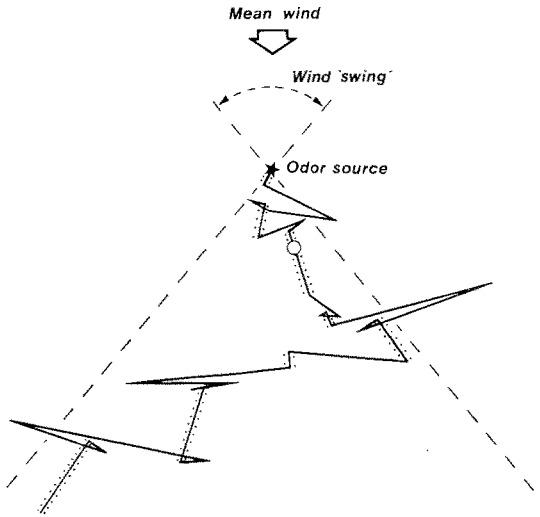

FIG. 5. Hypothetical, schematic track of a flying insect being guided to a small odor source, on the simplified assumptions that: (1) in odor-bearing air the insect flies upwind (lines flanked by dots); (2) in odor-free air it flies across wind in widening sweeps (plain lines), or hovers/lands (circle); (3) its airspeed always exceeds the wind speed; (4) the wind direction swings irregularly up to 80° causing the plume (not shown) to "snake" left and right.

These environmental and experimental objections to a mechanism that depends on flying along the plume apply not only to the chemotactic version of it called "aerial odor-trail following" (Shorey, 1973, 1976), but also to an alternative hypothesis, that the insect switches an obliquely upwind flight track over to the opposite side of the wind line in response to losing the scent, thereby turning back into a plume automatically on emerging from it (Kennedy, 1977; Marsh et al., 1978, 1981); this latter hypothesis has now been contradicted experimentally (Kennedy et al., 1980, 1981). These objections do not apply to the special chemoklinotactic mechanism that may come into play transiently during lulls in the wind (Baker and Kuenen, 1982).

We conclude that the image of insects flying along an odor plume in wind lacks field credibility as well as experimental support, whereas odor-modulated anemotaxis appears to be a mechanism that is well adapted to a range of wind directions. Whether it results in the flier following the plume continuously or meeting it only intermittently will depend on the rate of change of wind direction. When the wind direction is not constant, the plume meanders and the insect might be in contact with the plume for only a fraction of the total time taken to reach the source. The present need is for further field testing of the theory of odor-modulated anemotaxis.

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MICROFLORA AND VOLATILE FATTY ACIDS PRESENT IN INGUINAL POUCHES OF THE WILD RABBIT, *Oryctolagus cuniculus*, IN AUSTRALIA

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Abstract—Contents of inguinal pouches of the wild rabbit, *Oryctolagus cuniculus*, have been examined for the presence of microorganisms and volatile fatty acids. A total of 245 isolates were made from 167 samples taken from 75 rabbits, and microorganisms were obtained from 93% of individuals. The most common microorganism encountered was *Staphylococcus aureus*, followed by the yeast *Candida kruzei*. Other organisms isolated were *Bacillus subtilis*, *Escherichia coli*, and *Streptococcus faecalis*. Quantitative analysis of volatile fatty acids in scrapings from pouches showed considerable variation between individuals. Of the acids identified, acetic acid and isovaleric acid were the most abundant. In a liquid medium containing one of these acids, the growth of microorganisms was only slightly affected. However, growth became inhibited when these fatty acids were used in combination.

Key Words—Rabbits, *Oryctolagus cuniculus*, inguinal pouch, microorganisms, volatile fatty acids, acetic acid, isovaleric acid, odor signals.

INTRODUCTION

Human sweat secreted by apocrine glands is odorless when it appears on the skin surface in the axilla. It is only after a few hours, in the presence of microorganisms, that it acquires an individually characteristic smell (Shelley et al., 1953). The hairs in the axilla act as collecting sites, not only for

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glandular secretion and skin debris, but also for bacteria. Shaving and thorough washing of the axilla may eliminate odor for at least a few hours.

Similarly in other mammals, specially modified sites on the body offer ideal conditions for the collection of glandular secretions and the growth of bacteria which are involved in their fermentation (Mykytowycz, 1970).

Increasing interest in the function of the odoriferous glands in the communication of mammals stimulated a parallel concern for the role of bacteria in shaping the odor profiles.

The bacteriology of the skin gland secretions has already been studied in some species; e.g., the microflora have been described in the anal sacs of the red fox, *Vulpes vulpes* (Ware and Gosden, 1980); in the anal and castor glands of the beaver, *Castor canadensis* (Svendsen and Jollick, 1978); and in the anal pockets of the Indian mongoose, *Herpestes auropunctatus* (Gorman et al., 1974). It has been suggested that microorganisms play an important role in shaping odor signals in mammals and that volatile fatty acids which are frequently found in skin gland secretions could be the products of their fermentation (Albone et al., 1977; Albone and Perry, 1976; Albone, 1977).

The wild rabbit, *Oryctolagus cuniculus*, is well equipped with odor-producing skin glands which function specifically for communication purposes. The glands were the subject of prolonged interdisciplinary studies (Mykytowycz, 1968; Goodrich and Mykytowycz, 1972). The inguinal glands, which communicate identity and sex (Hesterman and Mykytowycz, 1982), secrete into bilateral pouches where the secretion and desquamated epithelium form a thick, waxy sebum which has a strong rabbity odor (Mykytowycz, 1966). These pouch characteristics seem to offer suitable conditions for microorganisms.

Preliminary studies of the chemical composition of inguinal pouch contents indicated the presence of volatile fatty acids. A number of synthetic volatile fatty acids, when presented to rabbits under experimental conditions, caused changes in heart rate (Hesterman et al., unpublished). In the present study an effort has been made to gain some information on the volatile fatty acid profiles and microorganisms in the inguinal pouches and their possible interaction.

METHODS AND MATERIALS

Animals

Of 75 wild-type rabbits sampled, 40 were males, which included four 10-day-old kittens, and 35 were females. All rabbits sampled had been in captivity for at least three months. Nineteen of them were born in captivity, while the others were caught in the field in various localities in the vicinity of

Canberra. Some were kept singly in small wire cages, others lived in 2 × 4-m outdoor pens in groups consisting of three or four individuals.

Sampling

Five bacteriological samplings were carried out at irregular intervals covering a period of 19 months and altogether 167 samples were taken. In the first three samplings only aerobic cultures were made. Both aerobic and anaerobic methods were used for the last two samplings.

The frequency of sampling of the pouch contents from individuals was also irregular, i.e., 84, 197, 231, and 560 days following first sampling. Material was taken once from 30 rabbits, twice from 22, three times from 4, four times from 14, and five times from 5 animals. On one occasion only, swabbings were made of the back and the belly areas of 11 rabbits of different sexes.

Bacteriological Procedures

Collection of Samples. During sampling rabbits were restrained manually. Since inguinal sebum is thick, waxy, and insoluble in water, irrigation by pipet or syringe and needle was impractical. Sterile cotton-wool swabs moistened immediately before use with sterile distilled water were employed to collect samples from pouches.

For all samples, blood agar, serum agar, and Mycosel agar plates, sown immediately from the swabs, were incubated aerobically at 37° C. During the last two samplings, a duplicate set of plates for each sample was incubated anaerobically at 37° C within 5 min of collection using the GAS PAK method and a self-heating catalyst. Following incubation, colonies were counted, and organisms were checked for morphology and Gram reaction. All organisms were immediately subcultured onto horse blood agar plates and subsequently identified by conventional methods.

Inhibition of Growth of Microogranisms by Volatile Fatty Acids. Dilutions of acetic acid or isovaleric acid from 1 : 10 to 1 : 10^6 (v/v) were made in nutrient broth. To each tube was added one standard drop (0.025 ml) of a 24-hr broth culture in the case of *Staphylococcus*, or a 48-hr broth culture in the case of *Candida*. The cultures were then incubated at 37° C for seven days with occasional shaking.

Volatile Fatty Acid Analysis

Sample Preparation. Samples of sebum from the inguinal pouches of individual animals (18 males and 18 females) were collected and stored 3–6 months at −25° C prior to use.

The scrapings (10–20 mg) were triturated with a mixture of 0.5 ml ether and 0.5 ml of 0.1 M hydrochloric acid, and the organic layer was separated

out. The aqueous layer was extracted a further three times with 0.5 ml ether and the combined ether extracts shaken twice with 0.5 ml of 0.05 M sodium hydroxide. The alkaline extract was acidified with 0.4 ml of 5 M sulfuric acid and then steam distilled into 2 ml of 0.05 M sodium hydroxide unitl 15 ml of distillate had been collected. The distillate was reduced to a small volume on a rotary evaporator, transferred to a small glass vial and evaporated to dryness at 40° C under a stream of nitrogen. Just prior to gas chromatographic analysis, 20 μl of water was added to the sample, followed by 30 μl of 7 M hydrochloric acid.

Gas Chromatography. A Varian Aerograph model 204B equipped with flame ionization detectors was used for the analysis of volatile fatty acids in the inguinal pouch scrapings. The test sample (0.5 μl) was injected through a Teflon-faced septum onto the front portion of a nickel column (2 m × 2 mm ID) packed with Chromosorb 101, 100–120 mesh (Johns-Manville). Temperatures were: injectors 220° C; detectors, 250° C; column, 180° C. Gas flow rates were: N_2 carrier gas, 25 ml/min; H_2, 25 ml/min; air, 150 ml/min. Tentative identification of the acids in the samples was based on comparisons of their retention times with those of the authentic compounds. Quantitative determination of the acids in sample solutions was based on comparisons of their peak areas to those of the standard solutions.

RESULTS

One or more species of microorganisms were isolated from the inguinal pouches of 70 (93%) of the 75 rabbits sampled. Cultures from the pouches of two males (5%) and three females (9%) failed to grow microorganisms. Bacteria were found in the inguinal pouches of all the 10-day-old nestlings.

Microorganisms were isolated from 155 (93%) of the total 167 samples. Altogether 245 isolates were cultured, representing six genera with the two most common being the facultative aerobes *Staphylococcus* and *Candida.* In addition to these organisms, *Bacillus subtilis*, *Escherichia coli*, and *Streptococcus faecalis* were occasionally isolated, with *Corynebacterium ovis* found only once.

Most frequently only one genus was cultured from a single sample (76%). Two different genera were found in 20% of samples and three in 4%. There were no obvious sex differences in this respect.

Repeated sampling revealed that the microflora in the inguinal pouches of an individual did not remain constant throughout the period of the study. Thus from the 19 females which were sampled at least twice, only three (16%) carried the same microorganism, and of the 22 males which were sampled at least twice, the same microorganisms were found in only seven (32%). Of the 22 samples cultured from the back and belly of 11 rabbits, a total of only 12 colonies grew.

Staphylococcus aureus. This was the most common isolate. It was cultured from 110 samples (66% of total), and occurred in 83% of all males and 77% of females. Primary culturing on serum agar produced most frequently a moderate growth, i.e., less than 100 colonies per plate. The same organism was also found in 73% of the anaerobic cultures.

Candida kruzei. This facultative aerobic yeast was the second most common organism encountered, being found in 60 samples (39%). It occurred in 50% of males and 60% of females. During primary culturing moderate growth was produced in 86% of the positive samples. From a total of 67 samples cultured anaerobically, 23 (34%) produced *C. kruzei.*

Bacillus subtilis. This organism was found only in nine (23%) males. It was isolated from 11 (7%) of total samples. The cultures yielded a light growth of this organism, except in one case where there was moderate growth.

Escherichia coli. This organism was isolated only during the last two samplings, and only from five females. It appeared in six (4%) of all samples, producing only light growth.

Streptococcus faecalis. This organism was found on only four occasions throughout the sampling period, twice in males and twice in females.

Corynebacterium ovis. This organism was found only in scrapings taken from one male with only three colonies isolated.

Volatile Fatty Acids. The relative quantities of eight volatile fatty acids in scrapings from 18 males and 18 females are shown in Table 1. These are expressed as a range because of the wide fluctuation of results between individuals.

Acetic acid was the most common, constituting on the average approximately 80% of total volatile fatty acids in males and 74% in females. The second most abundant was isovaleric acid (approximately 16% of total in males and 23% in females). Six other acids occurred in much smaller quantities.

The two most abundant acids—acetic and isovaleric—had an effect on the in vitro growth of the most common organisms, *S. aureus* and *C. kruzei.* Acetic and isovaleric acids when used together at dilutions down to 10^3 prevented the growth of the two most common isolates. *Staphylococcus* and *Candida. C. kruzei* appeared to be more affected by the mixture of two acids as there was some effect on growth at dilutions of 10^4. The two acids when used separately were less effective in inhibiting growth of these microorganisms (Table 2).

DISCUSSION

When the pattern of social behavior of the rabbit is considered (Mykytowycz, 1968), it is possible that more selective sampling could reveal a pattern of group, colony, or geographical distribution of specific microflora.

TABLE 1. VOLATILE FATTY ACID COMPOSITION OF INGUINAL POUCH SCRAPINGS FROM MALE AND FEMALE RABBITS, *Oryctolagus cuniculus*[a]

	Composition (mol %)							
	Acetic (C_2)	Propionic (C_3)	Isobutyric (iC_4)	*n*-Butyric (nC_4)	Isovaleric (iC_5)	*n*-Valeric (nC_5)	Isocaproic (iC_6)	*n*-Caproic (nC_6)
Males (N = 18)	79.7	2.3	1.1	0.3	16.1	0.1	0.2	0.3
	(16.5–98.9)	(0.3–9.3)	(0.1–8.3)	(0.1–2.0)	(0.7–84.0)	(0.1–2.1)	(0.1–1.9)	(0.2–4.4)
Females (N = 18)	73.8	0.6	1.0	0.1	23.0	0.9	0.5	0.2
	(13.1–99.1)	(0.1–3.9)	(0.1–7.6)	(0.1–0.4)	(0.1–85.7)	(0.2–11.9)	(0.4–3.3)	(0.3–1.9)

[a]The mean (and range) concentrations of C_2–C_6 acids in samples from 18 male and 18 female rabbits are shown.

TABLE 2. INHIBITORY EFFECT OF ACETIC ACID (C_2) AND ISOVALERIC ACID (iC_5) ON BROTH CULTURES OF *S. aureus* AND *C. kruzei* ISOLATED FROM INGUINAL POUCHES OF WILD RABBITS

Microorganism	Acid	Dilution factor				
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	C_2	–[a]	+	+	+	+
	iC_5	+	+	+	+	+
	$C_2 + iC_5$	–	–	+	+	+
C. kruzei	C_2	+	+	+	+	+
	iC_5	+	+	+	+	+
	$C_2 + iC_5$	–	–	±	+	+

[a] +, growth; ±, retarded growth; –, no growth.

The seemingly high incidence of *S. aureus* and *C. kruzei* found may be peculiar to the experimental colony involved in this study. If microorganisms such as *Staphylococcus*, *Candida*, etc., are indeed frequently carried by the rabbit in its natural environment, it could play some role in the epidemiology of diseases caused by these species in domestic animals.

At this stage one can only speculate that fermentation of the skin gland secretion is necessary for the formation of functional odor signals because the experimental data are inadequate to substantiate such a contention. In fact, observations during the present study and views on the anal glands of the fox (Albone and Perry, 1976) and the bacteriology of the beaver's scent glands (Svendsen and Jollick, 1978), suggest that the microbial populations, and consequently the fermentation of secretions, may not be sufficiently consistent to form a basis for the production of olfactory signals. It is possible, however, that conditions of captivity were responsible for the seemingly high incidence of *S. aureus* and *C. kruzei* and for frequent changes in the microflora profiles of animals used in these studies.

On the other hand, in some instances periodic alterations of the quality of personal odor may even be essential to communicate changes in the behavioral or physiological status of an individual. Thus microbial populations in the human vagina change in response to endocrine factors at puberty and menopause (Brown, 1978). Fluctuation of estrous cycles have been shown to influence short-term changes in microbial flora in the vagina of rats and rhesus monkeys, *Macaca mulatta*, and in the latter may also affect the quality and rate of production of copulins (Bonsall and Michael, 1980). The mongoose *Herpestes auropunctatus* can be trained to differentiate between mixtures of volatile fatty acids (Gorman, 1976). This finding reinforces the

speculation that different mixtures of acids in an animal's odor could be at least partly responsible for individual recognition.

The synergistic inhibitory effect of acetic and isovaleric acids on the growth of microorganisms suggests the existence of an additional mechanism for shaping the odor profiles of the inguinal pouches. Inclusion of other acids may increase the titer of the inhibitory effect. To test more fully the importance of this mechanism, it is necessary to study systematically the fluctuations in levels of the different acids in the same animal in relation to the density of populations of different microorganisms. However, there is a need first of all to establish whether the volatile fatty acids are produced in the glands or are a product of microbial fermentation. In addition, other constituents of the pouch and their contribution to the final odor signals would need to be investigated.

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CUTICULAR HYDROCARBONS OF *Reticulitermes virginicus* (BANKS)[1] AND THEIR ROLE AS POTENTIAL SPECIES- AND CASTE-RECOGNITION CUES

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Abstract—The cuticular hydrocarbon components of four castes of *Reticulitermes virginicus* (Banks) have been identified and quantitated. Components identified include *n*-alkanes; 2-, 3-, 11-, 13-, and 15-methylalkanes; 11,15-dimethylalkanes, (*Z*)-9-alkenes; (*Z,Z*)-7,9-dienes; and (*E*/*Z*)-6,9-dienes ranging in carbon number from C_{21} to C_{40}. All caste forms of *R. virginicus* contained the same components, but showed caste-specific proportions. Comparison of these hydrocarbons with those of the sympatric termite *R. flavipes* (Kollar) suggest that cuticular hydrocarbons might serve as species- and caste-recognition cues. A bioassay was developed to test this species-recognition hypothesis, with the experimental results supporting the hypothesis.

Key Words—Caste, methyl-branched hydrocarbons, *Reticulitermes virginicus, Reticulitermes flavipes*, Isoptera, termites, Rhinotermitidae, methoxy ethers, mass spectra, NMR spectra, bioassay.

[1]Isoptera: Rhinotermitidae.

INTRODUCTION

Hydrocarbons are major components of the cuticular lipids of many insects and have been postulated to function in water retention, protection from abrasion, as barriers to microorganisms, as pheromones, kairomones, and as defense chemicals (Howard and Blomquist, 1982). Complex mixtures of these chemicals, including *n*-alkanes; 2-, 3-, 4-, and 5-methylalkanes; internally branched mono-, di-, and trimethylalkanes; as well as simple-to-complex mixtures of olefinic components, occur in most insects (Jackson and Blomquist, 1976; Nelson, 1978). The only reports on the cuticular hydrocarbons of termites are Moore's (1969) preliminary note on the Australian species *Nasuititermes exitiosus* (Hill) (Termitidae) and our papers describing the hydrocarbons of the North American termites *Reticulitermes flavipes* (Kollar) (Rhinotermitidae) (Howard et al., 1978) and *Zootermopsis angusticollis* (Hagen) (Hodotermitidae) (Blomquist et al., 1979). The hydrocarbon compositions from these three species, representing three phylogenetically remote families, are different, and led us (Blomquist et al., 1979; Howard et al., 1980a) to postulate that these chemicals were being used as species- and caste-recognition cues. The availability of *Reticulitermes virginicus* (Banks), a closely related sympatric species of *R. flavipes*, provided us an opportunity to further test this hypothesis. This paper reports our results.

METHODS AND MATERIALS

Insects. Portions of three colonies of *R. virginicus* were collected from fallen logs in the DeSoto National Forest ca. 32 km north of Gulfport, Mississippi, during January 1977. The three sites were dominated by pine overstory and separated by at least 1.6 km. Termites were removed from the logs and segregated by caste: workers (undifferentiated larvae beyond the third instar), soldiers, nymphs (differentiated larvae beyond the third instar possessing external wing pads), and neotenic reproductives (one colony only) (Miller, 1969). No primary reproductives were found.

Chemical Methods and Materials. Cuticular lipids were extracted by immersing the insects in three successive portions of hexane for 10 min each. Combined samples were concentrated under nitrogen and the hydrocarbons isolated by chromatography on a 3-cm "minicolumn" of BioSil-A (Bio-Rad Labs, Richmond, California)[7] as described earlier (Howard et al., 1978). Alkenes, alkadienes, and alkanes were separated by preparative thin-layer chromatography (TLC) on 20% silver nitrate silica gel G plates developed in

[7] Mention of a company or trade name is solely for identification and does not imply endorsement by the U.S. Department of Agriculture.

hexane. Bands were made visible with Rhodamine 6G under UV light, scraped into a vial, 4 ml of hexane added, the vial centrifuged, and then the hexane solution transferred to another vial and taken to dryness under nitrogen. Straight-chain components were removed from portions of both the total hydrocarbon and alkane fractions by inclusion in Linde 5A molecular sieves in 2,2,4-trimethylpentane (O'Conner et al., 1962).

Hydrocarbons were analyzed by gas–liquid chromatography (GLC) using a Shimadzu GC-6AM gas chromatograph equipped with a dual flame ionization detector and dual 1.83-m × 3-mm ID stainless-steel columns packed with 3% SP-2100 on 100/120 mesh Supelcoport. All GLC analyses utilized temperature programing from 150 to 325° C at 5° / min, with a 3-min initial hold period and a final 10-min hold period. Retention times were compared to those of *n*-alkane standards.

Gas chromatography–mass spectrometry (GC-MS) analyses were performed on either a Hewlett Packard 5710A GC-5983A mass spectrometer interfaced to a Hewlett Packard 5933 data system or on a Varian 204 GC-MAT-CH5(DF) mass spectrometer interfaced to a Varian SS-100 data system. The Varian instrument was used for electron impact–mass spectrometry (EI-MS) and the Hewlett Packard instrument was used for chemical ionization–mass spectrometry (CI-MS). EI-MS were obtained at 70 eV. CI-MS were obtained using ultrapure methane (Airco, Inc.) at a flow rate of 13 ml/min as both carrier and ionizing gas, generating an internal ion source pressure of 0.5 torr. CI spectra were obtained at 200 eV.

Nuclear magnetic resonance (NMR) spectra were recorded with either a Bruker WH-250 spectrometer interfaced to an ASPECT-2000 data system (proton NMR) or a JOEL FX-100 Fourier transform spectrometer operated at 25 MHz ([^{13}C] NMR). Proton spectra were run in a 5-mm tube with $CDCl_3$ as solvent and $CHCl_3$ as internal standard. Carbon-13 spectra were run in a 1-mm sample tube with $CDCl_3$ as the solvent. Computer-generated spectra were produced with either a CDC Cyber-74 computer using the software program LAOCOON III (Bothner-By and Castellano, 1968) or an ASPECT-2000 data system using the software program PANIC.

Infrared spectra (IR) were obtained from neat films on KBr plates in a Perkin-Elmer model 337 grating infrared spectrometer. Positions of double bonds in alkenes and alkadienes were determined by EI-GC-MS analysis of the monomethoxyl derivatives (Howard et al., 1978; Blomquist et al., 1980).

Species Recognition Bioassay. Specimens of *R. virginicus* workers were fixed in formalin–acetic acid–alcohol (FAA) for at least 24 hr, dehydrated in acidified 2,2-dimethoxy-propane for ca. 30 min, and critical-point dried (CPD) with acetone as the transition fluid (Jones and La Fage, 1980). Individual CPD specimens were then either left untreated, treated with 1 μl acetone, or treated with a 1-μl acetone solution containing one termite

equivalent of either *R. flavipes* or *R. virginicus* cuticular hydrocarbons. After air-drying for 1 hr, these four classes of CPD termites were exposed to five groups each of 30 workers of *R. virginicus* for 1 hr, and the behavioral responses of the termites noted. Since no response other than an infrequent brief antennation was noted for CPD specimens either untreated or treated with acetone only, these treatments were left out of subsequent bioassays.

Behavioral responses of *R. virginicus* workers and soldiers to CPD specimens containing cuticular hydrocarbons were then quantified. Observational units consisted of 10 plastic cylinders 5.0 cm in diameter by 3.5 cm high, containing a single 47-mm-diameter cellulose filter pad (ca. 0.5 g, Gelman Instrument Co.) which had been moistened with 1 ml deionized water. A single hydrocarbon-treated CPD specimen was randomly assigned to each hemisphere of the pad in each cylinder and placed in as natural a posture as possible. Twenty-nine *R. virginicus* workers and one *R. virginicus* soldier were then added to each cylinder and allowed to acclimate for 10 min. One-minute observation periods were then begun, recording the interaction of the termites with one of the CPD specimens. The choice of which CPD specimen to begin the process with was determined by flipping a coin. The process was repeated three times for each experimental unit, producing a total of 30 one-minute paired observation sets. Responses quantified were number of antennations per minute, number of grooming events per minute, number of "pushes" per minute, and number of "bites" per minute. Pushing involved the *R. virginicus* worker or soldier antennating the CPD specimen briefly, backing up slightly, and then forcibly moving forward and striking the abdomen of the treated specimen with its head capsule. Since CPD specimens are somewhat lighter than live termites, they were frequently displaced from their original location when this behavior occurred. The data were analyzed using a paired t test (Sokal and Rohlf, 1981).

RESULTS

Each caste form of *R. virginicus* contains the same hydrocarbon components (Table 1), but the relative abundance of each component varies from caste to caste (Table 2). Eight classes of hydrocarbons are present: (1) *n*-alkanes; (2) 2-methylalkanes; (3) 3-methylalkanes; (4) internally branched 11-, 13-, and 15-methylalkanes; (5) 11,15-dimethylalkanes; (6) (*Z*)-9-monoenes; (7) (*Z,Z*)-7,9-dienes; and (8) (*E/Z*)-6,9-dienes.[8] The *n*-alkanes had retention times identical to those of standard *n*-alkanes, and were completely removed by the molecular-sieve treatment. They also gave EI and CI mass spectra identical to standard *n*-alkanes.

[8] The designation *E/Z* indicates that one double bond is *cis* and one is *trans*, but which is which is unknown.

TABLE 1. CUTICULAR HYDROCARBONS OF *Reticulitermes virginicus*

Component	Carbon number[a]	Diagnostic MS ions[b,c]
n-C_{21}	21	296
n-C_{22}	22	310
11-MeC_{22}	23	168/169, 182/183, 324
(*Z*)-9-C_{23}	23:1	322 (157, 171, 227, 241)
(*E*/*Z*)-6,9-C_{23}	23:2	320 (115, 129, 155, 169, 227, 241, 267, 281)
n-C_{23}	23	324
11-MeC_{23}	24	168/169, 196/197, 338
2-MeC_{23} + 3-MeC_{23}	24	294/295, 322/323, 338; 280/281, 308/309, 338
n-C_{24}	24	338
11-Me + 13-MeC_{24}	25	168/169, 210/211, 352; 182/183, 196/197, 352
2-MeC_{24}	25	308/309, 336/337, 352
(*E*/*Z*)-6,9-C_{25}	25:2	348 (115, 129, 155, 169, 255, 269, 295, 323)
(*Z*)-9-C_{25}	25:1	350 (157, 171, 255, 269)
n-C_{25}	25	352
11-Me + 13-MeC_{25}	26	168/169, 224/225, 366; 182/183, 210/211, 366
(*Z*,*Z*)-7,9-C_{25}	25:2	348 (129, 169, 255, 295)
2-Me + 3-MeC_{25}	26	322/323, 350/351, 366; 308/309, 336/337, 366
n-C_{26}	26	366
11-Me + 13-MeC_{26}	27	168/169, 238/239, 380; 182/183, 210/211, 380
2-Me + 3-MeC_{26}	27	336/337, 364/365, 380; 322/323, 350/351, 380
n-C_{27}	27	380
11-Me + 13-MeC_{27}	28	168/169, 252/253, 394; 182/183, 224/225, 394
n-C_{28}	28	394
11-MeC_{28}	29	168/169, 266/267, 408
n-C_{29}	29	408
11-MeC_{29}	30	168/169, 280/281, 408
11-Me + 13-Me + 15-MeC_{31}	32	168/169, 308/309, 450; 182/183, 280/281, 450; 224/225, 252/253, 450
11,15-diMeC_{31}	33	168/169, 238/239, 252/253, 322/323
11-Me + 13-MeC_{33}	34	168/169, 322/323; 182/183, 294/295
11,15-diMeC_{33}	35	168/169, 238/239, 280/281, 350/351
11-Me + 13-Me + 15-MeC_{35}	36	168/169, 350/351; 182/183, 308/309; 224/225, 280/281

TABLE 1. *(Continued)*

Component	Carbon number[a]	Diagnostic MS ions[b,c]
11,15-diMeC$_{35}$	37	168/169, 238/239, 308/309, 378/379
11-MeC$_{37}$	38	168/169, 378/379
11,15-diMeC$_{37}$	39	168/169, 238/239, 336/337, 406/407
11-MeC$_{39}$	40	168/169, 406/407

[a]Determined from CI-MS where $(M-1)^+$ is always the base peak.
[b]EI-MS.
[c]Ions in parentheses are for the monomethoxyl derivative of the olefin.

The monomethylalkanes and dimethylalkanes were readily characterized by mass spectrometry. CI-mass spectra of hydrocarbons have as their base peak the $(M-1)^+$ ion, thus directly giving the molecular weight of the branched alkane (Howard et al., 1980b). Location of the branch points was achieved by comparing EI-MS and CI-MS fragmentation patterns (Jackson and Blomquist, 1976; Nelson, 1978; Howard et al., 1980b). The diagnostic ion fragments are listed in Table 1.

The location of the double bonds in the three classes of olefins present in *R. virginicus* was determined by methoxymercuration–demercuration fol-

TABLE 2. AVERAGE PERCENT COMPOSITION AND SELECTED RATIOS[a] OF CUTICULAR HYDROCARBON CLASSES FROM THREE COLONIES OF *Reticulitermes virginicus* (BANKS)

	Caste			
Class	Worker	Soldier	Nymph	Neotenic[b]
Saturated alk.[c]	72.1 ± 11.6	56.8 ± 7.1	53.5 ± 14.0	81.8
Olefins[c]	28.0 ± 11.6	43.2 ± 7.1	46.6 ± 14.0	18.2
n-Alkanes	27.5 ± 3.7	27.8 ± 6.7	21.2 ± 5.1	30.7
Branched alk.	44.6 ± 8.6	29.1 ± 0.7	32.3 ± 8.9	51.2
Monomethyl alk.	39.5 ± 7.4	26.3 ± 0.7	28.6 ± 7.9	42.9
Dimethyl alk.	6.0 ± 1.8	2.7 ± 0.1	3.7 ± 1.0	8.3
Ratio: Br/*n*-alk.	1.6 ± 0.2	1.2 ± 0.2	1.5 ± 0.2	1.7
Ratio: MonoMe/DiMe	8.3 ± 1.1	11.8 ± 1.2	6.8 ± 0.5	5.4
Ratio: Sat/Unsat.	4.6 ± 1.7	1.6 ± 0.5	2.2 ± 1.3	4.6

[a]All values except those for neotenics reported as mean ± SD.
[b]Values from one colony only.
[c]Summation of saturated alkanes and olefins equals 100%.

lowed by EI-MS (Blomquist et al., 1980). All monoenes had a *cis* (*Z*) double bond (as evidenced by $AgNO_3$ TLC R_f values and absence of absorption at 970 cm^{-1} in the IR spectrum) between carbons 9 and 10. Two classes of dienes are present. The conjugated (*Z,Z*)-7,9 components are identical to those earlier reported from *R. flavipes* (Howard et al., 1978). The second class of diene, however, has not been previously reported from insects. Mass spectral (EI) examination of the total hydrocarbon mixture indicated that the GC peak containing 2-methyltetracosane and (*Z*)-9-pentacosene also contained a 25-carbon diene (mol wt = 348). The double bonds were not conjugated since the known (*Z,Z*)-7,9-pentacosadiene eluted at somewhat longer retention times. Also, the R_f value of the unknown diene on $AgNO_3$ TLC was intermediate between that of standard (*Z,Z*)-6,9-heptacosadiene and that of the (*Z,Z*)-7,9-C_{25}-diene. Infrared analysis of the TLC purified unknown C_{25} diene component showed moderate absorption at 3010 cm^{-1} (olefinic C—H), weak absorption at 1370 cm^{-1} (*cis* olefin) and moderate absorption at 970 cm^{-1} (*trans* olefin). These values were compatible with a *cis,trans* (*Z,E*) diene. Methoxymercuration–demercuration proceeded slowly, but ultimately yielded the desired monomethoxy adducts retaining one double bond.

Examination of these products by EI-MS showed that the double bonds

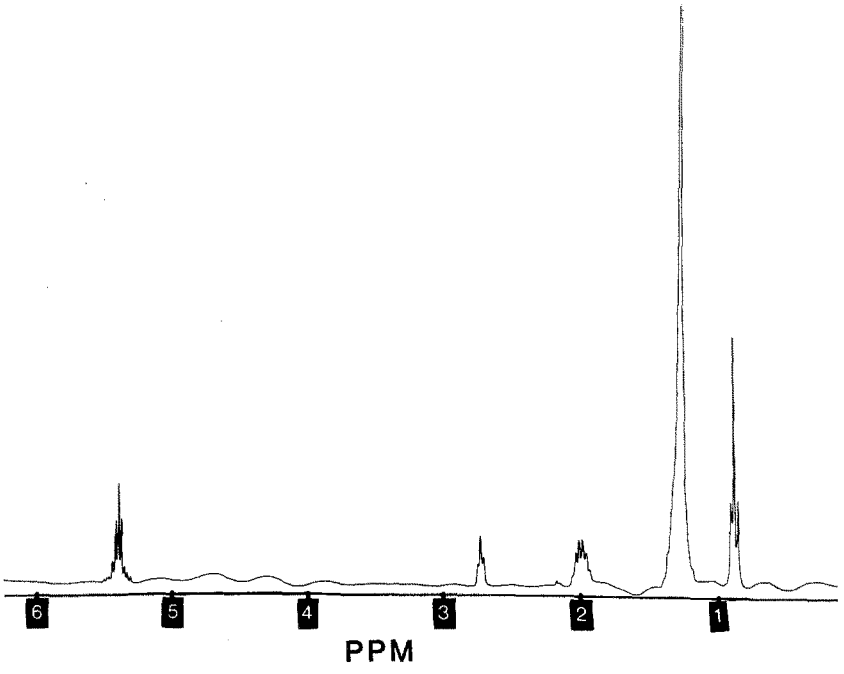


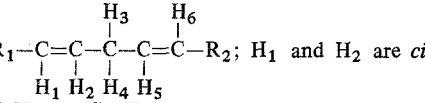
FIG. 1. 250 MHz PMR spectrum of (*E*/*Z*)-6,9-pentacosadiene.

were located between carbons 6 and 7 and 9 and 10 (Table 1). Confirmation of this was obtained by examination of both proton and [^{13}C]NMR spectra. The PMR spectrum (Figure 1) consisted of a 6-proton triplet at δ 0.88, a 32-proton broad singlet at δ 1.25, a 4-proton multiplet at δ 2.00, a 2-proton triplet at δ 2.72, and a 4-proton multiplet at δ 5.39. This spectrum is diagnostic for a long-chain hydrocarbon containing two double bonds separated by a single methylene group. Double-bond stereochemistries were determined by decoupling the internal methylene protons at δ 2.72 and the external methylene protons at δ 2.00, respectively. Chemical shifts and coupling constants were calculated from these spectra. Computer simulations were then carried out to confirm the values so obtained. Table 3 presents the experimental and computer-simulated values for the chemical shifts and coupling constants of the vinyl and allylic protons. The coupling constants for the vinyl protons (J = 11.0 Hz and J = 15.6 Hz) clearly indicate that one of the double bonds is *cis* and the other is *trans*. All of the coupling constants are $\pm$ 0.5 Hz because of the wide linewidths.

The proton-decoupled CMR spectrum of the unknown C-25 diene

TABLE 3. EXPERIMENTAL AND THEORETICAL CHEMICAL SHIFTS AND COUPLING CONSTANTS (Hz) FOR VINYLIC AND ALLYLIC PROTONS OF THE (*E*/*Z*)-6,9-PENTACOSADIENE ISOLATED FROM *Reticulitermes virginicus*

Assignment[a]	Experimental value[b]	Theoretical value[c]
H_1	1352.0	1352.1
H_2	1341.0	1341.0
H_3	680.7	680.7
H_4	680.7	680.7
H_5	1344.4	1344.4
H_6	1359.3	1359.3
$J_{1,2}$	11.0	11.0
$J_{2,3}$	6.3	6.3
$J_{2,4}$	6.3	6.3
$J_{3,5}$	6.3	6.3
$J_{4,6}$	6.3	6.3
$J_{5,6}$	15.6	15.6

[a] 

; H_1 and H_2 are *cis*; H_5 and H_6 are *trans*. R_1 and R_2 equal C_5H_{11}- or $C_{15}H_{31}$-.

[b] Determined by decoupling the internal methylene protons at δ 2.72 and the external methylene protons at δ 2.00.

[c] Generated by computer simulation using the software programs LAOCOON III or PANIC.

TABLE 4. CMR SPECTRAL DATA FOR (*Z,Z*)-6,9-HEPTACOSADIENE AND (*E/Z*)-6,9-PENTACOSADIENE

Carbon number	Chemical shift (ppm)	
	(*E/Z*)-6,9-C_{25}	(*Z,Z*)-6,9-C_{27}
1, 25(27)	14.2	13.9
2, 24(26)	22.8	22.8
3	31.5	31.7
4 (24)	29.4	29.5
5, 11	27.3, 32.6	27.4
6, 10	130.6, 130.9	130.3
7, 9	127.9, 128.5	128.1
8	30.5	25.8
12–22	29.7	29.8
23 (25)	32.1	32.1

contained 13 lines. The chemical shift and structural assignment of each line are shown in Table 4. For comparison, the proton-decoupled CMR spectral data of (*Z,Z*)-6,9-heptacosadiene are also presented in Table 4. The presence of two pairs of signals at ca. 128 and 130 ppm in the diene from *R. virginicus* is consistent with one of the double bonds having an *E* configuration and the other having a *Z* configuration. Only one line at ca. 128 ppm and one line at ca. 130 ppm is found in the (*Z,Z*)-C_{27}-diene. In addition, the signal from carbon 8 has shifted from 25.8 ppm in the (*Z,Z*)-6,9-C_{27}-diene to 30.6 ppm in the *R. virginicus* C_{25} diene, and the signal at 27.4 ppm from carbon 5 or 11 of (*Z,Z*)-C_{27}-diene has shifted to 32.1 ppm in the *R. virginicus* diene. These shifts are consistent with one of the double bonds in the termite diene having an *E* configuration, since it is known that carbons alpha to *E* double bonds have chemical shifts 4–6 ppm downfield from carbons alpha to a *Z* double bond (Stothers, 1972).

All of our evidence thus agrees that the *R. virginicus* diene is (*E/Z*)-6,9-pentacosadiene. Despite intensive efforts we have not yet been able to specify which double bond is *cis* and which is *trans*. Research continues on this problem.

Although all castes examined contained the same cuticular components, the relative proportions of branched to normal alkanes, monomethyl to dimethylalkanes, and saturated to unsaturated components appear to be caste-specific (Table 2).

Different behavioral responses were produced by termites exposed to conspecific CPD specimens containing their own or *R. flavipes* cuticular hydrocarbons. Antennation was the most frequently observed behavior, with 4.7 ± 2.49 ($\bar{X} \pm$ SD) events per minute occurring with *R. virginicus*

hydrocarbon-treated specimens, and 6.17 ± 2.83 events per minute occurring with *R. flavipes* hydrocarbon-treated specimens. These means are significantly different (paired *t* test, $t_s = 4.2328$, $P \geq 0.001$). Although not quantitated, the duration of each antennation event appeared to be somewhat greater with the *R. flavipes* hydrocarbon-treated specimens. Grooming behavior towards the *R. virginicus* hydrocarbon-treated CPD specimens occurred 0.37 ± 0.81 times per minute. No grooming behavior occurred towards the *R. flavipes* hydrocarbon-treated specimens. Pushing occurred 0.43 ± 0.97 times per minute with the *R. flavipes* hydrocarbon-treated specimens, but never with the *R. virginicus* hydrocarbon-treated specimens. Finally, a low frequency of actual biting of the CPD specimen by *R. virginicus* workers was noted in the *R. flavipes* cuticular hydrocarbon treatments (0.07 ± 0.37 times per minute), but never in the *R. virginicus* hydrocarbon treatments.

DISCUSSION

Subterranean termites live in a closed, low-volume system of interconnecting galleries distributed between the soil and substantial masses of wood (Howard and Haverty, 1980). Since the gallery system is totally dark, and essentially static with respect to air movement, the primary sensory modalities of the termites are likely to be tactile and chemosensory ones. From our comparisons of the cuticular hydrocarbons of three phylogenetically diverse termites, we earlier postulated (Blomquist et al., 1979; Howard et al., 1980a) that these chemicals might be serving as important species- and caste-recognition cues. None of these three species is sympatric with one another, and thus does not provide a good test of our hypothesis. *R. virginicus* and *R. flavipes*, however, are sympatric across much of their range and occupy similar ecological niches. Although *R. virginicus* and *R. flavipes* share some cuticular hydrocarbon components (*n*-alkanes, 2- and 3-methylalkanes, (*Z*)-9-alkenes, and (*Z,Z*)-7,9-dienes), they differ substantially in other components (presence or absence of internally branched monomethylalkanes, type of dimethylalkane, and presence or absence of (*E*/*Z*)-6,9-dienes) (Table 5). The differences probably are great enough to enable the termites to ascertain whether or not another termite is of the same species. Support for this assertion comes from our behavioral studies.

Unlike hymenopterous social insects, the responses of *Reticulitermes* spp. to nonconspecific intruders does not include immediate agonistic behaviors (at least under laboratory conditions). Instead, the termites, upon contacting an alien organism, briefly investigate it via antennation. This frequently causes the intruder to quickly draw back a short distance. These antennation–withdrawal interchanges may continue for intervals of up to 30–45 min, although each contact–investigation session by the termites

TABLE 5. CHARACTERISTIC CUTICULAR HYDROCARBON CLASSES OF *Reticulitermes flavipes* AND *Reticulitermes virginicus*

R. flavipes[a]	*R. virginicus*
n-Alkane	*n*-Alkanes
2-Methylalkanes	2-Methylalkanes
3-Methylalkanes	3-Methylalkanes
5-Methylalkanes	11-Methylalkanes
5,17-Dimethylalkanes	13-Methylalkanes
(*Z*)-9-Alkenes	15-Methylalkanes
(*Z*,*Z*)-7,9-Dienes	11,15-Dimethylalkanes
	(*Z*)-9-Alkenes
	(*Z*,*Z*)-7,9-Dienes
	(E/Z)-6,9-Dienes

[a]Howard et al. (1978, 1980a).

appears to last longer than the previous one. At some point in this sequence the workers (or soldiers) begin to aggressively interact with the intruder, pushing with their head capsule or attempting to bite with their mandibles. Alternatively, they may attempt to divert the alien organism into a side galley, where it can be walled off.

We have expended considerable effort in developing a species-recognition bioassay that would reflect the above behaviors. Our earlier efforts included the presentation of either dead termites with appropriate hydrocarbon treatments or various inert dummies with hydrocarbon treatments. The dead termites elicited either a feeding response (cannibalism) or a burial response (identical responses were obtained with either conspecific or alien termites). Responses to treated inert dummies never involved more than cursory examination via antennation. We thus concluded that any bioassay method must include a test object that rather closely mimicked the morphology of the termites, and that it must not contain chemicals which elicit a feeding or burial response. Critical-point dried termites seem to meet all of these criteria: they are morphologically correct and chemically inert. Indeed, application of cuticular hydrocarbon mixtures to the CPD specimens elicited the same behavioral responses noted towards either live conspecifics (antennation and grooming) or live aliens (antennation, "pushing," and "biting"). The relatively low frequency of the biting response is likely a result of the lack of movement by the CPD specimen. Unfortunately, these test specimens are fragile and, hence, not suitable for attaching to some device which could simulate movement.

Additional support for our assertion that these hydrocarbons are serving as species recognition cues comes from our finding (Howard et al., 1980a) that

the staphylinid beetle *Trichopsenius frosti* (Seevers) has achieved integration into the social structure of *R. flavipes* largely by chemical mimicry, i.e., its cuticular hydrocarbon components are identical to those of its host termites. We have, in addition, found that the three termitophiles (representing two subfamilies of Staphylinidae) associated with *R. virginicus* possess identical cuticular hydrocarbon components as their host termite (unpublished data).

Not only do these cuticular hydrocarbons serve as potential species-recognition cues, but they also are potentially important caste-recognition cues. In all termite species examined, every caste possesses distinctive proportions of each of the classes of hydrocarbons characteristic of its species. The castes of *Z. angusticollis* (Blomquist et al., 1979) can be characterized by the ratio of their branched to normal components. Those of *R. flavipes* (Howard et al., 1978) can be characterized by the ratios of branched to normal components and saturated to unsaturated components, and those of *R. virginicus* by the ratios of monomethyl-to dimethylalkanes and saturated to unsaturated components. A critical evaluation of the importance of cuticular hydrocarbon proportions as caste-recognition cues must await the development of behavioral studies to elucidate caste-specific behavioral patterns. We also cannot at present rule out the possibility that additional chemical or mechanical cues might also be of importance for both species and caste recognition.

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OLFACTORY RECOGNITION OF INDIVIDUALS BY MALE CAVIES (*Cavia aperea*)

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Abstract—In one study, using a habituation procedure, male South American cavies, *Cavia aperea*, distinguished individual differences in odors collected from three sources: perineal gland secretions, urine, and supracaudal gland secretions. In a second study, male cavies spent more noninvestigatory time and rested more on the side of a cage containing the odor of a familiar subordinate male as compared to the cage side containing the odor of a familiar dominant male. Since the odor source was a glass plate which had been left in the home cage of the donor males for three days, the actual odorous cue to which the test males responded is not known. These studies demonstrate that male cavies distinguish odors of individuals, that individual differences in odors are found in at least three different sources, and that cavies use chemical cues to distinguish between known individuals.

Key Words—Chemical communication, individuality, Caviidae, *Cavia aperea*, mammals, scent marking, odors.

INTRODUCTION

The ability to recognize conspecifics as individuals has several advantages to mammals. These include recognizing nongroup members, dominant individuals, potential mates, and strange young (see Brown, 1979). The use of olfactory cues by mammals for the identification of individuals has long been assumed, but conceptual difficulties have hampered interpretation of experimental demonstrations (see Brown, 1979; Müller-Schwarze, 1974; Johnson, 1973).

Beer (1970) and Snowdon and Cleveland (1980) listed three types of studies of individual recognition: (1) demonstrations of physical differences

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between individuals (usually chemical, visual, or vocal); (2) demonstrations that animals respond differently to specific individuals during spontaneous social interactions; and (3) presentations of isolated cues from different individuals and demonstration of patterns of differential responses to these cues. Studies which could be categorized as type 3 have examined visual (e.g., Marler, 1965) and auditory (e.g., Snowdon and Cleveland, 1980; Beer, 1970) cues and have provided examples of individual recognition.

For chemical communication, a variety of experimental paradigms, permitting a variety of conclusions, have been used within the broad context of type 3 studies. The ability of animals to discriminate among odors from different individuals has been demonstrated using habituation procedures. Here, the animal is habituated to odors from animal A and then a differential response to odors from animal B relative to odors from animal A proves discrimination (e.g., Schultz-Westrum, 1965; Krames, 1970). While this methodology clearly establishes the fact that an animal discriminates between samples of odors drawn from different individuals, it leaves open the role that familiarity plays in mediating this response. A variety of studies have demonstrated that animals respond differentially to novel vs. familiar stimuli (e.g., Bronson, 1968), and it may be difficult to determine whether the subject is making the distinction between animal A and animal B or between the class of familiar odors compared with unfamiliar odors. Studies on individual recognition which employ learning paradigms demonstrate that an animal can be trained to discriminate between odors of different individuals (e.g., Bowers and Alexander, 1967; Dagg and Windsor, 1971). Generally, the individual odors are of equal familiarity. However, in such studies, the biological significance of the discrimination of individual differences cannot be determined since the training procedures are generally far removed from those that might occur in a species-typical environment. Another group of studies has demonstrated that an animal behaves differently in the presence of an odor of an individual with which it has had an interaction compared with the odors of other (usually less familiar) animals (e.g., Carr et al., 1970; Epple, 1973; Nyby et al., 1970; however, Müller-Schwarze and Müller-Schwarze, 1972, did balance familiarity in their study of individual recognition in three male pronghorn antelopes). This paradigm comes closest to demonstrating that odor cues signaling individual identity are likely used under normal conditions. However, even among many of these studies, the familiarity of the odors of the two individual odor-donors was not balanced, thereby permitting a possible confusion in interpretation.

One of the important functions of an individual's odors may be the marking of a territory or home area (see Brown, 1979; Johnson, 1973). As noted by Johnson (1973), "It is . . . reasonable to suppose that in the course of repeated territorial encounters, scent marks of another animal might acquire

aversive properties through a learning process" (p. 528). Brown (1979) has suggested, "Animals losing a fight might benefit from avoiding the individual that won the fight, but would not need to avoid all other conspecifics. Likewise, territorial animals might learn the individual odor of their neighbors" (p. 112). Further, "A defeated male may avoid . . . the odor of the individual that defeated him, but might still show a great deal of investigation of this odor" (p. 112). Thus, a bioassay for individual recognition cannot simply measure olfactory investigation of individual odors, but must be sensitive to subsequent behaviors occurring as a consequence of the olfactory investigation.

The South American cavy, *Cavia aperea*, the wild congeneric of the domestic guinea pig, *C. porcellus*, was the subject of this investigation of individual recognition. Previous studies have indicated that these cavies are social and are able to distinguish individuals, as evidenced by a strict social hierarchy (Rood, 1972), and that the chemical composition of perineal secretions varies between individuals (Wellington et al., 1979). Individuals may vary also in the chemical makeup of their supracaudal gland secretions (see Martan, 1962). Previous studies have shown *C. porcellus* urine to carry much intraspecific information (Beauchamp, 1973, 1976; Beauchamp and Berüter, 1973; Beauchamp et al., 1979). Male *C. porcellus* can distinguish their own perineal secretions (Berüter et al., 1974) and urine (Beauchamp, 1973) from that of other males. This study presents evidence for the ability of *C. aperea* to distinguish between odorous cues isolated from different sources from different individuals, and for the ability of cavies to use olfactory information to recognize specific individuals.

METHODS AND MATERIALS

Experiment 1. The aim of this experiment was to test the ability of cavies to distinguish between isolated cues from individuals. This was determined through tests with urine and secretions from perineal and supracaudal glands. A habituation-preference methodology, similar to that of Schultze-Westrum (1965) and Halpin (1974), was employed.

A test group of 12 adult male cavies, all familiar with glass plates (7.5 by 15 cm) as a repository of conspecific odors, was used. These animals, born in captivity, were descended from 5 males and 11 females wild-trapped in the fall of 1974 outside Buenos Aires, Argentina. An equal number of males from the same colony was used as odor donors. During testing, each animal was presented with an odor stimulus which was placed in the center of a glass plate which remained in the animal's cage for one minute. Thirty seconds after removal of the plate, a fresh sample of the odor from the same donor was

presented on a clean glass plate for an additional minute. This procedure was repeated three times (habituation phase). Thirty seconds after the third presentation, the animal was presented with two glass plates, one containing the odor to which he had been habituated (familiar odor), the other containing the same odor source from another individual (novel odor). The amount of time spent investigating each odor, as measured by the time during which the animal's nose was within 1 cm of the test substance, was recorded during 2 min following presentation. Each donor animal was used with two different test animals, once as a source of the familiar odor, once as a source of the novel odor, thus eliminating any bias from a possibly more attractive or repellent donor. Pairings of donors with test animals and side of novel odor during the preference test were randomized. Each animal was tested once with each of the three odor sources. Differential responses to the odors in the preference phase demonstrates an ability to distinguish the two.

The group was initially tested with supracaudal gland secretions. One month later, they were tested with urine, and one month after that they were tested with perineal gland secretions.

The test population distinguished individual odors emanating from urine (correlated $t_{11} = 3.21$, $P < 0.01$), perineal gland ($t_9 = 3.26$, $P < 0.01$), and

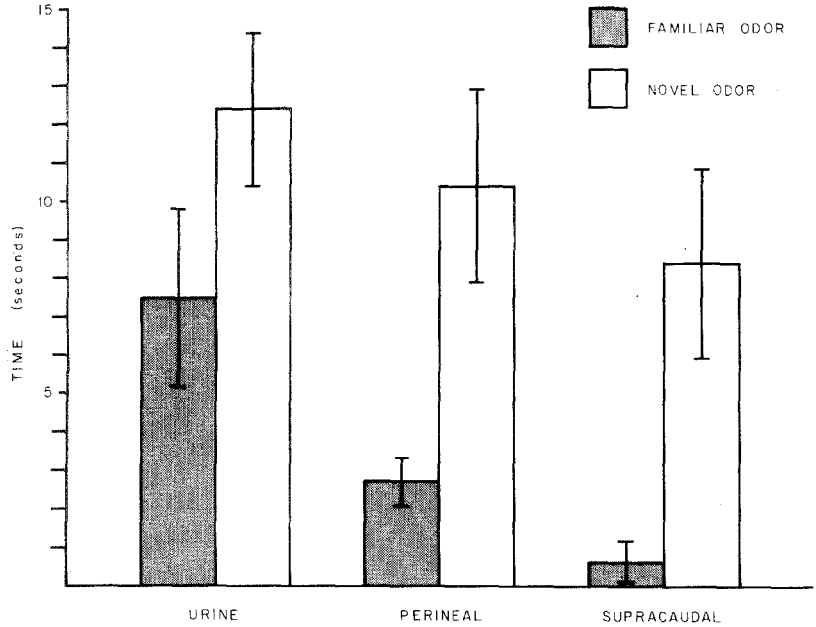

FIG. 1. Results of preference phase of habituation-preference testing of individual odors. The familiar odor is the one to which the test animals had been habituated. See text for details.

supracaudal gland ($t_{10} = 2.96$, $P < 0.01$) (Figure 1). In all cases, the novel odor elicited more investigation than did the familiar odor. The number of test animals varied from 12 due to death and addition of new members to the test group during the study period.

Cavies distinguished individual differences emanating from three odor sources. Each of the three substances could be used for marking and is generally investigated by conspecifics (see Rood, 1972; Beauchamp, 1973; Wellington et al., 1979). This experiment, like most previous studies of individual recognition, found that the cavies distinguished among samples of odors from different individuals. We performed experiment 2 to determine whether cavies responded to individual differences in odor cues in ways consistent with expected responses to the whole animal. Furthermore, this study was designed so that the familiarity of the odors of two different individuals tested was equalized and the differential response to the odors could be explained only on the basis of differential experiences with the sources of the odors.

Experiment 2. Wild male cavies are usually intolerant of conspecific males and exhibit a clear dominance hierachy (Rood, 1972). These dominance relationships were exploited to study individual recognition. We hypothesized that a cavy in a stressful situation would avoid dominant males and not avoid subordinate males, and that olfactory cues alone, in a stressful situation, would produce analogous results. If this were the case, then animals would be making a decision based on prior experience with odors of different individuals. This type of recognition and decision may be similar to what would be expected of territorial mammals coming across the scent marks of conspecifics in a natural situation. We also controlled for familiarity of donor animals by giving equal experience with dominant and subordinant donors.

Twelve adult cavies, not used in experiment 1, were selected from the captive colony. These males, isolated in 25 × 41 × 18-cm or 25 × 62 × 18-cm cages before puberty, ranged in age from approximately 6 to 11 months at the start of the study. These animals were given experience with various odors (e.g., female cavy urine, perineal gland secretions) to familiarize them with glass plates as a source for conspecific odors.

Test animals were paired in clean 25 × 62 × 18-cm cages for 15 min or until a fight ensued. Initial pairings were made by selecting animals of similar weights. Behavior patterns were recorded, and after five days of pairings, one male of each pair was judged to be dominant. Dominance was determined by the ability to displace the other male, prevent the other male from leaving a cage corner, or a chase of or attack upon the other male. Subsequent pairings were made by selecting two animals which were dominant in the previous pairings, or two which were subordinant. These subsequent pairings were identical to the initial pairings but were made during 3 or 4 days. Pairs were established until 10 of the 12 males had at least one animal to which they were

dominant and at least one animal to which they were subordinate. During the two days prior to testing, pairings were reestablished to refamiliarize each animal with the two individuals with whose odors he would be tested.

During testing, each of the 10 males was placed individually in a clean 25 × 62 × 18-cm cage, one side of which contained a glass plate (15 × 15 cm) which for three days had been in the home cage of the dominant male; the other side contained a plate from the subordinate male. The male was monitored for 4 min by two observers, during which total time spent investigating each plate and total time on each cage side was recorded. Analysis was performed on total noninvestigatory time on each cage side. The animals were observed for an additional 6 min to determine their final resting (nonambulatory for at least 30 sec) location, if any. Three days later, the procedure was repeated; however, the sides of the dominant and subordinate males' plates were reversed for each test animal.

Analyses were based on the totals from the two test sessions. The noninvestigatory time spent by the test males on the side containing the odor from the subordinate animal (218.7 ± 11.7 sec; mean ± SE) was significantly greater than the time spent on the side containing the odor from the dominant animal (170.7 ± 10.6; correlated $t_9 = 2.44$, $P < 0.05$) as hypothesized. However, investigation of the odors themselves was not different (49.10 ± 9.85 sec for dominants' odors, 41.55 ± 4.59 sec for subordinates' odors).

During the two test sessions, seven of the 10 test animals came to rest during the 10 min of observation. Four of these males rested only once, at all times over the plate of the animal to which they were dominant. Three males came to rest during both trials, two of these rested both times over the plate of the animals to which they were dominant, one animal rested once over the dominant and once over the subordinant side. Thus, 6 of the 10 animals rested exclusively on the side of the odor of the submissive animal while none rested exclusively on the side containing the odor of the dominant animal ($P = 0.016$; binomial test).

These results suggest that cavies recognize specific individuals given only olfactory cues. As hypothesized, the animals tended to avoid the odors from a dominant male and/or were attracted to the odors from a subordinant male. The methodology does not, however, allow us to distinguish avoidance from attraction. The animals' decisions could only be based on chemical cues. All animals investigated both odorous plates, and their resultant behavior (time on cage sides or area selected for rest) was necessarily based on previous experience with these odors. Furthermore, the distinction could not be based on the animals responding differentially to odors of different degrees of familiarity as the subjects had equal experience with both the dominant and subordinate animal prior to odor testing.

The actual cues to which the cavies responded in this experiment are

unknown. As shown in experiment 1, the cues may involve the odors of urine, perineal gland, or supracaudal gland, any combination of these odors, or even odors from other body areas. The cues normally used by cavies in a natural system may involve relatively complex mixtures of odors from various body sites (see Goodrich and Mykytowycz, 1972).

DISCUSSION

Previous studies of individual odor recognition often have not separated recognition of odors of differential familiarity from recognition of odors from different, but equally familiar individuals. For example, the results of Halpin (1976) and Mykytowycz (1975) may be the result of recognition of whether the odor is novel or familiar, or in the case of Epple (1973) distinction between familiar and less familiar odors. Carr et al. (1970) showed mice avoided the odor of a mouse which had recently defeated them, and Nyby et al. (1970) showed that gerbils avoided colony odors if they had recently been defeated by members of this colony. In both of these cases the odors of the victor were also the more familiar odors. Other studies, such as those of Schilling (1980), Schultze-Westrum (1965), Halpin (1974), and experiment 1 of this study specifically used differential familiarity (via habituation) to show that an olfactory distinction of individuals could be made by test animals. Such studies do not show that these cues are actually used by the animals.

In this report, experiment 2 demonstrates an olfactory recognition of individuals by cavies. Each test male had been equally familiar with the donor males but had experienced differential agonistic interactions with these animals. In another study, conducted identically to experiment 2 here, it was found (Martin, unpublished) that male cavies did not respond differentially, either in noninvestigatory time or resting location, to the odor of dominant and subordinate individuals with which the test animals had no experience. Taken together, these studies demonstrate a response based on olfactory cues of different individuals and not simply a response to the odors associated with the generalized classes of dominance and subordinance. Other studies (e.g., Epple, 1973; Lombardi and Vandenbergh, 1977), however, have demonstrated differential response to odors of dominant and subordinant individuals without the subjects having had prior experience with the particular individuals supplying the odors.

One set of recent studies implicates genetically controlled individual odors in regulation of mating preference in mice. Male mice differentially mate with female mice which differ only at the major histocompatibility locus (*H-2*) (Yamazaki et al., 1976). That olfactory recognition may be involved in this mating preference is demonstrated by subsequent studies showing that

mice can be trained to discriminate odors of mice differing only at *H-2* (Yamazaki et al., 1979; Yamaguchi et al., 1981).

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RANGE OF ACTION AND INTERACTION OF PHEROMONE TRAPS FOR THE SUMMERFRUIT TORTRIX MOTH, *Adoxophyes orana* (F.v.R.)[1]

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Abstract—In a spindle-tree apple orchard, sex pheromone traps for the summerfruit tortrix moth were distributed at different spacings. From the captures, information was derived about influence of prevailing wind direction and of trap spacing on interactions between traps. Prevailing wind direction in the experimental area had no appreciable influence on trap interaction, perhaps because of eddying within the planting. The relationship between density and capture of traps was first considered theoretically and interaction by overlap of active-space areas was distinguished from that by overlap of mere capturing areas, which depend also on distance of dispersal. The actual results were in accordance with these considerations and indicated that the diameter of the active-space areas averaged 15 m. The variation in the captures of the wider-spaced traps was too large to be conclusive about the average width of the capturing areas, but the diameter of these seemed to be more than about 45 m. These data have been related to adequate trap distances for monitoring and mass trapping.

Key Words—Lepidoptera, Tortricidae, *Adoxophyes orana*, pheromone traps, attraction, interaction, trap spacing, wind, monitoring, mass trapping.

INTRODUCTION

The summerfruit tortrix moth is one of the most serious lepidopterous pests in apple and pear orchards in the Netherlands. To monitor this species, the horticultural advisory services use sticky traps baited with a mixture of (*Z*)-9-tetradecenyl acetate and (*Z*)-11-tetradecenyl acetate in a ratio of 9:1

[1]Lepidoptera: Tortricidae.

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(Minks and De Jong, 1975). The use of such traps to investigate the (relative) population density and for mass trapping is still in the experimental stage. Captures from attractant traps depend on distance of attraction and of possible interaction among the traps, as well as other major factors. Firm information on these distances is rather scarce. Often no clear distinction is made between distance of attraction and distance of interaction, either in term or concept. Farkas and Shorey (1974) for instance, use "effective" to denote the distance of attraction, and Embody (1971) uses it to indicate mutual influence of traps. To avoid misunderstanding, the terms used here will first be defined. "Active" (Nakamura, 1976; Shorey, 1976) denotes "between an attractant source and the outer limits of the space within which males respond adequately to the emitted attractant," and "interfering" denotes that traps affect the capture by one another.

The limits of active space depend on trap and species characteristics and on many individual and environmental variables (Shorey, 1976). Trap interference depends on the active space of each trap and on the distance from which individuals on the move may enter it. Neither active space nor interfering distance are fixed features of a species.

To obtain workable values, Wolf et al. (1971) proposed the recapture of coded and released specimens, using special patterns of traps and release points. This paper presents the results of an experiment with attractant traps in the natural moth population of a typical Dutch apple orchard, aiming at an estimate of active space and interfering distance of the traps and the possible relationship with wind direction.

METHODS AND MATERIALS

The experimental site was an orchard of 5.7 hectares of Golden Delicious spindle trees, near Tiel in the Betuwe region of the Netherlands.It was largely isolated from other orchards, except for one along the middle third of the south border. The height of the trees was up to 3 m, the width between the rows 4 m and the tree distance 1.80 m on average.

The traps were triangular with sides 10 cm wide and 20 cm long (adapted from Minks and Voerman, 1973) and made of sheets of transparent polyvinyl chloride. The baits, suspended in the middle of the traps, consisted of polyethylene caps, containing 0.5 mg of a 9:1 mixture of (*Z*)-9-tetradecenyl acetate and (*Z*)-11-tetradecenyl acetate (purity $> 99\%$). The trap bottoms were coated with Tangle Trap® adhesive material to retain the captured moths.

The traps (100) were hung in the trees at a height of about 1.80 m and in the spatial pattern shown in Figure 1. The side rows of traps were kept as much

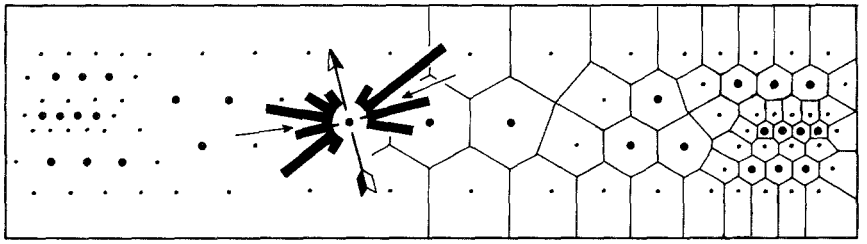

FIG. 1. Sketch map of the experimental orchard (125 × 450 m). Points: spatial pattern of traps; larger points indicate that traps are surrounded by six (sometimes five) other traps at equal distances. "Web" on right side: delimitation of "free areas" of the traps, as defined in the text. Arrows and bars: means and frequency distributions of the wind directions during periods of easterly and westerly winds.

as 25 m away from the orchard borders, to avoid possible interference of discontinuities, for instance, in population density and air currents.

The traps were inspected three times a week from May 31 until June 19, 1978, the first flight period of the moth. The total captures reached up to 1392 male moths.

Data about wind direction were obtained from two meteorological stations, one at De Bilt, near Utrecht, about 25 km to the northwest, and the other at Wageningen, about 18 km to the east-northeast of the experimental site.

RESULTS AND DISCUSSION

Wind Direction. The active space of a pheromone source is usually depicted as a long filamentous plume or, after averaging over time, as an elongated ellipsoid downwind. In this conception, mutual influence of traps might depend on their position in relation to wind direction. To investigate whether mutual influence within the experimental orchard depended also on prevailing wind outside it, distribution of captures was studied in relation to prevailing wind direction. To get usable data, we selected only trapping periods on nights when the wind direction was constant and similar at both weather stations. It may be taken for granted that during such periods prevailing wind direction in the experimental area half way between the meteorological stations was quite similar. In two such periods, enough moths were captured to allow further analysis. The mean wind directions during these periods are indicated in Figure 1 (arrows), together with frequency distributions of the constituent wind directions (bars). In the period with westerly winds, the number of moths captured was 321, with easterly winds 563.

Differences in captures are supposed to depend largely on the combined effect of position relative to other traps and wind direction, local differences in moth density, and the total number of moths that may respond to the attractant during a trapping period. A good comparative estimate for that number of moths is the mean number captured in many traps. Therefore capture data for the two periods under consideration were made equivalent by expressing trap captures as proportions of total capture.

Trap array (Figure 1) was designed in such a way that the trap arrangements on either side of the orchards were equal and similar, but reversely situated. Thus, each trap in one half of the orchard corresponds to a trap in the other half, and is similar in position relative to other traps. This similarity will also refer to wind direction for periods with opposite winds. During both trapping periods, mean wind directions were luckily quite opposite: more than 70% of the frequency distribution of the constituent wind directions of each was counterbalanced by its exact opposite. Therefore the (proportional) capture data of each trap from one half of the orchard during the period with one wind direction were combined with those from the other half during the period with opposite wind direction and vice versa. This procedure also compensated, at least partly, for possible differences in local moth densities. The resulting data were plotted on a sketch map of the trap pattern. To eliminate the effect of trap density on the data of each trap, an index for each trap was calculated by averaging the data of each pair of corresponding trap positions in this plot, and determining their deviations, relative to this average. The result is shown in Figure 2, together with the mean of the combined wind directions (arrow) and the distribution of their frequencies (bars). The mean direction is not far from the row direction, and about half of all frequencies are indeed very close to it.

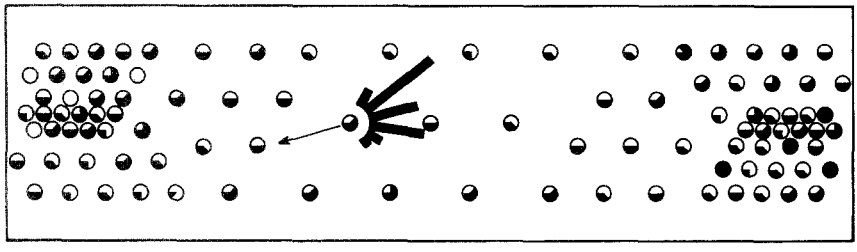

FIG. 2. Circles: indices of relative trap captures in relation to wind direction. For each trap position, the size of the black part indicates the value of the combined proportional capture data (see text) relative to that of the corresponding position on the other half of the pattern. Arrow and bars: mean and frequency distribution of the combined wind directions.

If interaction of traps is dependent on their mutual position in relation to wind direction, a certain trend in the indices along the rows might be expected. However, such a trend does not appear from Figure 2. In fact, in exactly half of all pairs of neighboring positions along rows the upwind index is larger and in the other half the downwind is. The visual impression of more "black" on the upwind side of the plot can be attributed completely to the lack of any capture in four downwind positions (all white), and therefore the relative overestimate of the corresponding values upwind (all black), though these were not really high, even well below the mean value. Thus, prevailing wind direction has no appreciable influence on trap interactions within the orchard.

The independence of wind direction is rather surprising, as Minks et al. (1971) and Minks and Noordink (1971) found that a large majority of the released *A. orana* were recaptured upwind. Barel (1973) also recaptured released moths mainly upwind but also mentioned "inexplicable" high downwind captures. However, large numbers of laboratory-reared moths, released at one point, might be poorly comparable with naturally distributed wild moths. Only Vollaard (1975) has tested an array of sex-attractant traps with natural populations of *A. orana*, using blocks of 3 × 3 traps, different distances apart. At the smaller distances (3.5 and 10 m), he found about twice as many captures in the downwind traps than in the others. At 25 and 40 m, this difference disappeared. His result, however, may be biased by lack of compensation for differences in total capture between periods with different wind directions. Wall and Perry (1978; 1980) tested lines of attractant traps for *Cydia nigricana* along the direction of the wind in open fields. They found that the upwind traps captured consistently more moths than the others.

In the orchard, however, the trees slow down the wind and cause it to eddy within the planting in such a way that air currents from different directions may be generated, thus wiping out the dominance of the prevailing wind and moving the active space of an odor source in different directions. Experiments by Lewis and Macaulay (1976) with smoke plumes indicate such effects even in a pea field because of wind turbulence. Thus the varying positions of the active space would, after some time, cover an area with a radius of about active distance around the source.

Trap Distance. Since the direction of prevailing wind appeared to be of minor, if any, importance for trap interactions in our experiment, it is justifiable to use the capture data of the whole trapping period, without respect to wind direction, to investigate the relation between spacing and interaction of traps.

The trap array was so designed that certain traps were surrounded by six (sometimes five) other traps at equal distances. These "central" traps are indicated in Figure 1 by larger points. Their mean captures are plotted in

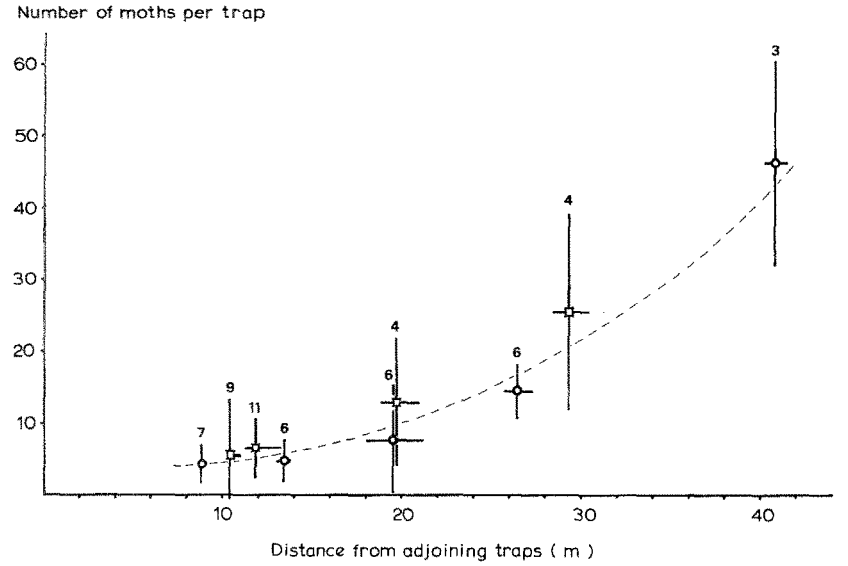

FIG. 3. Relationship between trap spacing and capture. Circles indicate mean values for groups of equally encircled traps (larger points in Figure 1); squares, those of less-uniformly surrounded traps (see text). Numbers above the points indicate number of traps per group; horizontal bars, range of trap distance within the group; vertical bars, standard deviation of the mean.

Figure 3 against distance to adjoining traps (circles). Many other traps (apart from those along the edges of the array) were also surrounded by other traps, although not at fixed distances. They were grouped on the basis of mean distance to the nearest five traps and are also plotted in Figure 3 (squares). If traps were only affected when their active spaces overlap, one might expect that the captures in traps further away would become less dependent on trap distance. Figure 3, however, shows an increasing rise in captures. So either the active distance is (far) greater than the largest experimental spacing (40 m), or the assumption does not hold.

The progressive increase along the plot suggests a possibly more linear relationship between capture and a quadratic function of distance, which can be called free area. This free area is defined here as the area around a trap, within which a moth is closer to that trap than to any other (similar to drawing range of Riedl and Croft, 1974) and thus limited by the middle lines between that trap and the adjoining ones, and sometimes the orchard boundary (Figure 1, right side). The array allows classing of traps by these free areas into distinct groups. The free areas of the traps within these groups were averaged, as were their captures (Figure 4).

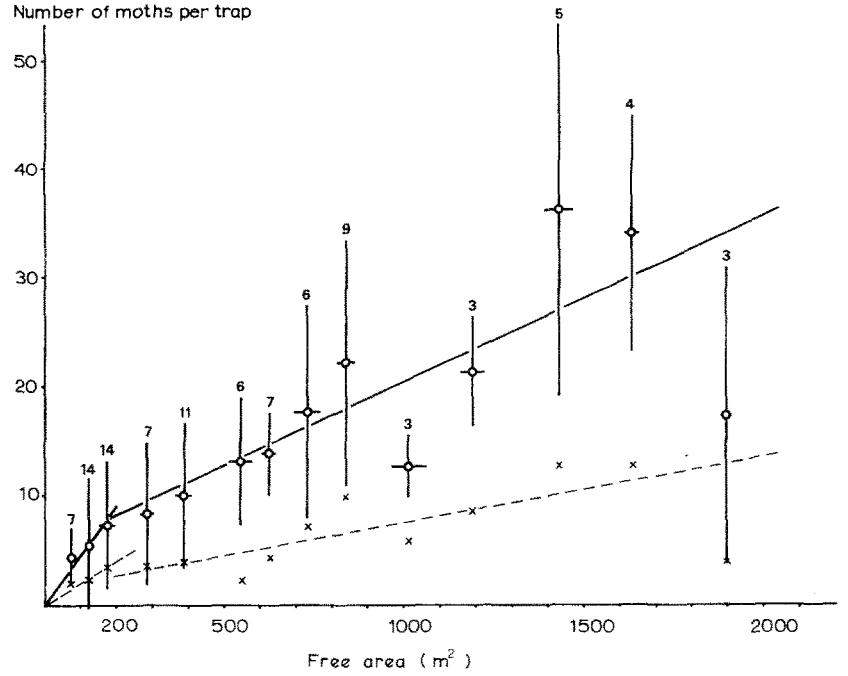

FIG. 4. Relationship between free area and trap capture. Numbers above the points indicate number of traps per group. o:total captures (mean + SD); linear regressions: a: free area < 220 m^2, $N = 35$, $y = 0.045x$ ($\bar{y}/\bar{x} = 0.045$); b: free area > 250 m^2, $N = 64$, $y = 5.44 + 0.015x$, $r = 0.57$. x:mean captures of one night (June 5–6): linear regressions: c:free area < 220 m^2, $N = 35$, $y = 0.018x$; d: free area > 250 m^2, $N = 64$, $y = 1.30 + 0.06x$, $r = 0.44$.

Theoretical Interpretation. To interpret Figure 4, let us first consider what kind of picture might be expected. A simplified situation is supposed, in which all trappable moths that wander into the active space of a trap are indeed captured, in addition to those already within it. This situation is analogous to that of pitfall traps for soil-dwelling animals: the active space around every trap acts as an aerial pitfall for the moths around it. The capture in an active space depends, therefore, on population density and on mobility of the moths: their locomotive pattern and especially the limits of their range of dispersal. Moths within a zone around the active space of a trap with a width equal to the maximum distance of dispersal risk entering that active space and thus being captured. So the outer limits of this zone define a trap's capturing area.

The trappability of a male moth depends on its responsiveness to the pheromone lure, which depends in turn on other things such as age, time of

day, and climatic factors. Thus active space of a trap does not act continuously as a pitfall to every moth. It might be expected that traps at high density would decrease local moth population and thus their forthcoming captures more than traps at low density. However, the share of closer spaced traps in the total capture (per trapping period) did not decrease with time. So it seems that the more intensive capture had no lasting effect on the local density of trappable moths. This may be caused by dispersal of moths left behind, development of responsiveness of other ones, or eclosion of new moths during the times of day without response. So distance of dispersal and capturing area should be understood as related only to the duration of a single period of responsiveness, i.e., a few hours every night.

For the relationship between trap density and mutual interference between traps, three situations may be distinguished. In situation 1, the free area is smaller than the active-space area, so all trappable moths will be captured, as active spaces overlap. The capture per trap will be inversely proportional to trap density and so directly proportional to free area.

In situation 2, the free area is intermediate between the active-space area and capturing area, and each trap will capture (apart from the moths within its active space) moths from the rest of its capturing area. At higher trap densities this remaining part will be smaller and therefore comprise less moths. On an average, however, these moths will be closer to active space, so that a larger proportion of them may wander into it and be captured, thus partly compensating for their smaller number. Hence the mean trap capture will consist of the mean number of moths within the active spaces plus a number coming from and positively correlated to the rest of the free areas.

In situation 3, the free area is larger than the capturing area so the traps are spaced too far apart for any interaction. The mean capture of the traps will be constant, corresponding to the number of trappable moths from within the active space plus a fixed proportion of those present in the rest of the capturing area.

On the basis of these considerations it may be expected that the points in Figure 4 would fit to a line consisting of three different parts, corresponding to situations 1, 2, and 3. The first part of this line should be straight and rise from the origin, the second part should still rise, but at a lower rate, as not all moths from the free areas will be captured, and the third part should be level. The transitions between these parts would coincide with the values of active-space area and capturing area. Active-space and capturing area, however, would vary with external and internal factors, so that these transitions may be gradual. In fact, such a division of the plot is not immediately evident. Nevertheless useful information is supplied. The first part of the plot should correspond to a straight line through the origin (situation 1). This condition is met rather well below 200 m^2 (trap spacing 15 m). The next points better fit a

line through the rest of the data, which rises less steeply and might correspond to situation 2. Whether this rising trend ends in our data is not clear because the variation in the captures is too large to be conclusive, particularly for free areas over 1000 m^2 (trap spacing 35 m). The rise certainly seems to continue to 1600 m^2 (trap spacing 45 m). Thus the active-space area of the traps seems to be at most 200 m^2, corresponding to an active distance of about 7.5 m, and the capturing area is probably about 1600 m^2 or more, corresponding to an average capturing distance of about 22.5 m or more. This indicates an average distance of dispersal (capturing distance minus active distance) of about 15 m or more.

As a check, captures in the only one-night trapping period, June 5–6, were also plotted in Figure 4. Again the captures for free areas up to 200 m^2 fit a straight line through the origin rather well, and the other data show an increase at a lower rate, similar to that of the total data. So the general picture corresponds very well.

This agrees well with what was brought out about the recurrent fading of local differences in numbers of trappable moths, which is presumably caused by different trapping intensities.

Quantitative data about active space from other sources are scarce. For our species, Minks (1975) suggests " possible overlapping attractancy when traps are placed 2–3 m apart in a square." McNally and Barnes (1981, Table 3) present data about the relation between captures and densities of codling moth (*Laspeyresia pomonella*) traps. When these data are plotted as in our Figure 4, an essentially similar picture appears: for free areas up to two trees (112.5 m^2), the points fit rather well to a line through the origin; for the larger free areas up to 32 trees (1800 m^2), they are exactly on a straight, less steeply rising line. These lines intersect at a free area of about two trees, corresponding to a trap distance of ± 11 m. So average active-space area may be supposed to be ± 112.5 m^2 and average active distance ± 5.5 m. For adult females of the tobacco cutworm moth (*Spodoptera litura*), the maximum active distance in an open field was estimated at 62 m (Nakamura, 1976), and the cabbage looper (*Trichoplusia ni*) may fly directly to a pheromone source 400 m away (Kishaba, 1970).

More is known about capturing and interfering areas. On the night of release, male moths of *A. orana* were recaptured 75 m away at most (Minks et al., 1971) and once 100 m away (Vollaard, 1975). Barel (1973) found that a male of that species could move in sustained flight at least 120 m. Our own estimate of the average capturing distance at more than about 22.5 m is compatible with those results. Capturing distances were assessed for the lesser peach tree borer (*Synanthedon pictipes*) at 800 m (Karandinos, 1974), for the cabbage looper at 244 m (Toba et al., 1970), and for the pea moth (*Cydia nigricana*) at 100 (200?) m (Wall and Perry, 1980). Interfering areas for pink

bollworm (*Pectinophora gossypiella*) were about 70,000 m^2 (Embody, 1971), and for codling moth (*Laspeyresia pomonella*) between 15,000 and 100,000 m^2 (Riedl and Croft, 1974).

The performance of *A. orana* seems rather poor compared with these other moth species. This may be partly attributable to a lower flight capacity and partly to the habitat. The dense foliage of an orchard with 1400 trees per hectare may help to account for the small distance of dispersal. It may also affect the active space of the traps in several ways. The attractant plume will, as it were, be sieved through it, and the resulting intense contact with leaves and branches may cause a large amount of the compounds to be adsorbed onto them. The foliage may also increase air turbulence and thus generate more and smaller eddies. These will speed up the transition of the odor plumes from the regime of molecular diffusion to that of turbulent dispersion in which the wispy structure of the plume is converted to a homogeneous haze and the peak concentrations fall off far more rapidly (Aylor, 1976). Under the constant stimulation of such a homogeneous attractant haze, the moths may adapt very fast and stop upwind movement (Kennedy et al., 1980, 1981). Thus the active space may be restricted.

The influence of dispersal of the moths on the interfering distance of traps is generally neglected in considerations on trap interference, and interfering distances are often interpreted as distances over which traps attract. A large distance of dispersal, however, as may be expected in the open field, will result in a large interfering distance, even when the active space of each trap is small. Recognition of the existence of dispersal makes phenomena conceivable which would otherwise be more difficult to explain. Thus one could explain larger interfering distances across the wind by assuming a wide cross-wind dispersal and without postulating an implausibly large width of active space. Wall and Perry (1978), for instance, assessed such interactions between traps for pea moths and assumed that the active space was spread across the wind in excess of the trap spacing (100 m). But the same interaction might be explained just as readily by assuming a much narrower active space combined with a wide cross-wind dispersal of the moths. If several traps are placed in a line with equal spacing smaller than the distance of dispersal of the target species, the intermediate traps will have multiple overlap of capturing areas, whereas the traps at the ends of the line would be largely free from overlap and therefore capture more moths. Indeed, Wall and Perry (1980) found such capture profiles for the pea moth in lines of traps 25 and 100 m apart. They suggested a multiple overlap of the trapping zones (= active spaces) and in consequence "a range of attraction of at least 400 m." Assumption of an average distance of dispersal of about 150 m, which seems quite acceptable in the open field, might offer an alternative explanation for their results, even with much smaller active spaces. More knowledge about all aspects of the

mate-finding behavior of a species may be essential for correct interpretation of capture results and for estimation of active-space values from them.

Knowledge about range of action and interaction of a certain type of attractant trap is needed to meet the different requirements for adequate spacing of them for mass trapping and monitoring. For mass trapping, efficiency is greatest if the maximum number of moths is captured with a minimum number of traps. Capture will be maximum when the area is completely covered by the active spaces of the traps. To do so with a minimum number of traps, trap density must be the reciprocal of active-space area. For *A. orana* in low orchards, one would need about 50 traps (of the type we used) per hectare (trap spacing 15 m). For monitoring of moth flight, efficiency is greatest when the amount of information per trap (number of moths captured) is maximum. This will be so when the capturing areas of the traps used do not overlap; under our conditions traps would need to be at least 45 m apart.

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DIRECTIONAL FLOW OF MALE SCENT RELEASED BY *Pseudaletia separata* Walker (Lepidoptera: Noctuidae) AND ITS REPELLENT EFFECT ON ADULTS AND LARVAE OF FOUR NOCTUID AND ONE PHYCITINE MOTH

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Abstract—Air movement around a male *Pseudaletia separata* Walker, observed using the smoke of an incense stick during wing-fanning, showed that the air moves away from the female and backwards. Moths moved or flew away immediately when vaporized benzaldehyde was blown over their antennae in a screen cage set in a greenhouse. This repellency lacked species, sex, or individual specificity. Male scent acts as an inhibitor to conspecific males and, at the same time, to other moths if they approach the male in courtship. Inhibition of ovipositional and larval locomotory behavior by benzaldehyde was also demonstrated.

Key Words—Lepidoptera, Noctuidae, *Pseudaletia separata*, male scent, repellent pheromone, repellent allomone, nonspecificity, sexual selection.

INTRODUCTION

It has long been known that adult males of some Lepidoptera have brush organs on parts of the body, from which some species release scent. It has been suggested that this male scent can act as an aphrodisiac to stimulate female receptivity during courtship (Birch, 1974). On the other hand, it has recently been proposed, based on a series of results from experiments and observations in the laboratory and field, that the male scent of *Pseudaletia* species acts as a male-to-male inhibitory pheromone during courtship (Hirai et al., 1978; Hirai, 1980a). This paper describes the direction of dissemination of the male scent of *Pseudaletia separata* Walker during courtship, its repellent activity

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against adults of *P. separata*, *Leucania loreyi* Duponchel, *L. striata* Leech, *Spodoptera litura* Fabricius, and *Etiella zinckenella* Treitschke; the ovipositional repellency of one component (benzaldehyde) to *E. zinckenella*; and the inhibition of feeding caused by benzaldehyde in larvae of *P. separata*, *S. litura*, and *Mamestra brassicae* Linne.

METHODS AND MATERIALS

The insects used in these studies were reared on an artificial medium (Hirai, 1979). Air movement around male *P. separata* during wing-fanning was observed with fixed moths by using the smoke of an incense stick. Moths were fixed by attaching the dried pith of an elder to the mesonotum with Alon Alpha® cement. The fixed moths appeared to fan their wings normally during observations.

Four noctuid moths (*P. separata, L. loreyi, L. striata, S. litura*) and one species of Phycitinae (*E. zinckenella*) were used in tests of the effect of benzaldehyde, which is only one of the components released by the male *P. separata* during courtship (Hirai, 1980b). In each test, 10 unmated moths 3–5 days old were released in a screen cage ($1.8 \times 1.8 \times 1.8$ m) placed in a greenhouse (ca. 20° C) at night from April to July 1980. In these experiments the control consisted of blowing air over the antennae from a distance of 15 cm by squeezing a plastic bottle (500 ml) and observing the moth's behavior. Then 7 ml of benzaldehyde (98+% pure by GLC) was added to absorbent cotton in a 500-ml plastic squeeze bottle and blown over the moth's antennae from the same distance as in the control. Moth responses were observed and recorded in each treatment. Extraction of benzaldehyde on 70 antennae with CS_2 within 20–30 sec after the blowing and subsequent GLC analyses showed that, on average, 0.26 ± 0.04 μg (SE) benzaldehyde had arrived to each antenna. This amount is considered to be not high for a moth when compared with the facts that a male moth of *P. unipuncta* has 11.2 μg benzaldehyde per scent brush and releases approximately 3.2 μg benzaldehyde during courtship (Hirai, 1980b).

The two following experiments were made to clarify the repellent effect of male scent on adults and larvae of several species from the practical standpoint. Inhibition of oviposition by benzaldehyde was investigated by using *E. zinckenella* and three bean species as the ovipositional sites, *Glycine max* Merrill, *G. soja* Sieb et Zucc. and *Vigna radiata* Wilcz. Six pairs of the moths were put in a wire screen cage ($25 \times 25 \times 25$ cm, ca. 20° C) in the laboratory. Two bean stems, 15–20 cm high with 6–13 pods each, were placed in two flasks 15 cm apart. Ten microliters of benzaldehyde was smeared on the bottom pod of one of the two bean stems. The number of eggs on the pods were counted every morning.

Repellency of benzaldehyde to the larvae of three noctuid species, (*P. separata*, *S. litura*, and *M. brassicae*), was observed at night in the laboratory (ca. 18°C). Each replicate consisted of 10 larvae of each species placed in the center of a polyethylene container (23 × 33 × 11 cm), in which two 0.7-g diet blocks were set on round filter papers separated by 13 cm. Ten microliters of benzaldehyde was smeared on the filter paper around one of the diet blocks. When the lid was replaced on the container, observations of larval feeding preference were made every hour.

RESULTS

Air movement around a male *P. separata* during wing-fanning is shown in Figure 1. Air to the front and above the moth in a range of 1.5 cm from the ventral side of the body and from the head was absorbed inside the fanning wings and flowed down backwards and behind the moth. Responses of adults to vaporized benzaldehyde are indicated in Table 1. Response types are as follows: Walking refers to movement of more than 1 cm including wallking and rotating. Flying means leaving the spot by flying away immediately after blowing. Large numbers of moths, which had been sitting on the screen slightly fanning their wings or assuming calling positions, irrespective of sex and species, moved or flew away immediately after the benzaldehyde was blown over them. The response was over 90% except for the females of *S. litura*. After stimulation by the benzaldehyde, the moths stroked their antennae with the forelegs as if to brush off the benzaldehyde. This repellency was observed even in the daytime. On the other hand, when air without odor was blown over the antennae, the moths clung to the screen, displaying

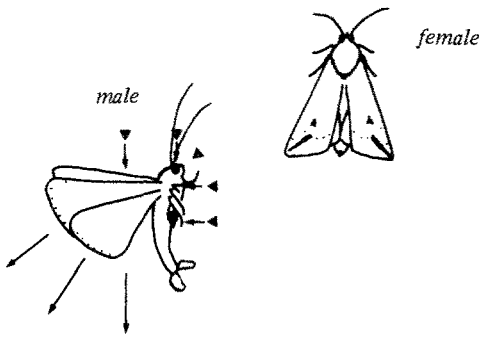

FIG. 1. Air movement around a male *Pseudaletia separata* during wing fanning. The male approaches a calling female from her rear and tries to copulate, thus the male scent released from the anterior abdominal scent brushes does not contact the female, but flows behind the male as indicated by the arrows.

TABLE 1. MOTH RESPONSES TO VAPORIZED BENZALDEHYDE (CHO) OR AIR (MEAN ± SE)[a]

Species		No. moths	Response type (%) None	Walking	Flying
Pseudaletia separata					
Female	Air	61	90.3 ± 4.1[b]	6.8 ± 3.2	3.0 ± 3.0
	CHO	61	0	3.3 ± 1.9a[c]	95.0 ± 3.4
Male	Air	65	95.0 ± 2.3	3.4 ± 2.1	1.7 ± 1.7
	CHO	65	4.8 ± 2.4	26.1 ± 9.4b[c]	67.3 ± 10.7
Spodoptera litura					
Female	Air	48	88.3 ± 7.9	11.7 ± 7.9	0
	CHO	48	16.0 ± 6.5	49.7 ± 8.8	32.8 ± 12.2
Male	Air	25	100	0	0
	CHO	25	6.7	38.4	55.0
Etiella zinckenella	Air	83	98.0 ± 2.0	2.0 ± 2.0	0
	CHO	83	5.2 ± 2.7	1.9 ± 1.9	93.0 ± 3.0
Leucania loreyi	Air	50	100	0	0
or *L. striata*	CHO	50	0	0	100

[a] Blowing experiments were carried out in a screen cage set in a green house (ca. 20°C) at night from April to July 1980.
[b] Means (None and walking + flying) between AIR and CHO in each of species and sex are significantly different at the 0.001 probability level according to χ^2 test.
[c] Wing raising is not tabled: (a) 1.7%; (b) 1.8%.

TABLE 2. OVIPOSITIONAL INHIBITION OF *Etiella zinckenella* BY BENZALDEHYDE (CHO) IN TWO-CHOICE EXPERIMENTS

Beans	No. pods	No. eggs/pod/night (mean ± SE) CHO[a]	Control
Glycine max	29(4)[b]	0.07 ± 0.05	1.6 ± 0.2[c]
Vigna radiata	8(3)	0	7.3 ± 2.4
Glycine soja	4(1)	0	4.5 ± 1.6

[a] CHO indicates that 10 μl benzaldehyde was smeared on one of the pods.
[b] Number of replicates (days) is shown in parentheses.
[c] Females laid significantly more eggs on the control than on the CHO-smeared pods at the 0.001 probability level (t test). In detail: *G. max* $0.001 < t$; *V. radiata* $0.05 < t$; and *G. soja* $0.10 < t$, respectively.

TABLE 3. NUMBER OF LARVAE THAT FED ON 10μl BENZALDEHYDE (CHO)-TREATED AND CONTROL DIETS AT DIFFERENT OBSERVATION TIMES AFTER RELEASE OF LARVAE IN TWO-CHOICE EXPERIMENTS

Species[a]	Time (hr)	Number of larvae (mean ± SE) CHO	Control	P^b
Pseudaletia separata (a)	1- 5	0	2.2 ± 0.3	$0.001 < t$
	8-14	0.8 ± 0.2	1.0 ± 0.2	
Spodoptera litura (b)	1	0	2.6 ± 0.4	$0.001 < t$
	2	0	1.5 ± 0.3	$0.005 < t < 0.001$
	3	0.3 ± 0.2	1.5 ± 0.5	$0.10 < t < 0.05$
	6	0.8 ± 0.3	1.9 ± 0.3	$0.001 < t$
	12	1.1 ± 0.3	2.2 ± 0.4	$0.2 < t < 0.1$
Mamestra brassicae (c)	1	0.05 ± 0.05	0.9 ± 0.3	$0.005 < t < 0.001$
	2	0.5 ± 0.2	1.3 ± 0.3	$0.025 < t < 0.01$
	3	0.3 ± 0.1	1.7 ± 0.3	$0.001 < t$

[a] Instar and replicates in respective species are as follows, (a) 4th, 20; (b) 5th, 10; (c) 5th and 6th, 19. Ten larvae were used for each replicate.
[b] Larvae fed significantly more on the control than on the CHO-treated diets at the respective probability level (t test).

resistance to the air current; moths which were slightly fanning their wings became stationary on the screen.

In the ovipositional inhibition tests, the moths did not land on the benzaldehyde-smeared plot during observations. Consequently the females laid significantly more eggs on the control than on the benzaldehyde-smeared pods (Table 2).

Responses of larvae to the benzaldehyde are presented in Table 3. Larvae started to move within 5 min after placement in the container. Most of the larvae moved towards the control diet. A few of them proceeded to the benzaldehyde-treated diet, but stopped 1–2 cm from the benzaldehyde-smeared spot, moving their heads from side to side, and then turning and leaving in the direction of the control diet. The result was that larvae fed significantly more on the control than on the benzaldehyde-smeared diet at the respective probability levels (t test) (Table 3). As time after placement of larvae passed, some of them started to eat the benzaldehyde-smeared diets. However, the results were obtained in a confined environment; if larvae were to meet benzaldehyde-smeared food in the field, they would clearly never approach it.

DISCUSSION

It is clear that the male scent released by male *P. separata* flows down backwards and away from the male during courtship. Moreover, judging

from the site of the scent brushes and the fact that a male approaches a calling female from the rear (Hirai, 1977), the male scent is not directed over the female. The blowing tests reaffirmed that the male scent probably acts as a repellent to other conspecific males (male-to-male inhibitory pheromone) as proposed in previous papers (Hirai et al., 1978; Hirai, 1980a). At the same time, it is apparent from the results of the blowing tests that other moths, irrespective of sex and species and including conspecific females, are also repelled by the male scent if they approach a courting male. The lack of species, sex, and individual specificity in the repellency of the male scent corresponds to the lack of species and sex specificity in EAG responsiveness to the male scent (the scent scales or authentic chemical samples) in other noctuid species (Birch, 1971; Grant, 1970, 1971; Grant et al., 1972). One exception is the sexual difference in EAG amplitude in response to benzaldehyde, the male *Pseudaletia unipuncta* Haworth being twice as responsive as the female (Seabrook et al., 1979). It is not known at present whether this disagreement between the EAG response and the behavioral response to benzaldehyde is due to the sexual differences in the types and number of olfactory cells responding, or in the threshold for behavior in the central nervous system. In addition, benzaldehyde is contained in the scent brushes of other noctuid species (Aplin and Birch, 1968, 1970; Bestmann et al., 1972; Grant et al., 1972) and is also present as a defensive substance in some millipedes, *Pachydesmus crassicutis* Wood (Blum and Woodring, 1962; Eisner et al., 1963) and in a harvester ant, *Veromessor pergandei* Mayr (Blum et al., 1969). Thus it is not surprising that the repellency of the male scent lacks species, sex, and individual specificity.

When the males and females of any animal have a general behavior but differ in structure, color, or ornament, such differences will have been mainly created by sexual selection (Darwin, 1872). Based upon this theory, the male scent of *P. separata* can be regarded as the product of sexual selection which acts between males in courtship. The repellency of male scent avoids fighting which might prevent successful copulation. This contrasts with the spectacular battles of hercules beetles, *Dynastes hercules* L., with their huge horns which both attract females and intimidate other males (Beebe, 1947), and with fights between males of the hemipteran, *Acanthocephala femorata* F., using their sexually dimorphic enlarged hind femora (Mitchell, 1980). The lack of species, sex, and individual specificity in the repellency of male scent emphasizes that there are two components of sexual selection: epigamic (male–female behavior) and intrasexual (male–male or female–female interaction) (Huxley, 1938) and also suggests that the product of sexual selection is not always grouped into the two components.

Another instance of the repellency of male scent is that of the male spruce budworm, *Choristoneura fumiferana* Clem. Sex attractant traps of this insect show a reduction in attractancy over time, apparently because the odor of the

male initially trapped repels other males (Sanders, 1978). In other animals, the female garter snake (*Thamnophis radix* L.) is courted en masse by the males when she emerges. It appears that once a single male intromits, the unsuccessful males abruptly leave the copulating pair. Further, recently mated females are usually not courted. These field observations suggest that the mating male deposits odor(s) during copulation which communicates to other males the mated status of the female (Ross and Crews, 1977). Male scent substances are found in the prawn, *Macrobrachium rosenbergii* (Peebles, personal communication). Other cases of male substances secreted during courtship which increase reproductive efficiency, as stated above, will probably be found by further observation and experiment to be general phenomena in the animal kingdom. From the standpoint of insect pest control, the male scents might serve as repellents for larval feeding and adult oviposition.

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ENERGETIC COSTS OF AMINO ACIDS EXUDATION IN THE INTERACTION BETWEEN THE PREDATOR *Gammarus pulex* L. AND THE PREY *Asellus aquaticus* L.

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Abstract—The amphipod *Gammarus pulex* L. displayed aggregative behavior when stimulated by exudates from the isopod *Asellus aquaticus* L. in laboratory experiments, while the isopod avoided its predator by chemotaxis. Nonvolatile exudates from the amphipod significantly increased the respiration rate of the isopod. Exudation of free amino acids was greatly enhanced when both species were present in the same water, and it was calculated that the increased exudation by the prey was equivalent to a loss of 4–5% of its assimilated energy.

Key Words—Amphipods, *gammarus pulex*, Isopoda, *asellus aquaticus*, aggregation attractant, repellent, amino acids.

INTRODUCTION

Since MacArthur's formulation of the optimality theory (MacArthur and Pianka, 1966), several pertinent contributions to behavioral ecology have been published (Krebs and Davies, 1978). The theory has been developed within the framework of natural selection, which tends to produce individuals that maximize their inclusive fitness by optimal behavior. One of the main problems an animal faces is to obtain enough food without becoming food itself. Behavior developed for such interactions with other organisms requires energy and adds to total respiratory costs. Visual courtship displays and aggressive encounters that are important for maximal gene survival may also represent considerable respiratory expenditures (Stiles and Wolf, 1970). The same holds for prey which are skillful at escaping, avoiding, and defending.

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Chemical defense developed by invertebrate and plant species is probably an evolutionarily optimal strategy, although the short-term energetic costs of developing specific biochemical defense mechanisms may be large.

Defense behavior may involve release of other chemical compounds than those used to recognize and avoid a potential predator. Dissolved organic compounds that are exuded to some extent by most organisms may suddenly escape during the interaction and add to the energetic costs. In the case of a predator–prey relationship, loss of organic exudates probably represents a secondary effect of the organism's physiological mobilization pattern. Unless the exuded compound is a repellent which promotes the survival of the individual, exudation is a net metabolic cost which influences the equilibrium between costs and benefits of the behavior.

While a rather extensive literature deals with alarm and escape responses of marine invertebrates to predatory starfish odor and carnivorous snails (e.g., Atema and Burn, 1975; Phillips, 1977, 1978), few, if any, studies have been made on limnic invertebrates. I report here a sudden increase in amino acid exudation due to behavioral interactions between the prey *Asellus aquaticus* L. and the predator *Gammarus pulex* L., two species that frequently coexist in temperate running water systems. Normally, 80–90% of the exuded amino nitrogen appears as ammonia and only minor amounts as amino acids (Dresel and Moyle, 1950). As the prey recognize the predator, however, a certain amount of amino acids is not metabolized but exuded from the body.

METHODS AND MATERIALS

Animals were caught in Höjeå, a small river in southern Sweden, brought to the laboratory, and acclimated for one week at 15° C in circulating stream water. The animals were starved for 24 hr in sterile, filtered stream water to remove gut contents and reduce amino acid excretion to a minimum. A three-way experimental design was carried out with 1000-ml bottles: 15 bottles contained 10 *Asellus aquaticus* (A), 15 bottles contained 10 *Gammarus pulex* (G), and 15 bottles contained five *Asellus* and five *Gammarus*. The mixed species set was duplicated so that, in one of the experiments, the species were separated in the bottles by a plastic net. The experiments were run in existing light (in darkness *Gammarus* consumed *Asellus*) in sterile, filtered, 15° C stream water. Animals were allowed to acclimate in groups of five to the bottle environment for 2 hr before the experiments. The stream water was removed with animals left untouched and new stream water added to about half the volume of the bottle. The animals in two bottles were then randomly combined (e.g., 5A + 5A, 5A + 5G) to give the composition above. After 1, 2, 24, 72, and 215 hr, three bottles in each group were removed and the water was

filtered (0.45 μm) and analyzed for free amino acids by gas chromatography (Bengtsson and Odham, 1979).

To distinguish between visual and chemical stimuli for the exudation, another experiment was performed. Ten *Asellus* or *Gammarus* were added to jars containing 500 ml stream water and incubated at 15° C for 5 hr. The water was removed and purged for 10 min, and 50 ml was distributed to each of 10 jars. Five of the jars containing amphipod-stream water received five isopods each, and the remaining five jars received five amphipods each. The same additions were made to jars containing isopod-stream water. In addition, five control jars containing stream water plus five animals were prepared for each species. All animals were previously acclimatized to stream water at 15° C. The jars were closed and incubated at 15° C for 5 hr. Oxygen concentrations were determined at the beginning and end of the experiment by a micro Winkler method.

A third group of experiments was conducted to compare the responses elicited by the prey when the predator was placed upstream and by the predator when the prey was placed upstream in a set of plastic tubes and troughs. In each experiment two troughs, 10 × 15 × 5 cm, were connected to each other by plastic tubing, 20 mm ID. Drains were positioned centrally in the troughs which were supplied with running tap water (10–20° C) in PVC tubing (5 mm ID). A section of wider stoppered PVC tubing, 20 mm ID, 60 mm length, containing 20 animals of the species to be tested upstream, was inserted in the inflow to one of the troughs. In one experiment two sections of the wider PVC tubing were inserted in the same inflow, one with 20 *Asellus* preceding one with 20 *Gammarus*. Water flow to both troughs was 10 ml/min. Fifteen animals of the species to be tested downstream, previously starved for 48 hr, were placed in each of the two troughs and observed for three nights. The number of animals in each trough was recorded at 6-hr intervals.

RESULTS AND DISCUSSION

During the first 2 hr of incubation a rapid increase in free amino acid concentrations was observed (Figure 1). The increase was especially pronounced in water with both species present (independent of the plastic net), and the concentration peak was at least twice as high as the sum of the peaks from the single species. This suggests that visual or chemical contact between the prey and the predator gave rise to an enhanced amino acid exudation. The extent of amino acid exudation in the single species indicated the relative contribution of each species to exudation during the interspecific interaction. The contribution of *Asellus* was especially evident for the more complex amino acids—lysine, arginine, histidine, and tryptophan (Table 1). Interest-

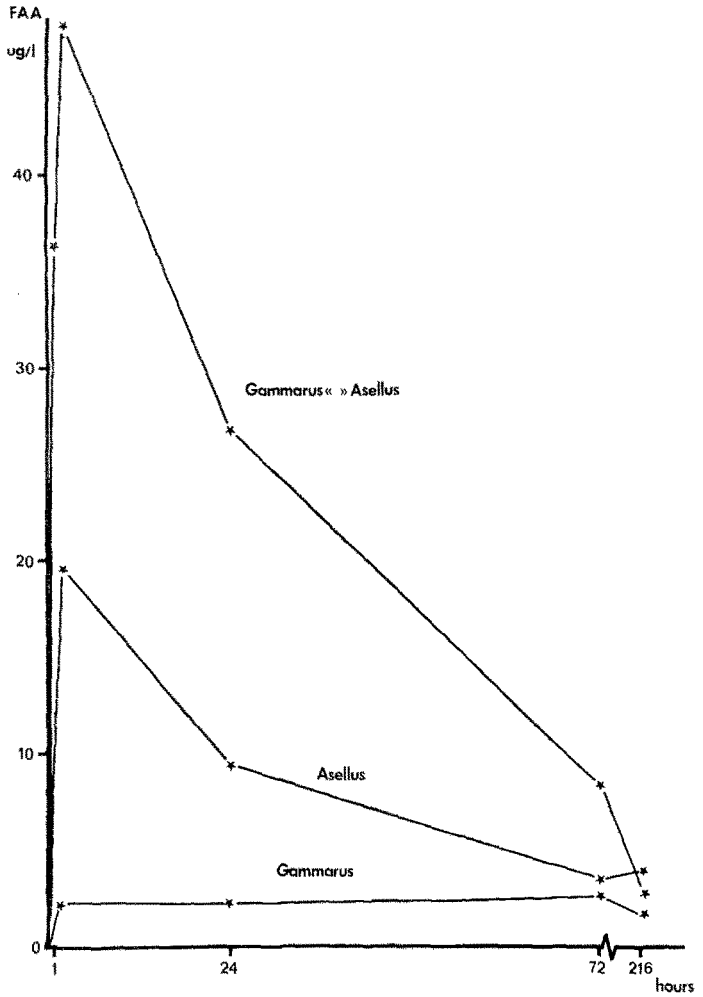

FIG. 1. Exudation of free amino acids (FAA) from *Asellus aquaticus L.* and *Gammarus pulex L.* Each point represents the sum of the individual amino acid concentrations at one sampling time.

ingly, animals usually lack the enzymes responsible for synthesis of several of the amino acids that predominated in the exudation (leucine, lysine, histidine) (Meister, 1972). Although this is an additional drawback of the exudation behavior when nutritional requirements are considered, the animals might compensate for this loss by utilizing hydrolyzed proteins synthesized by intestinal microorganisms.

This experiment does not explain the larger base level exudation from

TABLE 1. EXUDATION OF INDIVIDUAL AMINO ACIDS (NG/LITER) FROM *Asellus aquaticus* AND *Gammarus pulex* (MEAN, N = 3, ± SD)

	Time				
	1 hr	2 hr	24 hr	72 hr	9 days
Gammarus					
ala				83 ± 7	67 ± 4
gly				26 ± 3	19 ± 7
val					7 ± 2
thr			228 ± 16	140 ± 15	97 ± 11
ser		489 ± 22	414 ± 18	120 ± 8	134 ± 13
leu		7 ± 3	35 ± 6	42 ± 4	19 ± 2
ile					
pro		232 ± 15	75 ± 8	240 ± 16	87 ± 7
met			34 ± 2	47 ± 2	98 ± 4
asp			232 ± 18	215 ± 12	149 ± 8
phe		320 ± 19	341 ± 27	395 ± 18	315 ± 21
glu		95 ± 11	54 ± 5	147 ± 11	164 ± 15
tyr		60 ± 15	53 ± 4	24 ± 3	21 ± 1
lys			84 ± 4	80 ± 6	8 ± 2
arg		166 ± 25	340 ± 52	551 ± 65	160 ± 20
his		499 ± 37	215 ± 26	168 ± 18	135 ± 21
try		453 ± 32	146 ± 12	391 ± 28	217 ± 18
Asellus					
ala					37 ± 2
gly					
val					
thr					41 ± 5
ser			124 ± 8	118 ± 7	135 ± 8
leu		115 ± 6	7 ± 2	10 ± 4	12 ± 4
ile					
pro		181 ± 4	237 ± 16	74 ± 2	115 ± 7
met			62 ± 3	78 ± 5	143 ± 10
asp					143 ± 8
phe					
glu		320 ± 20	72 ± 6	69 ± 6	57 ± 6
tyr			156 ± 8	153 ± 10	142 ± 5
lys		5545 ± 105	1153 ± 63	35 ± 4	48 ± 6
arg		8022 ± 543	3273 ± 412	1059 ± 123	2416 ± 338
his		2035 ± 97	1787 ± 61	1114 ± 25	324 ± 11
try		3379 ± 183	2516 ± 175	841 ± 64	359 ± 17
G + A					
ala	280 ± 13	2625 ± 87	115 ± 6	141 ± 8	87 ± 5
gly	67 ± 5	521 ± 24	175 ± 15	34 ± 4	18 ± 3
val	252 ± 14	387 ± 18	88 ± 5	65 ± 4	73 ± 7
thr	80 ± 10	666 ± 42	103 ± 13	17 ± 8	22 ± 5

TABLE 1. *Continued*

	Time				
	1 hr	2 hr	24 hr	72 hr	9 days
ser	173 ± 12	4548 ± 104	302 ± 18	453 ± 32	298 ± 23
leu	50 ± 8	9873 ± 403	517 ± 34	389 ± 21	326 ± 27
ile					
pro	565 ± 21	705 ± 10	523 ± 23	248 ± 12	95 ± 6
met	168 ± 10	152 ± 12	183 ± 9	20 ± 3	25 ± 1
asp	54 ± 3	284 ± 8	50 ± 5	70 ± 4	76 ± 5
phe	163 ± 8	174 ± 10	269 ± 15	595 ± 22	612 ± 34
glu	224 ± 18	341 ± 16	171 ± 19	105 ± 11	36 ± 5
tyr			62 ± 7	364 ± 19	51 ± 6
lys	17290 ± 300	7460 ± 146	9312 ± 412	1573 ± 86	215 ± 14
arg	8537 ± 612	9600 ± 817	8932 ± 789	3239 ± 140	96 ± 8
his	8100 ± 367	6740 ± 303	3401 ± 257	338 ± 18	263 ± 13
try	431 ± 28	3517 ± 105	2493 ± 84	792 ± 36	413 ± 15

Asellus compared to *Gammarus*. It may depend on a combination of increased activity due to the handling of the material and differences in the exudation barrier of the exoskeleton. There are at least three possible reasons for the declining amino acid concentration following the rapid exudation in *Asellus* and *Asellus* + *Gammarus*: amino acids were consumed either by the animals, by microorganisms/protozoa attached to the exoskeleton (Nenninger, 1948), or by both groups. The equilibrium between exudation and consumption of amino acids that was reached within few hours in the *Gammarus* population may also have been influenced by microorganisms.

Both *Asellus* and *Gammarus* increased respiration rate when exposed to amphipod exudates (Table 2, $P < 0.01$ and $P < 0.05$, respectively, Kruskal Wallis test). Since volatiles were removed by purging, less volatile compounds seemed to be responsible for the respiration change. The behavior of *Asellus* in *Gammarus* exudates was in agreement with the result in free amino acid

TABLE 2. RESPIRATION OF *Asellus aquaticus* AND *Gammarus pulex* (MG O_2/LITER/DAY) IN MODIFIED STREAM WATER (MEAN, (N = 5) ± SD)

	Respiration of *Asellus*	Respiration of *Gammarus*
In *Asellus* exudates	7.35 ± 0.26	7.81 ± 0.28
In *Gammarus* exudates	9.48 ± 0.40	8.64 ± 0.45
Control (stream water)	7.22 ± 0.21	7.76 ± 0.17

TABLE 3. RESPONSE OF PREDATOR *Gammarus pulex* AND PREY *Asellus aquaticus* TO EACH OTHERS' EXUDATES[a]

Upstream test species	Downstream test species	Number of downstream test species preferring: Tapwater	Tapwater + exudates from upstream species	χ^2	P
Asellus aquaticus	*Gammarus pulex*	6.5 ± 1.1	21.5 ± 2.4	18.8	<0.002
Gammarus pulex	*Asellus aquaticus*	17.5 ± 4.2	11.0 ± 2.0	3.6	<0.5
Gammarus pulex[b]	*Asellus aquaticus*	26.5 ± 2.3	1.5 ± 1.8	54.5	$<1.6 \times 10^{-10}$

[a]Mean ± SD of six observations. Some individuals stayed in the connecting tube.
[b]Twenty *Asellus* were placed upstream from 20 *Gammarus*, which were placed upstream from the test animals.

exudation. The amphipods used in the experiment were of the same size but different sexes, which might explain the intraspecific dependence. The predator's lower contribution to amino acid exudation suggested in Figure 1 and Table 1 was confirmed by the insignificant increase in respiration rate of *Gammarus* in *Asellus* exudates.

The predator was significantly attracted by water passing the prey (Table 3). The prey displayed a weak response to water passing the predator, but the response was strong when a group of prey was placed upstream of the predator. This suggests that the exudation of chemical(s) by the predator, which stimulates the prey presumably to avoid capture, is largely initiated by the prey. The kind of chemical that emanates from the prey to stimulate the predator and from the predator to stimulate the prey is unknown but preliminary observations on the food preference in *Gammarus pulex* show that it is attracted by fungal mycelium rich in protein amino acids (Bengtsson, unpublished). Although the heavy exudation of free amino acids in *Asellus* is a consequence of the response to a chemical stimulus from the predator, the base level exudation may be sufficient to attract the predator.

The attraction behavior to a food source in response to a chemical stimulus like that shown by *Gammarus* has been found with, e.g., sea urchins (*Strongylocentrotus droebachienses*) (Garnick 1978) and with the mud snail *Nassarius obsoletus* in response to the bivalve *Modiolus demissus* and the gastropod *Littorina littorea* (Atema and Burd, 1975). Simple organic compounds may be responsible for such stimuli. Woodbridge (1978) found that periwinkles, *Littorina littorea*, which usually aggregate on pieces of decaying sea weed, were attracted by certain sugars, mainly glucose.

Few attempts have been made to isolate and characterize compounds that induce aggregation or escape in aquatic environments. Pfeiffer and Lemke (1973) showed that the molecular weight of the alarm substance of minnows (*Phoxinus laevis*) was below 500, while a preliminary chemical analysis of the alarm substance of the snail *Nassarius obsoletus* indicated a molecular weight of about 100,000 or greater (Atema and Stenzler, 1977). These examples and some few others reveal a tendency for molecular size distribution to be correlated to the specific habitat used. It may be hypothesized that these compounds belong to one of two categories: (1) Small, essentially hydrophilic compounds are used by pelagic animals. These animals require compounds that are rapidly dissolved and transported in the water, regardless of how fast the molecules are degraded. (2) Long-chain molecules contain hydrophobic side-chains that prevent rapid degradation and help keep the molecule associated with the bottom water which is rich in organic matter with polar properties. Behavioral compounds used by bottom-living animals should thus be larger molecules.

The loss of organic molecules such as amino acids that contain high

TABLE 4. COMPARISON OF CONSUMPTION, ASSIMILATION, AND EXUDATION ENERGY IN *Asellus aquaticus* AND *Gammarus pulex*

	Consumption ($\times 10^{-3}$ cal)	Assimilation ($\times 10^{-3}$ cal)	Exudation ($\times 10^{-3}$ cal) normal interaction	
Asellus aquaticus	51.3[a]	13.1[a]	0.3[c]	0.6[d]
Gammarus pulex	208[b]	83.3[b]	0.1[c]	

[a]From Prus (1971), assuming uncrowded conditions and consumption evenly distributed over the day.
[b]From Nilsson (1974), assuming animals larger than 10 mg.
[c]Calculated from ΔH_f^0 values in Cos and Pilcher (1970), based on exudation after 216 hr.
[d]Calculated from ΔH_f^0 values in Cos and Pilcher (1970), based on exudation after 2 hr.

potential energy because of relatively small entropy represents an energetic cost to the individual. In order to roughly estimate the importance of amino acid exudation in the animal's energy budget, the standard heat of formation (ΔH_f°) (Cos and Pilcher, 1970) for the different amino acids analyzed was used to calculate exudation energy. Assuming that only slight changes in volume and pressure occurred during the experiment, exudation energy may be compared with data on consumption and assimilation energy obtained with a bomb calorimeter (Table 4). Provided that at least half the exuded amino acids originate from *Asellus* (which is probably an underestimation), more than 1% of the consumed energy (4–5% of the assimilated) is lost during the course of interaction with the predator. The normal loss of exudation energy for *Asellus* is at least 0.2%, and for *Gammarus* 0.05%.

Usually both *Asellus aquaticus* and *Gammarus pulex* exploit the same food resource, hyphomycetes (Bärlocher and Kendrick, 1973, 1974; Kostalos and Seymour, 1976; Rossi and Fano, 1979), and at least in southern Sweden they have overlapping habitat niches. In laboratory experiments with different density levels and age groups, *Asellus* has been shown to be effectively preyed upon by *Gammarus* (Friberg, unpublished), and it is argued that *Asellus* is more important for *Gammarus* as prey than as competitor. However, it may be difficult to separate the effects of prey defenses from those of predator preferences (Phillips, 1977). *Gammarus* may prefer leaves with abundant mycelium over *Asellus* in laboratory choice experiments so that *Asellus* would be expected to be rare in its diet. Such food preference may be a result of the defensive responses of the mobile isopod.

Provided that *Asellus* can discover the presence of *Gammarus* at a short distance, the frequency of periods with enhanced exudation depends on the distribution and density of the populations. Although amino acids form an important part of the exuded material of all animals, not only due to their

energetic value but also to their importance as biochemical precursors and essential nitrogen sources, other exuded compounds such as carbohydrates and organic acids probably contribute substantially to the exudation expenditures. Whether it is a general phenomenon that different types of interactions promote the outbreak of a "cold sweat" behavior by a potential prey is not known. As long as individual prey have a high probability to escape the predator by some chemical communication system, relatively high behavioral costs may be accepted.

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THE INFLUENCE OF A QUEEN-PRODUCED SUBSTANCE, 9HDA, ON SWARM CLUSTERING BEHAVIOR IN THE HONEYBEE *Apis mellifera* L.

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Abstract—The effect of enantiomers of the queen-produced substance, 9-hydroxy-(*E*)-2-decenoic acid (9HDA) on swarm clustering behavior of the honeybee *Apis mellifera* was studied. Caged queens were removed from the swarms at the start of each test and were replaced with small Petri dishes containing one of the following treatments: 100 μg *S*(+) enantiomer of 9HDA, 100 μg *R*(−) enantiomer of 9HDA, 200 μg racemic (*R, S*) 9HDA, and a vehicle-treated control. Each swarm was considered to have dispersed when it had lost 50% of its starting weight. All treatments with 9HDA resulted in significantly longer swarm aggregation when compared with the control. Enantiomers were not shown to have different effects at the $P \leq 0.05$ level of significance. However, observations on swarm behavior indicated that the *R*(−) enantiomer was the most active in retarding swarm dispersal.

Key Words—Honeybee, swarming, 9-hydroxy-(*E*)-2-decenoic acid, pheromone, chirality, *Apis mellifera*, Hymenoptera, Apidae.

INTRODUCTION

Honeybee queens produce a number of pheromones which influence the behavior and physiology of worker bees. One of these is 9-hydroxy-(*E*)-2-decenoic acid (9HDA), which, like the better-known queen substance 9-keto-(*E*)-2-decenoic acid (90DA), is produced in the queen's mandibular gland (Callow et al., 1964). There has been some controversy, however, concerning the activity of 9HDA. In some studies it has been shown to be active either

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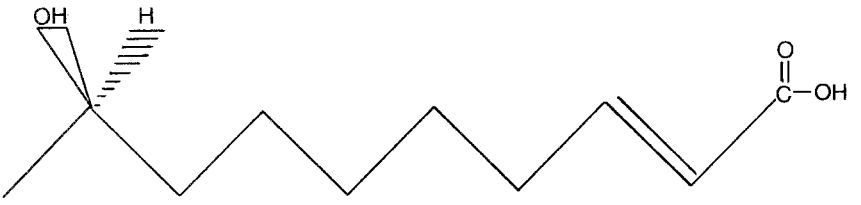
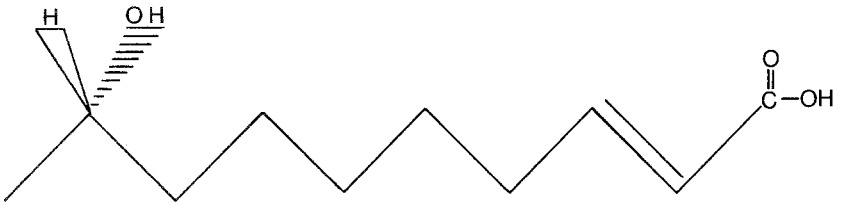


R(−)-9-hydroxy-(E)-2-decenoic acid



S(+)-9-hydroxy-(E)-2-decenoic acid

FIG. 1. Enantiomers of 9-hydroxy-(*E*)-2-decenoic acid.

alone or synergistically with 90DA in attracting workers to swarm clusters (Butler and Simpson, 1967; Ferguson et al., 1979), stabilizing swarm clusters (Butler et al., 1964; Butler and Fairey, 1964), inhibiting queen rearing (Butler and Callow, 1968), attracting drones (Butler and Fairey, 1964), and stimulating release of Nasonov pheromone at the hive entrance (Ferguson and Free, 1981). In contrast, other studies have shown little or no activity of 9HDA in attracting workers to swarms (Morse and Boch, 1971, Boch et al., 1975), stabilizing swarm clusters (Morse and Boch, 1971), inhibiting queen rearing (Boch and Lensky, 1976), and attracting drones (Blum et al., 1971; Boch et al., 1975).

The conflicting nature of these results has been further compounded by the disclosure that the purity of 9HDA used in some of the experiments was questionable (Boch et al., 1975). Also, the 9-hydroxyl function of 9HDA is chiral (Figure 1), and it is possible that the enantiomer used might influence the behavioral response, as is the case with several scolytid (Borden et al., 1980, Light and Birch 1979) and lymantriid (Cardé et al., 1977) species. Thus, the racemic 9HDA produced and used in previous studies might be a poor substitute for the natural pheromone. To investigate this possibility, a supply of both pure enantiomers and racemate was produced through a chiral synthesis from chiral methyl oxiranes (Kandil, unpublished observations). The purpose of this paper is to examine the activity of these enantiomers in stabilizing swarm clusters.

METHODS AND MATERIALS

Research was conducted at Simon Fraser University in Burnaby, British Columbia, Canada, from August 15, to September 15, 1981. To set up swarms, approximately 1 kg of workers was shaken from colonies into wire mesh packages with a caged queen, fed a 2:1 solution of sugar syrup, and left overnight. The following morning the caged queen was placed on a wooden cross covered with slotted plastic material to aid in clustering, and the workers were released from packages and allowed to cluster around the queen. The swarm cross was hung on a 2-kg scale so that swarm weight could be monitored continuously. Four swarms were set up in this manner 30 m apart, and two were used throughout the study. The third dwindled to less than 400 g midway through the study and was replaced by a new swarm. Another of the original swarms also diminished in size and was not used during the last two test days. The racemic mixture was not tested on those days. Swarms were fed sugar syrup at the conclusion of each day's test but not during testing.

Four mixtures were tested: (1) 100 μg of *S*-(+)-9HDA, (2) 100 μg of *R*-(−)-9HDA, (3) a racemic mixture containing 100 μg of each enantiomer, and (4) a blank control treated only with ether solvent. These quantities were chosen since they are similar to the quantity of 9HDA in the queen's mandibular glands (Callow et al., 1964). The test mixtures were put on glass Petri dishes 4 cm in diameter and the ether solvent allowed to evaporate.

Tests were begun between 1100 and 1300 hr when weather permitted. The queen was removed from each swarm and replaced with a Petri dish clipped to the swarm cross in the center of the cluster. A different test mixture was presented to each swarm daily. The weight of the swarm was noted at the beginning of each run and monitored at 15-min intervals or more frequently if a swarm cluster began dispersing. The swarm was considered to have dispersed when it had lost 50% of the starting weight; this was called the swarm-50 point (S_{50}). Once the S_{50} point was reached, the test dish was removed, the queen replaced, and the workers allowed to recluster. Tests were run for either 3 hr or 2 hr after the control swarm had dispersed, whichever was longer. On four of the days, the controls and test swarms did not disperse due to cold, rainy weather, and those tests were not included in data analysis.

RESULTS

The mean time during which swarms remained clustered was significantly different for all three 9HDA treatments and the control swarms (Table 1, $P < 0.01$, Kruskal-Wallis). The controls remained clustered for a mean of 81 min, significantly shorter than the mean time for *S*-(+)-9HDA (159 min, $P < 0.05$, Student-Neuman-Kuels test) racemic 9HDA (172 min, $P = 0.05$,

TABLE 1. TIME (MINUTES) SWARMS REMAINED CLUSTERED AFTER REMOVAL OF THEIR QUEENS[a]

Day	Control	Plus 9HDA	Racemic 9HDA	Minus 9HDA
1	67	60	*180	*180
2	128	*240	190	*240
3	85	*210	37	*210
4	190	110	*310	*310
5	40	*180		*180
6	5	135	93	*180
7	53	*180		*180
8	65	*180		*180
Mean ± SE	81.1 ± 6.4	159.3 ± 4.5	172.0 ± 6.9	211.4 ± 3.3
No. (and %) of times remained clustered	0/8 (0%)	5/8 (63%)	2/5 (40%)	8/8 (100%)

[a]Starred swarms remained clustered until tests were terminated. The swarms were considered to have dispersed when clusters had lost 50% of their starting weight.

SNK test), and *R*-(−)-9HDA (211 min, $P < 0.001$, SNK test). There were no significant differences between any of the 9HDA treatments ($P < 0.05$ in all cases, SNK tests). The control swarms dispersed in all eight tests, and the *R*-(−)-9HDA swarms remained clustered in all tests, a significantly different response ($P < 0.05$, signs test). The *S*-(+)- and racemic 9HDA treatments remained clustered in 63% ($N = 8$) and 40% ($N = 5$) of the tests and were not significantly different from either controls or *R*-(−)-9HDA treatments ($P < 0.05$, signs test).

DISCUSSION

The results of this study have shown that 9HDA is an active compound in maintaining swarm clusters after queen removal. All of the control swarms dispersed while 15 of 21 9HDA treatments remained clustered. The time during which swarms remained clustered was also significantly longer for all 9HDA treatments than for controls. This result is in agreement with Butler et al. (1964) and Butler and Fairey (1964), who also demonstrated that 9HDA stabilized swarm clusters.

The *R*-(−)-enantiomer of 9HDA was somewhat more active than either *S*-(+)-9HDA or racemic 9HDA. Swarms exposed the *R*-(−)-9HDA always remained clustered, while only 40–63% of the *S*-(+) and racemic treatments remained clustered within the test periods. The time during which these swarms remained clustered was longer for *R*-(−)-9HDA, although differences were not statistically significant. However, this lack of significance may reflect

the artificially imposed time limit. In addition, further observations suggested greater activity for the *R*-(−) enantiomer. For example, workers in swarms treated with *R*-(−)-9HDA were generally less agitated than those in *S*-(+) or racemic swarms. There appeared to be more workers exposing Nasonov glands and fanning in *R*-(−)-treated clusters, particularly on the Petri dish containing the bait. Finally, in two instances, swarm clusters with *R*-(−)-9HDA gained 200–300 g when workers from dispersing swarms fused with the stable *R*-(−)-treated clusters. No such gain was found in any of the other swarms. Thus, although all 9HDA treatments were active, *R*-(−) appeared more active than either *S*-(+) or racemic 9HDA.

Although a swarm clustering effect of 9HDA has been demonstrated in this work, several questions remain unanswered. The apparent diminished but demonstrable activity of both racemic and *S*-(+)-9HDA imply both *R*-(−) and *S*-(+) receptor sites for worker bees. The difference in activity was not likely due to impurities, since the synthetic methodology employed is expected to provide chiral purity in excess of 99% for both enantiomers (Kandil, unpublished observations). The possibility that a mixture of the two enantiomers, probably rich in *R*-(−), is the most stimulating cannot be overlooked. Electroantennogram studies will be conducted in conjunction with a determination of the chirality of queen-produced 9HDA to provide a better understanding of these phenomena. Also, the natural concentrations of 9HDA present during swarming need to be investigated.

The apparent differences in activity of 9HDA enantiomers in swarm clustering may well have had a part in the variable results reported for 9HDA in the past. The activity of 9HDA in other functions such as queen-rearing inhibition and drone attraction clearly must be reevaluated when the natural enantiomeric composition of 9HDA is established.

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TANNIN-MEASURING TECHNIQUES: A Review

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Abstract—Techniques used in different biological and industrial fields for the detection and quantification of tannin compounds in plants are discussed. Emphasis is placed on the choice of method for ecological investigations, which may benefit from the use of a method that measures relative astringency of plant extracts at reduced costs in time and equipment, permitting the analysis of larger samples than may be feasible with more sophisticated analytical techniques.

Key Words—analytical techniques, astringency, chromatography, secondary plant compounds, spectrophotometry, tannins.

INTRODUCTION

Techniques for measuring the tannin content of plants were first developed because of the economic importance of tannins to the leather industry (Morfit, 1852; Farnsworth, 1966; Haslam, 1966). Increasing interest in phytochemistry has led to the development of more sophisticated and precise analytical methods to identify tannin constituents. In addition to the use of polyphenols in biochemical systematics, the role tannins play in plant defense against pathogens and herbivores and in the formation of humus has attracted increasing attention from ecologists (Benoit, 1965; Swain and Cooper-Driver, 1973; Rhoades, 1979; Swain, 1979; Cooper-Driver, 1980). The following brief review will collect techniques now scattered throughout the literature in order to facilitate the choice of method(s) appropriate to a particular investigation.

Tannins are phenolic polymers (molecular weight 500–3000) which, through hydrogen bonding with peptide linkages, precipitate proteins from

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aqueous solution, rendering plant proteins relatively indigestible and reducing the activity of enzymes (Goldstein and Swain, 1965; Van Sumere et al., 1975). Until recently, tannins were divided into two main groups: (1) the hydrolyzable tannins, which are esters of glucose with gallic, *m*-digallic, or hexahydroxydiphenic acid; and (2) the condensed tannins, which are polymers of flavan-3-ol or flavan-3, 4-diol phenolics joined by C–C or C–O–C bonds (Nierenstein, 1934; Basaraba, 1960; Benoit, 1965; Farnsworth, 1966; Haslam, 1966). Swain (1979) distinguished four tannin classes: (1) hydrolyzable tannins, as described above; (2) proanthocyanidins (equivalent to the older category "condensed tannins," condensations of flavan-3-ols that produce catechin, epicatechin, and gallocatechin); (3) oxytannins (formed upon injury to the plant by oxidation of the catechins); and (4) beta-tannins (a diverse group of compounds of lower molecular weight than in the other categories, but which are capable of precipitating proteins). Older chemical techniques used the protein-precipitating ability of tannins or the phenolic reaction with ferric salts, for instance; the more recent use of chromatographic and spectrophotometric techniques has made it possible to identify in plant species not only classes of tannins but individual tannin compounds as well (Haslam, 1966; Swain and Cooper-Driver, 1973).

TANNIN-MEASURING TECHNIQUES

Techniques for measuring tannins can be separated into those that measure tannin directly and those that measure it by subtraction. The following categories of methods generally include both types.

Volumetric Titration. An oxidizing agent, permanganate, is used to oxidize known concentrations of a standard tannin solution, producing a standard curve. The tannin content of the unknown is then expressed as equivalents of the appropriate hydrolyzable or condensed tannin (the names given often refer to the source of the tannin-yielding extract, e.g., quercitannin, quebracho).

Direct titration of the tannin-containing extract is used in the official method of analysis of tannin in allspice and cloves specified in the Association of Official Agricultural Chemists (1965) *Official Methods*. After the essential oils are extracted from the spice with anhydrous ether, potassium permanganate solution, standardized against 0.1 N oxalic acid, is used to titrate a water infusion of the sample. Sodium indigotin disulfonate is the indicator. The difference between the volume of permanganate used to titrate the sample and that used to titrate a blank, consisting of the indicator and water, is multiplied by the slope of the standard curve of ml permanganate vs. mg quercitannic acid. This method assumes that tannin is the only oxidizable substance present in the extract. Since most plant materials containing tannin

also contain nontannic phenols, direct oxidative titration methods are best regarded as assays for total phenols unless tannins are known to constitute the large majority of phenolic constituents present.

Generally, oxidative titrimetric tannin assays should involve a fractionation step, specific for tannins, to separate them from the other phenolics. Laurent (1975) precipitated tannin from the raw extract of fern prothalli with ammoniated zinc acetate. The precipitate was redissolved in dilute sulfuric acid, and this solution was titrated with $KMnO_4$, the tannin content being expressed in catechin equivalents.

Alternatively, tannin concentration can be obtained by subtraction, by titrating the total raw extract and then the extract after tannin has been removed from solution. The AOAC (1965) method of analysis of tannin in tea uses this technique. The titration procedure is similar to that used for cloves and allspice, but the reagents are acidic, and the indicator used is indigo blue rather than sodium indigotin disulfonate. Tannin is removed from the raw extract (a tea infusion made with boiling water) by precipitation with an acidic gelatin–salt solution. The volume of $KMnO_4$ used to titrate this tannin-free solution is subtracted from that used to titrate the total raw extract and the difference is multiplied by the slope of the standard curve (permanganate vs. gallotannic acid). The method assumes that all of the tannin and only the tannin is removed by precipitation with gelatin.

Gravimetric Techniques. Tannin is precipitated from solution and weighed, or the raw and tannin-free extracts are concentrated to dryness and weighed.

Bradfield and Penney (1944) used lead acetate to precipitate phenolic compounds from infusions of black tea. Burns (1963) suggested formaldehyde and HCl. Laurent (1975) used hide powder according to the specifications of the leather industry official method to remove tannin from solution and obtained tannin weight by subtraction. Feeny and Bostock (1968) precipitated the acetone layer of their water–acetone oak leaf extracts with ethanol and ether. They obtained the weight of tannin directly, after dialysis of the precipitate to remove nontannic phenols followed by freeze-drying.

Colorimetric Techniques. The density of color produced by various reactions is read spectrophotometrically and compared with a standard.

Burns (1963) describes the use of ferric ammonium citrate, which produces a blue color in the presence of phenols and hydroxamic acid, without prior precipitation of tannin from solution. The nonspecificity of this reaction makes it of doubtful value for the measurement of tannin if nontannic phenols are present in the plant extract, as they usually are.

Another oxidative technique for measuring total phenols was first described by Folin and Denis (1912) and has since been used in a variety of ways. Folin–Denis reagent is an aqueous solution of sodium tungstate, phosphomolybdic acid, and phosphoric acid, which is reduced to a blue

complex of tungsten and molybdenum oxides by phenolics. The reagent is added to the sample with an excess of saturated sodium carbonate solution. The color is allowed to develop for a constant time period and is read at 725 nm (Swain and Hillis, 1959) or at 760 nm (AOAC, 1965; Laurent, 1975). Hillis and Swain (1959) used Folin–Denis reagent to measure total phenols in the tissue of the Victoria plum tree. Laurent (1975) measured total phenols in solution before and after separation of tannin with nylon powder; in addition, she measured total phenols in the supernatant and in the redissolved precipitate in a tannin separation procedure using polyvinylpyrrolidone. (She found that both these substances effectively remove tannin from solution but also remove some nontannic phenols, leading to overestimation of tannin content). The AOAC (1965) method of analysis of tannin in wine and distilled spirits uses Folin–Denis reagent without tannin separation. The assumption is made that no nontannic phenols are present. Burns (1963) also describes the Folin–Denis technique, stating that although it is not specific for tannins, it is often used to measure tannin in forage crops because of its ease and consistency.

More specific color reactions exist which can be used to measure condensed tannins and their precursors. The proanthocyanidin tannins yield small amounts of anthocyanidins when treated with hot mineral acid, hence their name (Swain, 1979); anthocyanidins are colored compounds and the amount produced can be quantified spectrophotometrically. Heating the plant extract with HCl and *n*-butanol produces a red color which is read at 530–550 nm (Swain and Hillis, 1959; Feeny and Bostock, 1968; Cooper-Driver et al., 1977; Balick et al., 1978; Swain, 1979). Vanillin dissolved in sulfuric acid (Swain and Hillis, 1959; Laurent, 1975) or in hydrochloric acid (Burns, 1963) reacts with resorcinol-type phenols to produce a rose color. The reagent is added quickly to the sample, and the color is read at 500 nm after a constant time period of 15 or 20 min.

Bate-Smith (1973a) has introduced a spectrophotometric technique for measuring the relative astringency of tannins which he calls hemanalysis. The plant extract is mixed with dilute finger blood, and the optical density of the hemoglobin is measured after the tannin–hemoglobin precipitate has been removed by centrifugation.

Goldstein and Swain (1965) developed another technique for measuring relative astringency spectrophotometrically. They measured the inhibition of enzymes such as β-glucosidase by tannic acid and condensed tannins through determining the amount of residual enzyme activity in the supernatant after centrifugation of the tannin–enzyme complex. β-glucosidase acts on the glycoside substrate aesculin to produce an aglycone, aesculetin, which forms a colored chelate with aluminum chloride. The reading at 385 nm for 10 min of the rise in absorbancy gives the amount of aesculetin produced, and,

therefore, the activity of the enzyme left in the supernatant, i.e., the amount of enzyme not complexed with the tannin. The level of enzyme inhibition is expressed as percentage of activity of the control.

Chromatographic Techniques. Chromatographic techniques employing an *n*-butanol–acetic acid–water solvent and a variety of phenolic reagents (vanillin HCl, potassium ferricyanide, diazotized *p*-nitroaniline, etc.) have been used to identify tannin constituents of plant extracts (Haslam, 1966; Feeny and Bostock, 1968; Laurent, 1975; Swain, 1979). Chromatography can also be used to quantify the amount of tannin in solution. Laurent (1975) separated tannin from nontannic phenols on a one-way chromatogram in aqueous acetic butanol solvent, eluted the nontannic phenols from the paper, and used Folin–Denis reagent to measure the total phenols in the eluate and in the raw extract to obtain tannin content by subtraction. She stated that this was the best technique of those she tested for measuring tannins accurately without inadvertently measuring nontannic phenols.

USES OF TANNIN-MEASURING TECHNIQUES

There are various reasons for studying plant tannins. Of the studies cited above, several were concerned with tannin content from the point of view of its economic importance. Bradfield and Penney (1944) wished to correlate tannin content with tea quality as determined by professional tea tasters. Burns (1963) investigated a variety of techniques in use for the estimation of tannin content of plants with respect to the selection, breeding, and management of potential forage crops. The AOAC (1965) methods are standardized methods of analysis used by government agencies that enforce food standard control laws. A detailed study of phenolic constituents of the Victoria plum tree was made to clarify the relationship of tannins to a canning problem known as "gummosis" of the fruit (Hillis and Swain, 1959; Swain and Hillis, 1959).

The use of chemical data in plant systematics relies on rigorous methodology and the ability to describe molecular structures; for this, specific spectrophotometric and chromatographic techniques are necessary (Bate-Smith, 1972a, b, 1973b, c, 1977; Heywood, 1973; Swain, 1979, 1980; Swain and Cooper-Driver, 1973). On the other hand, attempts to investigate the ecological relationships of plant tannins to herbivores can usefully be carried out on less, as well as on more, exhaustive levels (Feeny and Bostock, 1968; Cooper-Driver et al., 1977; Balick et al., 1978).

As interest in the adaptive significance of plant secondary compounds and in the testing of evolutionary models has risen (Rhoades, 1979; Swain, 1980), the relationships among tannins and herbivores have joined the

traditional concerns of physiology, biochemistry, pedology, phytopathology, food science, and industrial technology (Haslam, 1966; Van Sumere et al., 1975). In herbivory studies it is generally desirable to examine a fairly large number of plants; genotypic and phenotypic variability among individuals will make it difficult to detect real correlations of damage with tannin concentration in a small sample. The tannin-measuring technique chosen for such a study should therefore be as simple as possible to reduce time and labor costs. Additionally, the method should measure a component of tannin content which is ecologically significant. By this I mean that it is less important to know whether a given species contains ellagitannins or catechins than it is to know how palatable or digestible it is relative to other species, or how these characteristics change over time. Bate-Smith (1975, 1977) has shown that astringency (protein-precipitating capacity) varies with molecular structure within the proanthocyanidins. The *n*-butanol HCl method of measuring proanthocyanidins used alone (Cooper-Driver et al., 1977; Balick et al., 1978) may not yield results that can be related directly to plant palatability. Although a failure to find strong correlations between tannin content of plants and herbivore damage in field studies might be due to other factors, it would probably be as well to use some method of measuring plant astringency in tannin–herbivory studies.

Protein-precipitating capacity has traditionally been measured using hide powder or gelatin. Laurent (1975) claims that the results obtained with the hide powder technique are variable, and attributes this to the long, involved procedure which may result in tannin loss, particularly when dealing with low concentrations. Bate-Smith (1973a) also comments on the laboriousness of the procedure, and points out that the protein of skin is not the same as the protein of saliva, the precipitation of which accounts for the "puckery" sensation induced by tannin. The use of gelatin is discussed by Farnsworth (1966) and by Nierenstein (1934), who states that gelatin precipitates phenols other than tannins, such as hydroxyhydroquinone, gallic acid, and protocatechuic acid. Swain (1979) notes that this is also a problem when hide powder or polyvinylpyrrolidone are used in high concentrations.

Bate-Smith's hemanalysis (Bate-Smith, 1973a) avoids this difficulty by using a minimal amount of protein. It is a microtechnique, requiring the addition of 1 ml of plant extract to 5 ml diluted blood, and is very sensitive within the range of 0.3–0.8 mg tannic acid equivalents. This creates its own difficulties, however. The extract used in the test is not the original 50% methanolic plant extract, but the residue of that extract after evaporation, redissolved in the minimum volume of water. This concentrated extract may exceed the threshold of protein precipitation even though the original extract was below the threshold concentration; in any event, should the extract cause complete hemoglobin precipitation, it must be diluted and retested. Further-

more, changes in body chemistry may lead to callibration problems when using finger blood.

Despite the possibility of overestimation of tannin when using gelatin to measure astringency, the AOAC method of analysis for tea tannin has certain advantages, discussed below, that may make it a suitable alternative to hemanalysis for testing relative palatability or digestibility.

The official method calls for extracting tea tannin by making an infusion of 5 g dry wt tea in 400 ml boiling water, then diluting to 500 ml with water. When working with plants containing proanthocyanidins, 50% methanol is the preferred solvent (Farnsworth, 1966; Laurent, 1975; Swain, 1979). I found that the use of the methanolic extract had no effect on the AOAC procedure, interfering with neither the precipitation of gelatin nor the permanganate titration. The original procedure, calling for 5 g dry wt plant material per sample, is not suitable for studies where less biomass is available. In using this method to measure the relative astringency of bracken fern (*Pteridium aquilinum*) fronds, I found that by reducing all reagent quantities by a factor of five I was able to achieve sensitivity comparable at the low end of the range to that of the hemanalysis technique and extending rather farther at the high end. A drawback of this procedure is the high degree of precision needed in delivering the indicator to replicates. Small variations in the volume of indicator added will result in variable titration results which become magnified as the dilution factors are multiplied. This restricts the sensitivity at low concentrations, but levels of tannin as low as 0.28 mg in 2 ml extract can be detected without titration error (within-sample variability). Standard curves of ml 0.1 N $KMnO_4$ against mg tannin titrated yield straight-line relationships between 0 and 10 mg for both gallotannic acid (AOAC, 1965) and quebracho tannin (Figure 1).

An advantage of the method is the minimal amount of equipment required. No spectrophotometer or centrifuge is necessary. The original plant extract does not have to be processed prior to analysis, which represents a reduction in time as well as in equipment compared with the hemanalysis technique. The lack of precision resulting from the absorption by gelatin of some nontannic phenols will be less important in comparative within-species studies, where the error involved may be constant from sample to sample. (I note in passing that within-species variability of tannin concentration seems to have been largely ignored in the ecological literature, one value per species or per sampling date usually being given.) Furthermore, although phenolics of low molecular weight are too small to form the effective cross-links between proteins which result in precipitation of stable complexes, many of these have been shown to inhibit enzyme activity and to bond to proteins (Van Sumere et al., 1975). A method which includes them is therefore probably more biologically relevant than one which does not, since what is of interest in a

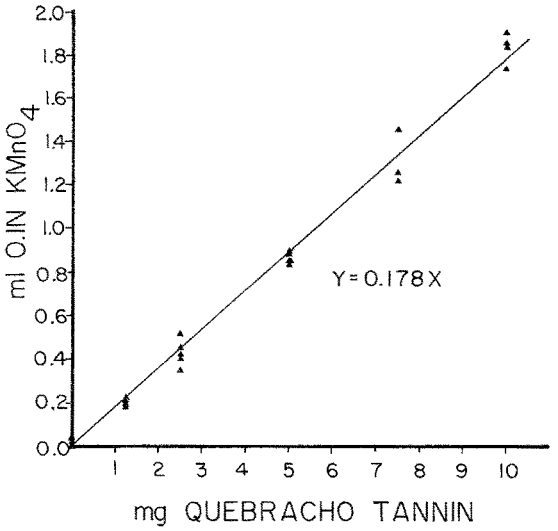

FIG. 1. Standard curve for titration of quebracho tannin (a condensed tannin) with potassium permanganate.

study of tannin–herbivore interactions is the protein-complexing capacity of the plant extract rather than the concentration of an arbitrarily defined class of phenolics.

CONCLUSIONS

For those interested in an exhaustive characterization of the tannin constituents in a plant, the chromatographic and spectrophotometric techniques reviewed by Swain (1979, 1980) probably offer the best methods currently available. But for those interested in a relatively simple method of estimating plant palatability for use in tannin–herbivore studies, the AOAC tea tannin method has a good deal to recommend it, in that it measures an ecologically relevant parameter of plant phenolics at a relatively low cost in time, equipment, and reagents. This advantage permits the extensive sampling needed to characterize variability in natural plant populations.

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ETHYL NICOTINATE: A Chemical Attractant for *Thrips obscuratus* (Thysanoptera: Thripidae) in Stonefruit in New Zealand

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Abstract—Ethyl nicotinate was found to be a potent attractant for *Thrips obscuratus* in peaches and apricots. The male-to-female ratio in traps was 1:8. The chemical was more attractive than ripe fruit, with peak catches at harvest. Season-long trapping showed the efficiency of the chemical compared to unbaited traps. The chemical remained attractive for at least 2 weeks when 50 μl were placed in open 2-ml vial caps. Various pyralids, geometrids (Lepidoptera), and chironomids (Diptera) were also attracted in low numbers to ethyl nicotinate.

Key Words—*Thrips obscuratus*, Thysanoptera, Thripidae, attractant, ethyl nicotinate, apricots, peaches.

INTRODUCTION

Chemical attractants are now in widespread use with many insect pests for improved spray timing or direct control. To date, little information is available on chemical attractants for thrips (Lewis, 1973), with most sampling techniques being based on physical attraction. Chemical attractants could greatly assist sampling programs for these highly mobile insects.

Thrips obscuratus (Crawford) is an indigenous species of New Zealand. It is strongly attracted to ripe peaches which led us to investigate the attractive properties of various chemicals possibly associated with fruit ripening.

METHODS AND MATERIALS

Small-scale field experiments at Lincoln College had previously indicated the attractive properties of some simple organic chemicals to insects.

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Several esters of nicotinic acid were attractive to some lepidopteran species and reevaluation of trap catches indicated positive attraction of *T. obscuratus* to ethyl nicotinate in particular. More extensive experiments were conducted to confirm the attractive properties of this chemical in apricots at Lincoln College, Canterbury, and peaches in Alexandra, Otago, New Zealand.

1979–1980. Folded cardboard traps, similar to Zoecon Pherocon 1C® traps, with the 30 × 25-cm base of each trap covered by a thin layer of Tack Trap®, were used to monitor thrip catches. In view of the previously noted attraction of *T. obscuratus* to ripening fruit, both ripe peaches and apricots were placed in traps, in addition to unbaited controls. One milliliter of ethyl nicotinate was placed in a 2-ml-capacity plastic vial cap filled with a cottonwool wick. The cap was placed in the center of the trap. Four replicates of each were placed singly in trees 1.5 m above ground level and at ground level. Traps were removed after 48 hr.

1980–1981. Experiments were confined to ethyl nicotinate in apricots at Lincoln College. A season-long trapping program ran from October 10 to May 13, using 50 μl of ethyl nicotinate in each vial cap. Traps were renewed weekly, and all traps were placed singly in trees 1.5 m above ground level. Four replicates of each treatment were prepared. The attractiveness of ethyl nicotinate aged over 5 weeks was also compared with weekly catches using fresh caps.

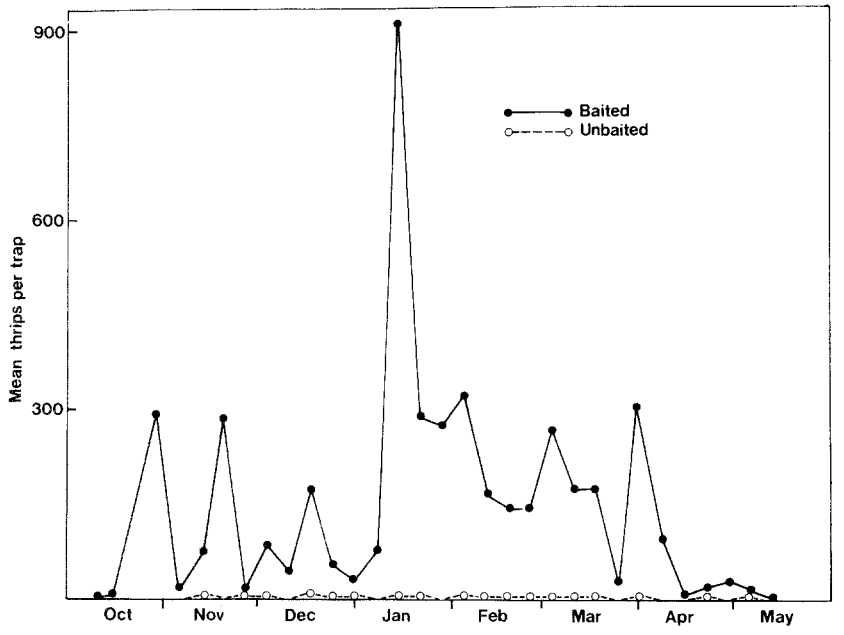

FIG. 1. Seasonal record (1980–1981) of abundance of *Thrips obscuratus* measured by ethyl nicotinate baited and unbaited traps (mean of 4 traps).

RESULTS

1979–1980. Ethyl nicotinate was a significant attractant for *T. obscuratus* compared with the attractiveness of either ripe peaches or apricots. In peaches at Alexandra (Jan. 20, 1980), 48-hr catches were 95.0 ± 20.3 and 81.5 ± 11.8 per trap (mean of 4 replicates) with ethyl nicotinate at 1.5 m and ground level, respectively. Comparative catches with ripe peaches were 13.8 ± 5.7 and 17.5 ± 8.0, and for the controls, 11.8 ± 7.5 and 3.8 ± 1.1. Experiments at Lincoln College (Jan. 23, 1980) in apricots gave 48-hr catches of 772 ± 387 and 358 ± 98 at 1.5 m and ground level, respectively, with ethyl nicotinate, compared with 6.5 ± 0.5 and 0 for ripe apricots, and 28.8 ± 13.4 and 13.8 ± 4.9 for the controls.

1980–1981. The season-long trapping program using 50 μl of ethyl nicotinate per cap began with the first catch of *T. obscuratus* by October 15, 1980 (Figure 1). The positive attraction of ethyl nicotinate for *T. obscuratus* is clearly shown in comparison with unbaited controls. No chemical sprays were applied for thrip control, so the peaks in trap catches may coincide with generation or flight times. Maximum trap catch coincided with the ripening of the apricots. The corresponding decline in thrip catches mirrors the harvest of the ripe fruit since harvest commenced on January 7, and all fruit was

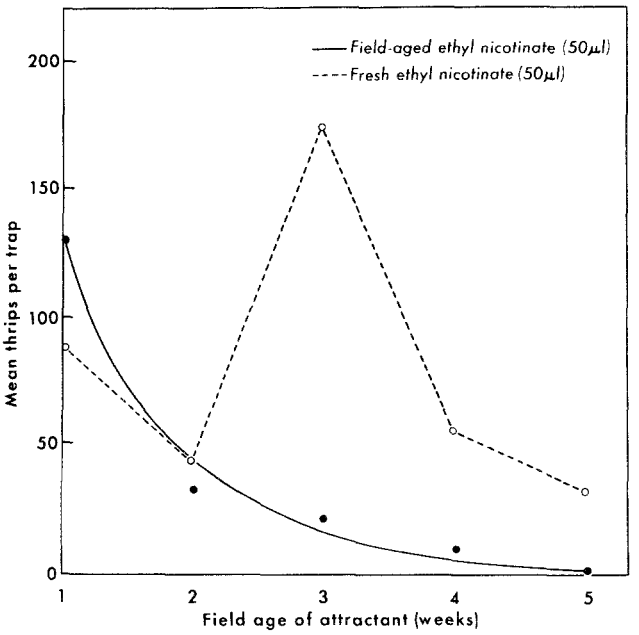

FIG. 2. Attraction of *Thrips obscuratus* to field-aged caps of ethyl nicotinate compared with attraction to fresh caps.

removed from the experimental trees by January 23. Examination of ripe apricots ($N = 30$) over the harvest period showed a mean at 160.7 ± 1.8 *T. obscuratus* per fruit. In spite of this, trap catches suggest that ethyl nicotinate remained a potent attractant over the period of fruit ripening.

Sex ratios in the natural population were not determined but trap catches gave an 8:1 ratio of females over males.

Formulation of the attractant has received little attention. However, a comparison of trap catches between field-aged and fresh caps showed a similar level of attraction with caps up to 2 weeks old (Figure 2). Daily measurement of ethyl nicotinate caps held at 25°C in an enclosed controlled-temperature room showed a decline in weight from 55 mg to 4 mg after 6 days, suggesting there is potential for further reduction in the amount of ethyl nicotinate per cap.

Other insects were positively attracted in low numbers to ethyl nicotinate-baited traps compared with unbaited traps. In particular, the lepidopteran families Pyralidae and Geometridae were regularly trapped, and Chironomidae (Diptera) were trapped late in the season. Identification of trap catches were made to genus, and representative trap catches are shown in Table 1.

The utility of ethyl nicotinate as an attractant has yet to be resolved. In our trial, no *T. obscuratus* were trapped during the blossom period. Whether

TABLE 1. CATCHES OF INSECTS IN ETHYL NICOTINATE-BAITED TRAPS ON REPRESENTATIVE DATES[a]

Insects	Date 1980–1981					
	Nov. 19	Dec. 17	Jan. 14	Feb. 18	Mar. 18	Apr. 22
Lepidoptera						
Family: Pyralidae						
Mnesictena flavidalis	1.5	0.5	NA[b]	0.25	0.25	1.0
Eudonia spp.	5.75	0.5	NA	1.25	0.75	0.25
Orocrambus spp.	0	0.5	0.5	2.5	0.75	0.25
Scoparia spp.	0.75	0	NA	0.25	0.25	0
Family: Geometridae						
Helastia spp.	5.75	0.75	0	0.5	0.75	1.75
Diptera						
Family: Chironomidae	0	0	0	2.75	3.5	2.5
Thysanoptera						
Family: Thripidae						
Thrips obscuratus	289	174	919	147	176	18

[a]Means of 4 traps, weekly catch.
[b]NA = not available.

this was a factor of low population density or a possible change in food preferences has not been determined. However, for the remainder of the season, ethyl nicotinate was a potent attractant for *T. obscuratus*, being more than competitive with ripening fruit. Similarly, trap design requires further investigation and preliminary trials have suggested that attaching an attractant-filled cap to a 75-mm-diameter plastic disk covered in Tack Trap was equally as effective as the Pherocon 1-type trap. Ethyl nicotinate is a powerful dermal irritant, so care is necessary in its handling.

Achnolegements—We thank Dr. W.L. Roelofs, New York State Agricultural Experiment Station, for his review of the manuscript.

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SEX PHEROMONE COMPONENTS OF THE TURNIP MOTH, *Agrotis segetum*[1]: Chemical Identification, Electrophysiological Evaluation and Behavioral Activity[2]

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Abstract—Analysis of female abdominal tips of *Agrotis segetum* by means of GC-MS showed the presence of 13 aliphatic acetates and alcohols. (*Z*)-7-Dodecenyl acetate was found to be the main component in the extracts at amounts of about 1 ng/female. (*Z*)-9-Tetradecenyl acetate and (*Z*)-7-dodecenol were present to the extent of 49 and 19%, respectively, of the main component. Minor components could be identified as decyl acetate, (*Z*)-5-decenyl acetate, dodecyl acetate, (*Z*)-9-dodecenyl acetate, tetradecyl acetate, a tetradecenyl acetate, hexadecyl acetate, a hexadecenyl acetate, (*Z*)-5-decenol, and (*Z*)-9-tetradecenol. The presence and biological activity of decyl acetate, (*Z*)-5-decenyl acetate, and (*Z*)-7-dodecenyl acetate in the extracts could be detected by GC-EAD. Tested by EAG (*Z*)-5-decenyl acetate evoked the highest response among pheromone candidates, followed by (*E*)-5-decenyl acetate and (*Z*)-7-dodecenyl acetate. Single-cell recordings from 100 male antennal sensilla trichodea revealed receptor cells highly sensitive to (*Z*)-5-decenyl, (*Z*)-7-dodecenyl, (*Z*)-8-dodecenyl, and (*Z*)-9-tetradecenyl acetate as well as (*Z*)-5-decenol. The (*Z*)-5-decenyl, (*Z*)-7-dodecenyl, and (*Z*)-9-tetradecenyl acetate receptors were activated significantly also by female extracts. When tested in a tube olfactometer, a blend of decyl, (*Z*)-5-decenyl, (*Z*)-7-dodecenyl, and (*Z*)-9-tetradecenyl acetate evoked the same male response as did female glands.

[1]Schiff., Lepidoptera: Noctuidae.
[2]This study was made within the Swedish project "Odour Signals for Control of Pest Insects."

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Tested in the field, this blend was more attractive than virgin females. Other authors previously reported many of the compounds identified in the present study. However, both quantitative and qualitative discrepancies exist among the various investigations, possibly due to the existence of geographical races.

Key Words—*Agrotis segetum*, turnip moth, Lepidoptera, Noctuidae, sex pheromone, straight-chain acetates, single-cell recordings, electroantennography, behavior, gas chromatography–mass spectrometry, entrainment.

INTRODUCTION

Multicomponent sex pheromones seem to be more the rule than the exception in moths (Silverstein and Young, 1976; Tamaki, 1979). The sex pheromone of the turnip moth *Agrotis segetum* (Schiff.) (Lep., Noctuidae) is a recent example. Bestmann et al. (1978) reported (*Z*)-5-decenyl acetate (*Z*5–10:Ac) to be a sex attractant of *A. segetum*. Tóth et al. (1980) described the pheromone of *A. segetum* as composed of a 4:1 mixture of *Z*7–12:Ac and *Z*9–14:Ac, while Arn and coworkers (1980) reported the pheromone of the species to consist of a blend of *Z*5–10:Ac, *Z*7–12:Ac, *Z*9–12:Ac, *E*5–12:Ac, and 12:Ac. However, the behavioral significance of the latter three compounds was unclear. Another compound, *Z*8–12:Ac, was identified from the female pheromone gland, but it was assigned an inhibitory effect on male attraction.

In addition to the studies on European turnip moths, Wakamura (1978, 1980) identified a mixture of *Z*5–10:Ac and *Z*7–10:Ac as the pheromone of the Japanese *Agrotis fucosa*, which was synonymized recently by taxonomists with *A. segetum* (S. Wakamura, personal communication).

The present study was initiated in 1980 when we failed to trap *A. segetum* in the field with *Z*5–10:Ac, the sex attractant reported by Bestmann et al. (1978). We then started a detailed GC-MS examination of the pheromone of *A. segetum*. The chemical studies were accompanied by investigations of the electrophysiological and behavioral significance of the identified compounds.

METHODS AND MATERIALS

Insect Material. A. segetum moths were obtained from a culture permanently maintained at the Ecology Building, University of Lund, and based on insects originating from southern Sweden and Denmark. The culture had not been fortified with wild insects since 1978. Thus insects used for this study were inbred for about 20 generations. Larvae were reared on a Hinks and Byers' (1976) diet, using potato instead of pea beans. The culture was maintained on a 17-hr light–7-hr dark cycle with photophase temperature

23°C and scotophase temperature 16°C for both pupae and adults. Larvae were kept under the same light regime but at a temperature of 26°C.

The insects were sexed as pupae and placed in separate compartments. After emergence, 1–3 adults were kept in 250-ml plastic jars and fed a 5% honey solution. Females used for preparation of extracts were fed a 5% sucrose solution.

Observation of Calling Behavior. Female moths used for studies of calling behavior were placed individually in 250-ml plastic jars covered with a nylon screen. Red darkroom lamps (ca. 10 lux at moths) were used to permit observation during the scotophase. The insects were observed during 10 nights after emergence. To determine the calling period, observations were made during the entire dark period at 30-min intervals, and the number of calling females was recorded. Once the calling period had been ascertained observations were made from 1 hr before to 1 hr after calling.

Females were regarded as calling when they clung to the walls or to the screen cover and exposed their ovipositors. Their pheromone glands were then readily visible.

The mean time of day of calling activity (T) was calculated as follows:

$$T = \frac{\Sigma[(\text{time}) \times (\text{No. of females calling at that time})]}{\text{Total No. of callings recorded}}$$

The time is measured as the number of hours into the dark period.

Preparation of Extracts. Extracts were prepared from 2- to 5-day-old females within a 2-hr period of maximum calling activity (see Figure 1). The abdominal tip was stripped off immediately anterior to the pheromone gland with a pair of forceps (Otto et al., 1976). Before immersion in a sharply coned dissection vial containing 100–500 μl solvent (pentane or hexane), gut contents and hemolymph were removed by adsorption on filter paper. The dissection vial was kept at −20° C during preparation and afterwards stored in the dark at −20° C. The solvent was recovered with a syringe 24 hr following the last preparation. It was stored in a sealed ampoule at −20° C until analysis.

Entrainment. Female effluvium was examined using charcoal filters similar to those described by Grob and Zürcher (1976). One to three moths were placed in a glass entrainment chamber (250 ml) before the beginning of the dark period. Air (charcoal-filtered and humidified) was passed through a chamber at a rate of 1.3 liters/min. Volatiles were entrained on the same filters for 5 hr starting from 1 hr into the dark period. Entrainment from one female obtained in this way was called "one female calling night."

Adsorbed material was extracted with three portions of carbon disulfide (15 + 10 + 10 μl).

GC-MC. Gas chromatographic–mass spectrometric analyses were performed on a Finnigan 4021 GC-MS equipped with an Incos data system.

Pentane and hexane extracts of 20–80 female abdominal tips were concentrated to approximately 20 μl by careful heating. Approximately a fourth of this sample was injected, splitless, on a WCOT capillary column (60 m × 0.24–0.30 mm ID) coated with Superox FA (Alltech Assoc.). The linear helium flow through the column was 26 cm/sec at 60°C. The injector temperature was 210°C. The split valve was opened 0.5 min after injection. Column temperature was maintained at 60°C for 5 min following injection, and then heated to 220°C using a program of 10°C/min.

In certain analyses 10:Ac, 12:Ac, 14:Ac, 16:Ac, and 18:Ac were added as references for quantifications or for the calculation of retention values relative to the straight-chain acetates [equivalent chain length (ECL) values]. ECL values from the same substance never differed more than two ECL units in subsequent analyses.

GC-FID and GC-EAD. Gas chromatographic analyses were performed on a Hewlett Packard 5835 gas chromatograph. An adjustable micro needle-valve effluent split (Scientific Glass Engineering, Inc.) was inserted between the column and the detector to permit simultaneous flame ionization detection (FID) and electroantennographic detection (EAD) (Arn et al., 1975). A split ratio of 4:1 was used.

Aliquots of sample (1–5 μl) were injected, splitless, on a WCOT fused silica capillary column (20 m × 0.19 mm ID) coated with SP 1000 (Supelco, Inc.). The nitrogen flow through the column at 60°C was 13 cm/sec. The injector temperature was 250°C. The split valve was opened 0.5 min after injection. Column temperature was maintained at 60°C for 5 min following injection, and then raised to 200°C using a program of 8°C/min. Under these conditions, standard samples of most long-chain olefinic acetates could be resolved. The chromatographic conditions allowed flame ionization detection of ca. 200 pg. Although no actual quantification was undertaken, electroantennographic detection on the order of 1 pg of Z5–10:Ac using a male *A. segetum* antenna as detector was possible.

Ozonolysis. One extract obtained from approximately 50 females was fractionated according to chain length of acetates and alcohols on a Carlo Erba 4160 GC equipped with a Superox FA SCOT column. Ozonolysis according to Beroza and Bierl (1967) was performed on the fractions. Separation and identification of the ozonolysis products were obtained by GC-MS.

Electrophysiology. Electroantennogram (EAG) and single-cell experiments were performed on excised antennae of male *A. segetum* moths. The experimental arrangement used in the EAG measurements was essentially the same as described by Van Der Pers (1981). Single-cell recordings were made using the tip recording technique as applied by Van Der Pers and Den Otter (1978).

In both EAG and single-cell experiments the odor sources were

disposable syringes containing a piece of filter paper (13 × 13 mm) onto which 1 μg of a given test substance had been pipetted. The test substances are given in Figure 4.

The maximum EAG amplitude was used as a measure of response in the EAG experiments and expressed as a percentage relative to the reference response.

Tube Olfactometry. Male behavioral response to different stimuli was studied in a tube olfactometer similar to that described by Sower et al. (1973). The glass tube was 45 mm in diameter and 450 mm long with a glass filter in the upwind end and a nylon screen at the outlet. Filtered, compressed air was delivered to the tube at a rate of 0.3 m/sec.

The tube was lighted uniformly from below with red light (650 nm peak emission, 3 lux) through white transluscent Perspex. A male, 2–4 nights old, was introduced downwind in the tube at least 10 min before the onset of the stimulation. The tube was connected to the air supply 2 min prior to stimulation.

Stimuli were inserted 25 mm from the upwind end of the tube. Female glands to be tested for activity were excised from calling females and held in the air stream with a pair of forceps. Synthetic chemicals were applied in hexane (1 μl) to a glass rod applicator, which was inserted into the tube as soon as the solvent had evaporated. Male activity was observed during 2 min after the stimulation was started. The frequencies of the following male behavioral steps (cf. Baker et al., 1976) were recorded on a tape recorder: (1) orientation into the 5-cm upwind end of the tube; (2) wing-fanning while walking or flying short distances; (3) extension of their genital claspers; and (4) clinging to the applicator, trying to copulate with it.

The tubes were successively washed with ethanol, acetone, and heptane and subsequently heated to 170°C for at least 1 hr between experiments.

Field Attractancy Test. Between June 26 and July 14, 1981, field tests were conducted close to Lund (southern Sweden), using traps similar to those described by Kärnestam (1979). The traps consisted of a plastic tube (8 cm diam. × 40 cm long) attached to a wind vane which was free to rotate on a pole 1.2 m above ground. A conical metal screen on each end directed males inside the tube but prevented their escape. Fourteen pairs of traps were spaced at least 60 m from each other in lettuce, potato, and carrot fields. The distance between traps within each pair was 4 m. One trap of a pair was loaded with a newly emerged virgin female from a laboratory culture and the other with a synthetic pheromone blend. When baited with a virgin female, a metal screen cage inside the tube contained the insect. The insect was fed sucrose solution and replaced every week. When baited with synthetic chemicals, the insect cage was changed for a 1-ml polyethylene capsule (36 × 8 mm, 1.5 mm wall thickness). The capsule was loaded with chemicals dissolved in pentane/hexane. The solvent was allowed to evaporate before the capsule was closed.

Synthetic baits were not changed during the experimental period. Traps were checked every third or fourth day.

Chemicals. *Z*4-10:Ac, *E*5-10:Ac, *Z*5-10:Ac, *Z*6-10:Ac, *E*7-12:Ac, *Z*7-12:Ac, and *Z*9-14:Ac were synthesized via the corresponding alkynes, which were hydrogenated with Lindlar catalyst and Na/NH_3 for *Z* and *E* isomers, respectively. After purification (Houx et al., 1974), these compounds were more than 99.9% pure with respect to positional and geometrical isomers.

Other compounds used were commercially available or gifts from several laboratories.

RESULTS

Female Calling Rhythm. Female calling activity was at a maximum 4.5 hr after the beginning of the dark period. The mean time of calling was 3.9 ± 0.9 hr ($N = 86$) into the dark period (Figure 1). Differences in the percentage of females calling and diurnal rhythm of calling between the second and the fifth night after emergence were minor.

GC-MS Analyses and Ozonolysis. A typical reconstructed total ion chromatogram from an analysis of 9 female equivalents is shown in figure 2A. Figure 2B shows the corresponding mass chromatograms (extracted ion

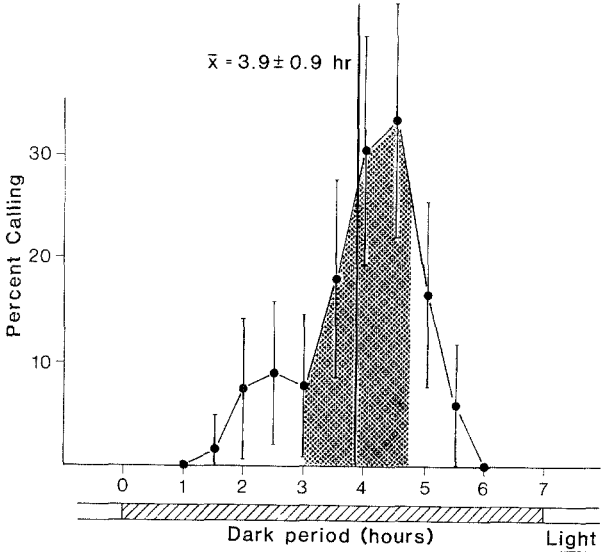

FIG. 1. Percent calling by female *Agrotis segetum* on their fifth night after emergence as calculated from observations every 30 min ($N = 66$). Vertical lines indicate 95% confidence intervals. $\bar{X}$ is the mean time of day of calling (±SD, shaded area).

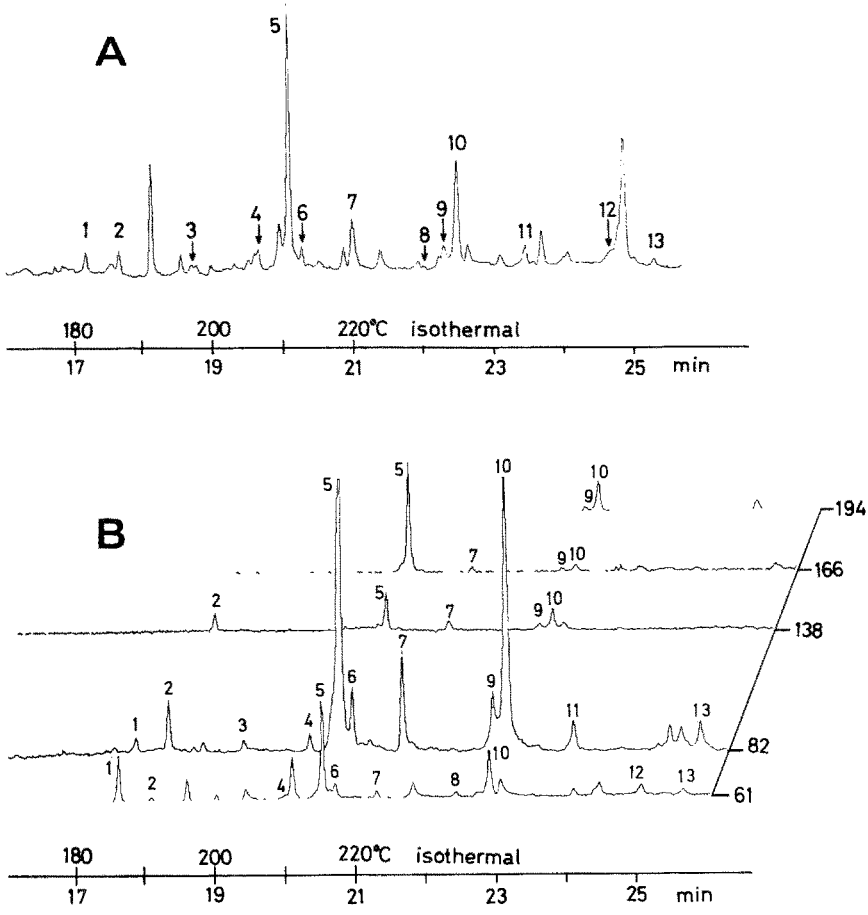

FIG. 2. (A) Total ion chromatogram, m/z 30–31, 33–300 and (B) mass chromatograms (extracted ion current profiles) of m/z 61, 82, 138, 166, and 194 of an extract corresponding to 9 female *A. segetum* abdominal tips. m/z 61 is a typical fragment of acetates, and m/z 82 indicates unsaturated straight-chain compounds. m/z 138, 166, and 194 correspond to M^+-CH_3COOH of decenyl, dodecenyl, and tetradecenyl acetate respectively, and to M^+-H_2O of decenol, dodecenol, and tetradecenol. Peak numbers refer to compounds listed in Table 1.

current profiles) for some fragments of diagnostic value. Compounds found with chemical structure similar to moth pheromones identified earlier can be divided into the following three groups:

1. Saturated Acetates. The compounds corresponding to peaks 1, 4, 8, and 12 were identified as 10:Ac, 12:Ac, 14:Ac, and 16:Ac, respectively, from their mass spectra and retention values.

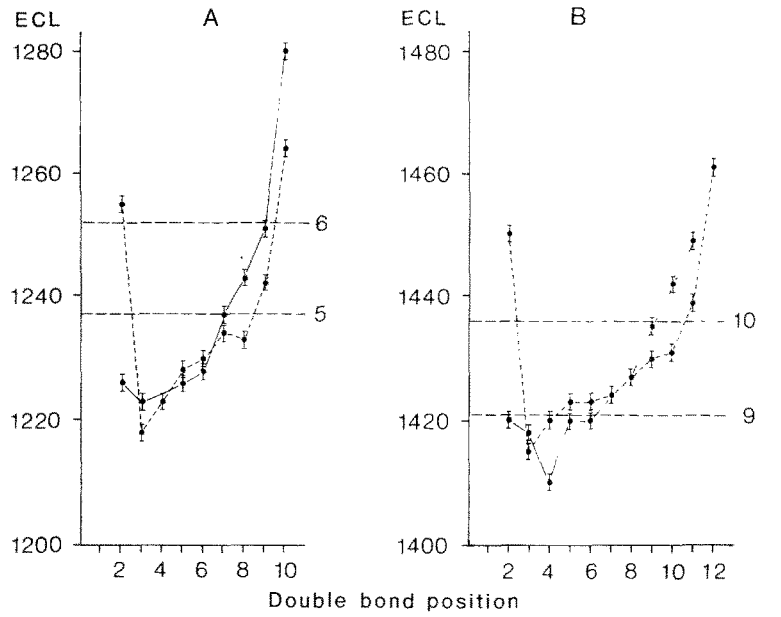

FIG. 3. Variation in equivalent chain length (ECL) with the position of the double bond; dodecyl acetate = 1200, tetradecyl acetate = 1400, on a superox FA WCOT capillary column. (A) dodecenyl acetates, (B) tetradecenyl acetates; – – – – *E* isomers, ——— *Z* isomers. Vertical lines indicate maximum spread (±2 ECL units). Compounds 5, 6, 9, and 10 refer to substances isolated from *A. segetum* females.

2. Monounsaturated Acetates. From their mass spectra the following assignments were made: compound 2 was a decenyl acetate, compounds 5 and 6 dodecenyl acetates, compounds 9 and 10 tetradecenyl acetates, and compound 13 a hexadecenyl acetate.

The calculated ECL (1037) of compound 2 is within the range of both *E*5–10:Ac and *Z*5–10:Ac. These two isomers were not separated by GC. Other decenyl acetate references available were *Z*4 (1020), *Z*6 (1043), *E*7 (1045), and *Z*7 (1052).

The ECL of compound 5 (1237) corresponds to that of synthetic *Z*7–12:Ac (Figure 3A). Compound 6 (1252) could be either the *E*2 or the *Z*9 isomer. However, the mass spectra of these compounds differ: *E*2 shows significantly less abundant fragments in the high mass range. Thus compound 6 was identified as *Z*9–12:Ac.

Concerning the tetradecenyl acetates, compound 9 could probably be any of the following isomers: *E*4, *E*5, *E*6, *Z*5, or *Z*6. The calculated ECL (1436) of peak 10 is identical to that of synthetic *Z*9–14:Ac (Figure 3B). This assignment was confirmed by ozonolysis. On the other hand, the isolated

TABLE 1. AMOUNTS OF SUBSTANCES RELATIVE TO AMOUNT OF *Z*7-12:Ac ISOLATED FROM *Agrotis segetum* FEMALE ABDOMINAL TIP EXTRACTS

Assignment	No.	Relative amount
10:Ac	1	8
*Z*5-10:Ac	2	6
*Z*5-10:OH	3	3
12:Ac	4	5
*Z*7-12:Ac	5	100[a]
*Z*9-12:Ac	6	7
*Z*7-12:OH	7	19
14:Ac	8	1
Z/*E*?14:Ac[b]	9	6
*Z*9-14:Ac	10	49
*Z*9-14:OH	11	8
16:Ac	12	2
Z/*E*?16:Ac[c]	13	3

[a] The amount of *Z*7-12:Ac isolated per female was approximately 1 ng.
[b] This tetradecenyl acetate isomer was probably one of the following: *E*4, *E*5, *E*6, *Z*5, or *Z*6 (see text).
[c] Double-bond isomerism was not determined.

amount of compound 9 (about 3 ng from 50 females Table 1) was too low for ozonolysis.

The isomerism of the hexadecenyl acetate was not determined.

3. Monounsaturated Alcohols. The mass spectra of peaks 3 (1122), 7 (1313), and 11 (1509) correspond to those of a decenol, a dodecenol, and a tetradecenol, respectively. These ECL values correspond with those of synthetic *Z*5-10:OH, *Z*7-12:OH, and *Z*9-14:OH. However, only a limited number of references was available (one decenol, five dodecenols, four tetradecenols) and correspondence with other isomers cannot be ruled out.

Simultaneous GC-FID and GC-EAD of Female Extracts and Entrained Material. GC-EAD of female extracts with a male *A. segetum* antenna as the detector gave an EAD chromatogram with three reproducible peaks. The retention times of these compounds corresponded to the retention times of synthetic 10:Ac, *Z*5-10:Ac, and *Z*7-12:Ac, respectively. The *A. segetum* antenna did not give a significant response to *Z*9-14:Ac which was shown by GC-MS and ozonolysis analysis to be one of the major compounds in the female gland extract. However, when a male *Adoxophyes orana* (*F.v.R.*) antenna was used as the detector, the presence of *Z*9-14:Ac in the *A. segetum* female extract was confirmed. The antenna of *A. orana* is known to be highly sensitive to *Z*9- and *Z*11-14:Ac (Den Otter, 1977). With this antenna, a strong

EAD response (2 mV) was obtained from the extract of an *A. segetum* female gland at the same retention time as that of synthetic *Z*9–14:Ac.

A sample containing the entrained volatiles from half a female calling night gave rise to an EAD chromatogram similar to that of a female gland extract. The decyl acetate and (*Z*)-5-decenyl acetate peaks were of the same magnitude as in several analyses of gland extracts, while the *Z*7–12:Ac was smaller but still significant. The FID signal of *Z*7–12:Ac corresponded to somewhat less than 1 ng/female calling night. In a single experiment using a *A. orana* antenna it was not possible to detect *Z*9–14:Ac in the entrained volatiles.

Electrophysiology. Several compounds identified by means of GC-MS or GC-EAD in this study or mentioned earlier in the literature (Arn et al., 1980; Wakamura, 1978) can be considered as *A. segetum* pheromone candidates. Of these *Z*5–10:Ac showed the highest EAG activity (Figure 4): two times higher than *E*5–10:Ac. The following compounds elicited lower EAG activity: 10:Ac, *Z*5–10:OH, *Z*7–12:Ac, and *Z*8–12:Ac. However, the responses were significantly higher than those obtained from the other compounds tested.

The single-cell responses of 100 sensilla trichodea to the same set of substances (with the exception of *E*5–10:Ac) are summarized in Table 2. Receptor cells very sensitive to *Z*5–10:Ac, *Z*7–12:Ac, *Z*9–14:Ac, *Z*5–10:OH, and *Z*8–12:Ac were present. The first three compounds activated cells

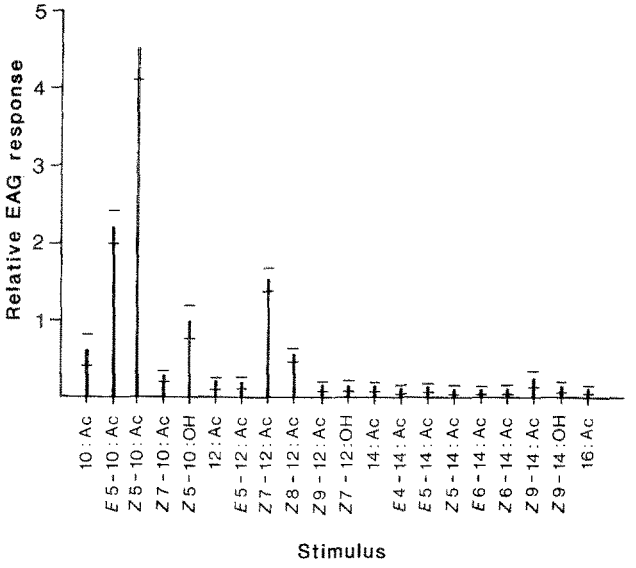

FIG. 4. Relative EAG responses to compounds tested as *A. segetum* pheromone component candidates.

TABLE 2. SINGLE-CELL SCREENING OF 100 SENSILLA TRICHODEA ON MALE *A. segetum* ANTENNAE[a]

Sensillum	Reaction to	%	Reaction to gland extract
Type A	Z5-10:Ac (large spike amplitude) Z5-10:OH (small spike amplitude) Z8-12:Ac (small spike amplitude)	68	Activated
Type B	Z7-12:Ac (large spike amplitude)	28	Activated
Type C	Z9-14:Ac (large spike amplitude)	2	Activated
Type D	"Silent type" (no reaction)	2	
		100	

[a]All compounds listed in Figure 4 except ES-10:Ac were tested. In addition, every sensillum type was tested for activation by a female abdominal tip extract (25 FE).

with large spike amplitudes; the following two components activated cells with small spike amplitudes. The omission of *E*5-10:Ac from the 100 sensilla trichodea series was rectified afterwards to some extent by testing a limited number of the A and B type sensilla (Table 2). However, *E*5-10:Ac did not elicit any single-cell activity from these sensilla.

Three different cells identical to the earlier-mentioned *Z*5-10:Ac, *Z*7-12:Ac, and *Z*9-14:Ac receptor were activated by the female extract.

Tube Olfactometry. Hexane, female glands, and a selection of 12 synthetic stimuli were tested for behavioral activity. The selection of synthetic compounds to be tested was based on the chemical analyses and electrophysiology (Figure 4, Table 2).

Neither *Z*5-10:Ac nor *Z*7-12:Ac applied in dilution series elicited a significant behavioral response, with the exception of wing-fanning (Table 3). The lowest amount tested approximately corresponded to the content of that specific compound in one excised female gland. The concentration was subsequently increased in decadic steps.

Contrary to single substances, combinations of compounds in proportions similar to those found in female glands gave rise to significant extension of genital claspers and copulation efforts. The activity of a four-component blend consisting of *Z*5-10:Ac, 10:Ac, *Z*7-12:Ac, and *Z*9-14:Ac was as high as that of a female gland (Table 3).

Field Attractancy Test. The most active laboratory stimulus was compared with the attractivity of virgin females in the field. Traps baited with 10:Ac (1.25 μg), *Z*5-10:Ac (1.0 μg), *Z*7-12:Ac (12 μg), and *Z*9-14:Ac (10 μg) caught significantly more moths than traps baited with living females ($P < 0.01$, Wilcoxon matched-pairs signed-ranks test, two-tailed). The average catch per trap and night was 3.5 and 1.3 males, respectively. The

TABLE 3. NUMBER OF MALE *A. segetum* RESPONDING TO DIFFERENT STIMULI IN A TUBE OLFACTOMETER (N = 10)[a]

Stimulus and amount (ng)	Wing-fanning	Orientation	Extension of genital claspers	"Copulation"
Hexane	4 a	4 abc	0 a	0 a
*Z*5-10:Ac				
0.1	5 a	2 a	1 ab	1 ab
1	9 a	5 abc	0 a	0 a
10	6 a	7 bc	0 a	0 a
100	7 a	5 abc	0 a	0 a
*Z*7-12:Ac				
1	9 a	6 abc	0 a	0 a
10	8 a	4 abc	0 a	0 a
100	7 a	3 abc	0 a	0 a
*Z*5-10:Ac (0.02) + *Z*7-12:Ac (1)	5 a	3 abc	1 ab	0 a
*Z*5-10:Ac (0.02) + *Z*7-12:Ac (1) + 10:Ac (0.04)	4 a	4 abc	2 ab	1 ab
*Z*5-10:Ac (0.02) + *Z*7-12:Ac (1) + *Z*9-14:Ac (1)	8 a	1 a	0 a	0 a
*Z*7-12:Ac (1) + *Z*9-14:Ac (1) + 10:Ac (0.04)	9 a	2 ac	0 a	1 ab
*Z*5-10:Ac (0.02) + *Z*7-12:Ac (1) + *Z*9-14:Ac (1) + 10:Ac (0.04)	9 a	7 bc	6 b	4 b
Female gland	8 a	8 b	5 b	4 b

[a]Numbers in the same column followed by the same letter are not significantly different at the 5% level (chi-square test with Yates correction).

number of moths trapped which belonged to other species in relation to the number of *A. segetum* males trapped was 12.5% in the female-baited traps and 6.3% in the traps baited with synthetic pheromone.

DISCUSSION

In the present study 13 pheromone candidates were isolated from female gland extracts. Seven of these compounds could be characterized unequivocally by chemical means: 10:Ac, 12:Ac, 14:Ac, 16:Ac, *Z*7-12:Ac, *Z*9-12:Ac, and *Z*9-14:Ac. In addition, EAG and single-cell experiments firmly sup-

ported the presence of Z5–10:Ac. While Z- and E5–10:Ac could not be resolved by GC, only receptor cells specialized to Z5–10:Ac were found on the male antenna. The second tetradecenyl acetate found (compound 9, Figure 2, Table 1) was present in small amounts. No specialized receptors were found on the male antenna for the five candidate isomers suggested by GC-MS. Therefore no further chemical characterization was attempted. The hexadecenyl acetate isomer was identified with certainty only in the final stage of our work, and no trials to determine the double-bond configuration were made.

The chemical analysis results are consistent with those of Tóth and coworkers (1980), who identified Z7–12:Ac and Z9–14:Ac, in the ratio 4:1, as the two major straight-chain monoolefinic acetates in *Agrotis segetum* female pheromone glands. Bestmann et al. (1978) reported Z5–10:Ac which is quantitatively a minor component in our animals.

On the other hand, there are both quantitative and qualitative discrepancies between the results obtained by Arn et al. (1980) and our results. Arn and coworkers reported Z9–12:Ac to be the most abundant dodecenyl acetate isomer, although of no certain behavioral function. In our study this isomer only amounts of 7% of the major compound, Z7–12:Ac. Moreover, the 12:Ac is a minor compound compared to Z7–12:Ac. We could not detect E5–12:Ac and Z8–12:Ac, which were among the seven compounds found by Arn et al. The detection limit of our general analytical procedure is about 100 pg. On the other hand it is possible that small amounts of Z8–12:Ac eluted close to the major compound Z7–12:Ac on the columns used for this study and were therefore unresolved. We know of no well-documented cases, however, where the major pheromone-like compound in the gland extract is not also a primary sex pheromone component (Roelofs and Cardé, 1977). Such is the case for *A. segetum* where Z7–12:Ac was shown to be the major component.

Contrary to the work of Tóth et al. (1980), we have not shown Z9–14:Ac to be present in the female effluvium. It is possible that the number of females used for entrainment was too low. Decreasing yield for compounds of lower volatility was also a problem. Using a male *A. segetum* anenna for GC-EAD analysis of potential Z9–14:Ac-containing samples had no advantage compared to GC-FID because of the low antennal sensitivity to that compound.

Specialized receptors on the male antenna were found for only five of the 20 compounds listed in Figure 4. Three of these receptor cells reacted with large spike amplitudes to Z5–10:Ac, Z7–12:Ac, and Z9–14:Ac, respectively, and they were all activated by the female extract. In tube olfactometry, a combination of these three compounds with the addition of 10:Ac, at low concentrations, elicited strong close-range behavioral responses such as extension of genital claspers and copulation efforts towards the stimulus applicator. Thus we conclude that a mixture of 10:Ac, Z5–10:Ac, Z7–12:Ac, and Z9–14:Ac in a ratio similar to that found in calling females is a good

approximation of the pheromone of *A. segetum*. Although no specialized receptors were found in single-cell screening, 10:Ac seemed to be essential for full behavioral activity in the laboratory behavioral bioassay. In the field, a combination of the compounds in the same ratio (with the absolute amounts based upon 1 μg of *Z*5–10:Ac) was three times more attractive than virgin females. This blend should be compared with that recommended by Arn et al. (1980). The activity of their blend (also based upon 1 μg *Z*5–10:Ac) was reported as being the same as of virgin females.

The ratio of substances in our synthetic blend used in the field experiments was based on a preliminary GC-MS quantification in an early stage of our work. Compared to the final GC-MS figures reported in this study, the proportion of *Z*9–14:Ac in the lures was probably too high. For *Z*7–12:Ac and *Z*5–10:Ac Arn et al. (1980) reported that a ratio higher than 2:1 was inhibitory. We have used a ratio of 12:1 in field experiments and still obtained very high activity. Unpublished field experiments (Löfqvist and Löfstedt, in progress) indicate that the optimal ratio between *Z*7–12:Ac and *Z*5–10:Ac is higher than 2:1 but lower than 12:1. However, different geographical races might produce and respond to different pheromone blends.

In *A. segetum* we could not find *Z*7–10:Ac, which has been reported to constitute 47% of the amount of the major pheromone component *Z*5–10:Ac in *A. fucosa* (Wakamura, 1978, 1980). Neither a significant EAG activity nor specialized receptors were revealed when male *A. segetum* antennae were examined. This suggests that the two species, although very similar according to Steck et al. (1979), may be reproductively isolated under natural conditions. It is possible that the gene flow between the two populations is limited by the differences in the composition of their pheromone. In this case one should reconsider whether *A. fucosa* and *A. segetum* are synonymous.

Sex pheromones or sexual attractants have been reported for six species belonging to the genus *Agrotis* (*Scotia*) (Table 4). All components described are acetates with 10-, 12-, or 14-carbon atom chains in their alcohol moiety. Moreover, with the probable exception of *Agrotis segetum* pheromone components, all reported compounds are monounsaturated. The present paper strongly indicates that the pheromones of *A. segetum* is a combination of one saturated and several monounsaturated compounds. Other examples of such a combination are *Argyrotaenia velutinana* (Roelofs et al., 1975), *Adoxophyes* sp. (Tamaki, 1979), and *Archips argyrospilus* (Roelofs et al., 1974).

The pheromone of *A. segetum* proposed in this study has been successfully used in a monitoring program for the turnip moth during the summer of 1981.

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TABLE 4. COMPARISON OF SEX PHEROMONE AND SEX ATTRACTANT COMPONENTS IN MOTHS BELONGING TO THE GENUS *Agrotis*

Species	10 : Ac	*Z*5–10 : Ac	*Z*7–10 : Ac	*Z*5–12 : Ac	*Z*7–12 : Ac	*Z*5–14 : Ac	*Z*9–14 : Ac	Reference
A. segetum[a]	X[c]	X	O	O	X	?	X	This study
A. fucosa[a]	–	X	X	–	–	–	–	Wakamura, 1978, 1980
A. ipsilon[a]	–	–	–	–	X	–	X	Hill et al., 1979
A. orthogonia[b]	–	–	–	X	X	–	–	Strubel and Swailes, 1978
A. venerabilis[b]	–	X	–	–	X	–	–	Steck et al., 1979
A. exclamationis[a]	–	–	–	–	–	X	X	Bestmann et al., 1980

[a] Compounds isolated from females.
[b] Compounds identified by field screening.
[c] X = compound present, O = compound not found, – = not reported, ? = compound might be present.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS. XIII. Kairomonal Activity for *Trichogramma* spp.[1] of Abdominal Tips, Excretion, and a Synthetic Sex Pheromone Blend of *Heliothis zea* (Boddie)[2] Moths[3,4]

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Abstract—Volatile chemicals emanating from an excretion (apparently meconium) and abdominal tips of female *Heliothis zea* (Boddie) moths mediated increased rates of parasitization of *H. zea* eggs by *Trichogramma pretiosum* Riley. A blend of synthetic chemicals, consisting of hexadecanal, (*Z*)-7-hexadecenal, (*Z*)-9-hexadecenal, and (*Z*)-11-hexadecenal, which has been identified as the sex pheromone of and from the abdominal tip of female *H. zea* moths, also increased rates of parasitization of *H. zea* eggs by *T. pretiosum* in greenhouse experiments. In addition, parasitization of *H. zea* eggs by wild *Trichogramma* spp., in field plots of cotton, *Gossypium hirsutum* L., treated with a similar blend of chemicals, in Conrel fibers, was more than double that in untreated plots.

Key Words—Kairomone, pheromone, *Trichogramma, Heliothis zea.*

INTRODUCTION

The involvement of kairomones in the host-seeking behavior of *Trichogamma* spp. has been known for some time. Laing (1937), for example, found that *Trichogramma evanescens* Westwood females perceived an "odor" left at

oviposition sites by adult *Sitotroga cerealella* (Olivier). Lewis et al. (1971) demonstrated that a factor(s) associated with the presence of adult *Heliothis zea* (Boddie) increased rates of parasitization by *T. evanescens*. Lewis et al. (1972) demonstrated that scales from *H. zea* moths were a source of kairomone(s) that elicited increased rates of parasitization and subsequently this interaction has received considerable study (Lewis et al., 1975, 1979; Nordlund et al., 1976; Beevers et al., 1981; Gross et al., 1975). We found that contact with these kairomones stimulated an intensive search behavior and that the kairomone could be used in the field to increase rates of parasitization.

Recently, we demonstrated the involvement of volatile chemicals from plants in the host-seeking behavior of laboratory-reared *Trichogramma pretiosom* and wild *Trichogramma* sp. (Altieri et al., 1981). The finding that *Trichogramma* responded to these plant-produced chemicals, along with other evidence for the existence of long-range mediators, led us to examine *H. zea* moths for active volatile chemicals. In this paper, we discuss the finding that increased rates of parasitization by *Trichogramma* are elicited by volatile materials from *H. zea* abdominal tips and from a "pinkish" excretion of the moths (believed to be meconium since it is present in the gut of pupal stage *H. zea* and absent in the gut of moths soon after emergence) and by blends of synthetic chemicals known to be present in the abdominal tips of female *H. zea* adults and which have been identified as the sex pheromone of *H. zea* (Klun et al., 1980).

METHODS AND MATERIALS

The *Trichogramma* stock used in the greenhouse experiments originated from Hermosilla, Mexico. It was found to cross successfully with a stock from Los Mochis, Mexico (Gonzales and Allen, 1975; Division of Biological Control, University of California, Riverside, California 92501, unpublished results), that was identified as *Trichogramma pretiosum* Riley (Oatman et al., 1970). These parasitoids were reared in our laboratory, according to the procedure of Lewis and Redlinger (1969), in *H. zea* eggs at ca. 26° C and 70% relative humidity, and used on the day of emergence.

The *H. zea* eggs used in the studies (and also for rearing the *T. pretiosum*) were obtained from laboratory cultures, washed with sodium hypochlorite as described by Burton (1969), irradiated with 25 krad (^{60}CO source) when 8–36 hr old, and stored at ca. 10° C.

In the greenhouse experiments, cotyledonous-stage, pink-eyed purple-hull cow peas, grown in 22.8-cm pie pans, were used. Eggs were applied to cotyledons using a camel's hair brush moistened with saliva. Newly emerged *T. pretiosum* were released from 2-dram shell vials.

The synthetic blend used in experiment 3 consisted of (*Z*)-11-hexadecenal, (*Z*)-9-hexadecenal, (*Z*)-7-hexadecenal, and hexadecanal in an 87:3:2:8 ratio (by weight) (Klun et al., 1980, Carpenter et al., 1981). The components used in both the greenhouse and field experiments were obtained from Chemical Samples Company, Columbus, Ohio. For the field experiment, the components were purified by high-performance liquid chromatography on a 25 × 2.5-cm (OD) $AgNO_3$-coated silica column eluted with toluene (Heath and Sonnet, 1980). Each compound collected from the $AgNO_3$ column was filtered through a 10 × 1-cm (ID) column of NaCl and then through a silica gel (60–120 mesh, BDH Chemicals) column of the same dimensions, and solvent was removed by evaporation. The compounds were then analyzed by gas chromatography on a 66-m × 0.25-mm (ID) glass capillary column coated with SP2340 (Supelco, Bellefonte, Pennsylvania) (Teal et al., 1981). This column separates both geometrical and positional isomers of the compounds of interest. The purity of the compounds used in this formulation was greater than 98%. Conrel black hollow fibers (Albany International, Controlled Release Division, Needham Heights, Massachusetts 02194) were evacuated and filled with a blend of compounds consisting of hexadecanal, (*Z*)-7-hexadecenal, (*Z*)-9-hexadecenal, (*Z*)-11-hexadecenal (4%, 1%, 2%, and 92%, respectively, by weight) and 1% BHT [2,6-bis(1,1-dimethylethyl)-4-methyl phenol], an antioxidant, dissolved in an equal volume of *n*-hexane (nanograde, Mallinckrodt) at ca. 208 μg/fiber.

Percentage of parasitization was determined by dissecting the host eggs according to the procedure of Lewis and Redlinger (1969).

Arcsin transformations were conducted on percentages prior to analysis. A paired *t* test was used to determine significance. Means are given $\pm 1\ S\bar{x}$ in parentheses.

RESULTS

Greenhouse Experiments

Experiment 1—Effect of Moth Excretion. This experiment was conducted on individual pans of peas, ca. 1.5 m apart. *H. zea* eggs (10/pan) were applied to the cotyledons in each pan as previously described. Portions (ca. 1 cm^2) of *H. zea* cage liners with a "pinkish" excretion from the moths were clipped from the liners. The liners were obtained from cages of moths less than 36 hr of age. These pieces of liner were placed in the bottom of 2-dram shell vials. Seven vials were then placed, open end up, under the canopy and on the bottom of each treated pan. The control pans received no treatment. Six female *T. pretiosum* were released in each pan and allowed to function for ca. 2 hr. The eggs were then collected and dissected. This experiment was conducted on three different days with 12–20 replications per day, for a total

of 47 replications. The mean percent parasitization in the treated pans was 74.2% (±10.2), which was significantly higher ($P < 0.05$) than the mean percent parasitization of 46.8% (±7.0) for the control pans. Periodic examination indicated that no *Trichogramma* were visiting the vials and thus the *Trichogramma* appear to have detected volatiles emanating from the vials and were stimulated to increase parasitization without direct contact with the chemical source.

Laboratory observations were made of 15 *Trichogramma* females exposed to pieces of the excretion carefully scraped from the liners, so as to contain no other material, and placed on clean filter paper. All the *Trichogramma* individuals became highly excited when they approached the material and began an intensive and fast-paced, weaving search of the substrate. These data demonstrated that there is a chemical(s) in the "pinkish" excretion of adult *H. zea* that enhances search by *T. pretiosum* females and the chemical appears to be volatile.

Experiment 2—Effect of Abdominal Tip Components. In this experiment, pans of peas were set up individually ca. 1.5 m apart. *H. zea* eggs (10/pan) were applied to the cotyledons in each pan, as previously described. *H. zea* female abdominal tips were cut from virgin females and one tip was squashed in a piece of Whatman No. 1 filter paper (ca. 6 cm^2). Two of these papers were placed on the substrate under the canopy of each of the treated pans. The control pans received no such treatment. Six female *T. pretiosum* were released in each pan and allowed to search for ca. 2 hr. Again, the impregnated papers were periodically examined to confirm that no contact stimulation was occurring. The eggs were then collected and dissected, as previously described. This experiment was conducted on six different days with 10 replications per day for a total of 60 replications.

The mean percent parasitization in the treated group was 55.0% (±7.0), which was significantly higher ($P < 0.05$) than the mean for the control group, which was 42.5% (±5.9). These data indicate that some volatile chemical(s) associated with the abdominal tips of *H. zea* moths increased rates of parasitization by *T. pretiosum*.

Experiment 3—Use of Synthetic Pheromone Blend. In this experiment, pans of peas were arranged in groups of three (close enough together that the foliage touched) with ca. 0.75 m between groups on greenhouse tables. *H. zea* eggs (10/pan) were placed on the cotyledons in each pan as previously described. The synthetic pheromone blend was applied to two cotton rolls (#2 Medium, Uni-Disco Inc., P.O. Box 4450, Detroit, Michigan 48228) at the rate of 5.0 μg/roll in 0.5 ml of hexane. Two rolls were placed on the table in the center of each group of treated pans. The control groups received no such treatment. Two vials of 6 female *T. pretiosum* each were released under the foliage, on opposite sides of each group. The parasitoids were allowed to

search for ca. 3 hr. The eggs were then collected and dissected. This test was conducted on 3 days with 6 replications each day, for a total of 18 replications.

The mean rate of parasitization in the treated groups was 52.8% (±12.5), which was significantly higher ($P < 0.05$) than the mean of 40.8% (±10.1) for the control groups. These data demonstrated that the sex pheromone blend of *H. zea* can increase parasitization by *T. pretiosum.*

Field Experiment Using Synthetic Pheromone

Pairs of plots, 1 row by 1.5 m, were set up in a field of cotton, *Gossypium hirsutum* L. (variety: Delthine 61), that was ca. 0.75 m high. Plots were separated from each other by a minimum of 15 m. In the treated plots, Conrel fibers loaded with the pheromone blend, as previously described, were placed 3 each on a piece of Scotch Doublestick Tape (Minnesota Mining and Manufacturing Co., St. Paul, Minnesota 55101) and attached to a stem in the upper third of the plant at four equally spaced locations (total 12 fibers/plot). The control plots were untreated. All plots were egged with 20 *H. zea* eggs, using a camel's hair brush and Plantgard as an adhesive (Nordlund et al., 1974). After ca. 4 hr exposure, the eggs were collected and dissected as described. The test was run for 2 days with one reading each day. No parasitoids were released, thus the parasitization that occurred resulted from naturally occurring *Trichogramma.* This experiment was replicated 10 times on two different occasions (at different locations), for a total of 20 replications.

The results are given in Table 1 and demonstrate that the presence of the sex pheromone blend of *H. zea* elicits a dramatic increase in the rate of parasitization by naturally occurring *Trichogramma* in the field. The parasitization in the sex-pheromone-treated plots was more than double that

TABLE 1. MEAN PERCENTAGE PARASITIZATION (±1$\bar{x}$) OF *H. zea* EGGS BY NATURALLY OCCURRING *Trichogramma*, IN FIELD PLOTS TREATED WITH CONREL FIBERS LOADED WITH SYNTHETIC *H. zea* SEX PHEROMONE[a]

	Treated	Control
Reading 1 (day 1)[b]	24.4 (±5.9)	18.6 (±4.8)
Reading 2 (day 2)[c]	45.5 (±10.2)	20.2 (±5.0)
Means of both readings[d]	35.6 (±8.1)	20.6 (±4.9)

[a]Data from 20 replications.
[b]Means not significantly different ($P < 0.05$).
[c]Means significantly different ($P < 0.01$).
[d]Means significantly different ($P < 0.001$).

of the control plots during the second day. The significantly higher parasitization in the treated plots on the second day of the test as compared to the slight difference on the first day indicates that the increase was a result of the attraction and resulting redistribution of the parasitoids rather than simply stimulation of parasitoids that were already present.

DISCUSSION

Previous studies reported earlier in this series and elsewhere have demonstrated that the contact stimuli in the scales of *H. zea* moths serve a vital role in the host-finding behavior of *Trichogramma* spp. and that these chemicals can be used to manipulate the behavior and increase the field performance of *Trichogramma* (Lewis et al., 1975, 1979). However, questions remained as to whether these stimuli were, in fact, the only stimuli to which *Trichogramma* respond. Earlier preliminary screening, using various olfactometers, for attraction of *Trichogramma* to long-range volatile chemicals resulted in no positive results. However, evidence for the existence of such mediators with a very strong influence on the foraging behavior of *Trichogramma* continued to mount. Field releases of *H. zea* moths (wings partially clipped to prevent migration from the field) into a 5-acre cotton field at Tifton, Georgia, resulted in a dramatic increase of natural *Trichogramma* within 2–3 days (Morrison and Lewis, unpublished data) indicating the keen ability of *Trichogramma* to detect the presence or activity of *H. zea* moths. Studies with biweekly releases of ca. 50,000 *Trichogramma* in a large scale study in Portland, Arkansas, during the 1981 growing season, reflected increased parasitization as the moth population increased but a rapid drop in rates of parasitization at the end of and between generations, indicating a keen ability of the *Trichogramma* to perceive and quickly respond to changes in moth populations (King et al., unpublished data). Similar *Heliothis–Trichogramma* relations were noted by Lingren (personal communications) in various *Trichogramma* studies in cotton-growing areas of Texas. The data reported here demonstrate the ability of *Trichogramma* to utilize the sex pheromone and other volatile chemicals from *H. zea* moths as kairomones for quick and effective detection of habitats containing host eggs and offer an explanation for the responses cited above.

Such a relationship is of ecological significance in that it provides a mechanism for *Trichogramma* to synchronize with the initial upsurge of moth populations and maximum egg deposition, thereby avoiding the competition with predators that tends to build up later in a generation. Host eggs freshly parasitized by *Trichogramma* are readily consumed by egg predators. However, after the host egg has been parasitized for 3–4 days, it becomes less desirable for the predators (unpublished data).

The identity of the chemical(s) in the "pinkish" excretion that elicits the

response by *Trichogramma* is unknown and needs immediate further attention. The majority of the tissue, obtained when cutting the abdominal tips, was the epidermal gland from which Klun et al. (1980) identified the sex pheromone components used in experiment 3 and the field experiment. Additional studies will be required to determine if other active materials are present in the gland.

The fact that various pheromones or components of pheromones also serve as kairomones for predators or parasitoids has been demonstrated on several occasions. Rice (1969), for example, found that the predators *Enoclerus lecontei* (Wolc.) and *Temnochila virescens chlorodia* (Mann.) and the parasitoid *Tomicobia tibialis* Ashm. respond to the aggregation pheromone of their host or prey, *Ips* spp. Vité and Williamson (1970), found that frontalin, the major component of the aggregation pheromone of *Dendroctonus frontalis* Zimm. also attracts the predator *Thanasimus dubius* (F.). *Aphytis melinus* DeBach and *Aphytis coheni* DeBack, two parasitoids of *Aonidiella aurantii* (Mask.), are attracted to the sex pheromone of *A. Aurantii* (Sternlicht, 1973). Kennedy (1979) found that *Cheriopchus colon* (L.), *Entedon leucogramma* (Ratz.), and *Dendrosoter protuberans* Nees, parasitoids of *Scolytus multistriatus* (Marsham) and *Cerocephala rufa* (Walker), a reported hyperparasitoid of *D. protuberans*, are attracted to multilure, the sex pheromone of *S. multistriatus*. Male *Nazara viridula* (L.) produce a sex pheromone that is highly attractive to females of the same species and to the tachinid parasitoid *Trichopoda pennepes* (F.) (Mitchell and Mau, 1971). Also, Corbet (1971) found that the mandibular gland secretion of *Anagasta kuehniella* (Zeller) functions as an epideictic pheromone and elicits ovipositor probing by the parasitoid *Venturia canescens* (Grav.).

The use of volatiles, as demonstrated in these studies, to enhance the field performance of *Trichogramma*, should require considerably less concern with application patterns than is the case with contact stimuli (Lewis et al., 1979; Beevers et al., 1981). The technology developed for use of sex pheromones for mating disruption should contribute readily to their application for enhancing the performance of *Trichogramma*.

These new discoveries of the overlapping roles of chemical cues in the mating behavior of *H. zea* and host-search behavior of *Trichogramma* open exciting possibilities for integration of augmentation–manipulation of entomophages and mating disruption into a potentially powerful pest management system for *H. zea* and similar pests.

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ELECTROANTENNOGRAM RESPONSES OF *Scolytus multistriatus* (COLEOPTERA: SCOLYTIDAE) TO ITS PHEROMONE COMPONENTS AND TO ASSOCIATED COMPOUNDS[1]

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Abstract—Electroantennograms were recorded from *Scolytus multistriatus* in response to 4-methyl-3-heptanol, the four geometric isomers of multistriatin, and cubeb oil. Charateristic dose–response curves for response amplitude and the time required for the voltage trace to return to 1/2 baseline (recovery rate) were established. Recovery rates were significantly more rapid following stimulation with 4-methyl-3-heptanol or cubeb oil than with the multistriatin isomers. At most intensities, α-multistriatin, the isomer that evokes behavioral response, gave significantly larger EAGs with significantly longer recovery rates than the other isomers. Results of differential adaptation experiments suggested that 4-methyl-3-heptanol interacted with the processes involving multistriatin and cubeb oil activity. However, cross-activity of acceptors for these compounds seems unlikely; single sensillum recordings are needed to ascertain the response spectra for individual receptor neurons.

Key Words—Coleoptera, Scolytidae, *Scolytus multistriatus*, pheromone, insect olfaction, electroantennogram, electrophysiology, differential adaptation, multistriatin, 4-methyl-3-heptanol, α-cubebene.

INTRODUCTION

The European elm bark beetle, *Scolytus multistriatus* (Marsham) is the principal vector of the Dutch elm disease pathogen, *Ceratocystis ulmi*

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(Buisman) C. Moreau, in most of the United States. The beetle was first reported in this country in 1909 (Chapman, 1910), but it was not until 1930 when *C. ulmi* was introduced that the insect became a serious economic pest. Since the elm has significant value as an urban shade tree, considerable research has been conducted during the past three decades in an effort to develop methods of controlling the beetle and the disease.

As early as 1935, several researchers noted aggregations of the beetle on certain elm trees (Felt, 1935; Martin, 1936). Meyer and Norris (1967) demonstrated an increase in attractiveness of beetle-infested elm bolts versus unattacked elm, but they attributed this increase to the release of host volatiles caused by the beetles' mining. Peacock et al. (1971) demonstrated that, although the host odors were involved, a pheromone produced by the virgin female beetles boring in the elm was the major attractant. The pheromone system was found to be composed of three compounds (Figure 1): (−)-4-

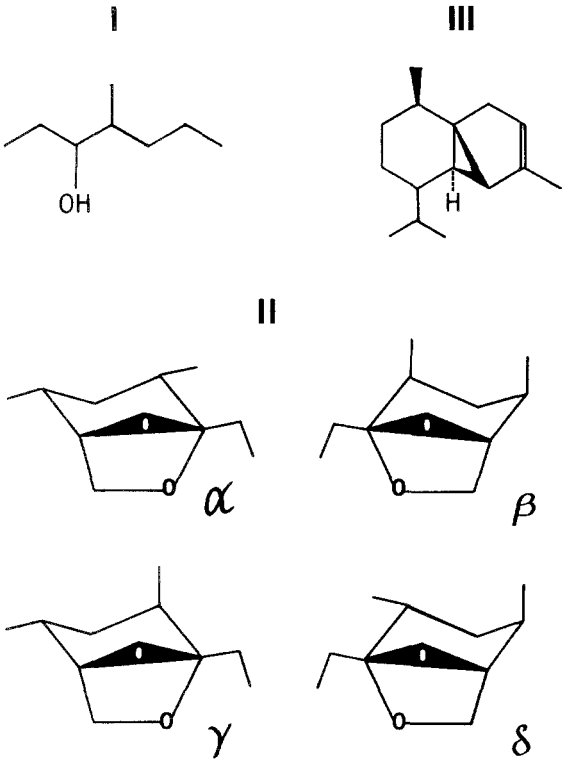

FIG. 1. *Scolytus multistriatus* pheromone compounds and associated isomers. 4-Methyl-3-heptanol (I), isomers of multistriatin (II); α-cubebene (III).

methyl-3-heptanol (I), (−)-2,4-dimethyl-5-ethyl-6,8-dioxabicyclo[3.2.1]octane [(−)α-multistriatin] (II), and (−)-α-cubebene (III) (Pearce et al., 1975). Components I and II are produced by the female beetle, whereas III is produced by the host tree. In bioassay and field tests, beetles responded much more strongly to the three components combined than to their doublet combinations or to individual compounds (Pearce et al., 1975; Lanier et al., 1977).

Multistriatin can exist as four geometric isomers (Figure 1). In the United States, beetles are attracted only to the α-isomer (Lanier et al., 1977). In Germany, it was reported that *S. multistriatus* were attracted most strongly to a combination of I, II plus δ-multistriatin and they appeared to be inhibited by the α-isomer (Gerken et al., 1978). However, later reports state that α-multistriatin is most active (Klimetzek et al., 1981; Angst et al., 1982). Each of the geometric isomers has two enantiomeric forms; in the United States, only the (−)-α-multistriatin was active (Elliott et al., 1979). In this report we present the results of a study of electroantennograms (EAGs) of *S. multistriatus* in response to known components of its aggregation pheromone and the four structural isomers of multistriatin.

METHODS AND MATERIALS

Materials. The *S. multistriatus* emerged in the laboratory from field-collected (Syracuse, New York) brood wood. Testing occurred within 5 days of adult emergence.

The chemicals used were either synthesized at the SUNY College of Environmental Science and Forestry or commercially acquired. The four isomers of multistriatin were separated by GLC. The 4-methyl-3-heptanol was purchased from Aldrich Chemical Company. With the exception of α-cubebene, all samples were purified to at least 99% by preparative gas chromatography. Due to the difficulty in purification of α-cubebene, we chose to use cubeb oil (69.9% α-cubebene). Serial dilutions of the compounds were made in purified hexane and stored at 0° until used.

Preparation. A technique modified from Payne (1970) and Angst and Lanier (1979) was used to secure the beetle for recording. A 2-mm hole was drilled through the center of a Plexiglas block (2.8 × 2.8 × 1.5 cm) and a 1-mm slot was machined so that it extended from this hole to the edge of the block. The beetle was placed into one end of a rubber tube (3 mm OD, 1.5 mm ID, 5 cm long), with its head exposed. The tube was then slid through the slot into the hole and gently positioned so that the end of the tube was flush with the surface of the block. This left only the beetle's head exposed. A metal plate was then positioned to frame the insect's head through a 2-mm hole and to restrict movement. The antenna was extended against the metal surface. The

block was placed on a magnetic holder with a universal joint that allowed angular positioning of the preparation.

Recording. The electrodes were glass capillaries pulled to tip diameters of approximately 10 μm and filled with a weak saline solution (NaCl, 3.75 g; $CaCl_2$, 0.105 g; KCL, 0.175 g; $NaHCO_3$, 0.100 g; H_2O, 500 ml) (Roelofs, 1976) Chlorinated silver wires connected the electrodes to a Grass P-18 differential amplifier. The recording electrode was inserted into the distal third of the antennal club. The indifferent electrode was inserted through the intersegmental membrane between head and thorax. Response potentials were displayed on a storage oscilloscope. Two parameters were measured directly from the stored oscilloscope image for each response: (1) the maximum response amplitude; and (2) the time required for the potential to return halfway to the baseline (recovery rate).

Stimulation. Presentation of chemical stimuli was accomplished by injecting a "puff" of pheromone-laden air into a pure airstream (1000 ml/min) that continuously passed over the preparation. The puff was delivered in a second airstream (1000 ml/min) through an electronically controlled solenoid valve that opened for approximately 0.3 sec. This puff of air passed through a pipet containing the compound and into the continuously moving stream. To minimize background contaminants, all streams were filtered through a molecular sieve (PM-100^r, Hewlett-Packard) and dehydrated with indicating silica gel. A vacuum immediately behind the preparation removed contaminated air.

Stimulation pipets were prepared by depositing 5 μl of desired solution onto a strip of No. 1 Whatman filter paper (1 × 3.5 cm). After the hexane solvent was allowed to evaporate for 5 min, the filter paper was placed inside a shortened disposable pipet (4.75 cm long). Both ends of the pipet were capped with Teflon® and glass stoppers. Pipets were stored at −40° C and allowed to come to room temperature for 2 hr prior to use.

Between stimulations, the beetles were allowed to recover for 5–10 min, depending on the intensity of the preceding response. Following a completed series of tests, the beetle was allowed to recover for 2–3 hr, after which a second series of tests was performed. Twenty series of tests on ten beetles (five males and five females) were used to establish the dose–response curves for the multistriatin isomers, 4-methyl-3-heptanol, and cubeb oil. Comparisons among test series were faciliated by normalizing EAG amplitudes and recovery rates to an index that arbitrarily assigned a value of 100 to the response to 250 μg of α-multistriatin (Payne, 1975). The data were analyzed using analysis of variance and compared with the least-significant-difference test.

For differential adaptation experiments, two airstreams, each controlled by a solenoid valve, were employed. A timer allowed the closing of one valve to trigger the opening of a second. Thus, the preparation could be exposed to

one compound for a duration of approximately 0.5 sec, followed by an exposure to a second stimulus. The basic premise of the differential adaptation technique is that the exposure of the antenna to a very high concentration of a compound causes adaptation of the receptor mechanism involved with that compound. Subsequent stimulation by the same compound within a short time interval may not elicit further depolarization because all the receptor mechanisms continue to be fully adapted. If the adapting stimulus is followed quickly by stimulation with a second compound that uses a different receptor mechanism, a further depolarization may occur. A comparison of the second response with an unadapted response estimates the degree to which receptor mechanisms overlap in their recognition of different compounds. Our differential adaption data are based on 12 series of tests on six additional beetles (three males and three females). To ensure saturation of the system, 5 μl of pure compound was placed in the pipet to be used for the adapting stimulus. Mixed isomers of multistriatin were used in place of pure α-isomer.

To establish the points at which the EAG saturates for 4-methyl-3-heptanol, α-multistriatin, and cubeb oil, separate tests were performed on another six beetles (three males and three females). All intensity values expressed in this paper represent the amount of material loaded into the disposable pipets. Ideally, intensity should be expressed as the number of molecules actually interacting with the receptor mechanism.

RESULTS

The electroantennogram (EAG) elicited in response to a chemical stimulus was characterized by a slow negative potential that reached a peak approximately 0.3 sec after response onset. The peak amplitude of the potential varies from 0.1 to 6.5 mV, depending on the compound and concentration tested. Recovery rates varied from 0.5 to 45 sec, depending on the stimulus compound and concentration.

Responses of males and females to the various compounds did not differ significantly for either peak response amplitude or the recovery rate. In all cases, an increase in stimulus intensity resulted in an increase in response amplitude (Figure 2) and prolonged recovery (Figure 3). The hexane control stimulation produced a response of 9.6% of the standard (250 μg α-multistriatin). The point at which further increase in stimulus intensity failed to increase EAG response was established for α-multistriatin (ca. 250 μg), 4-methyl-3-heptanol (ca. 1000 μg), and cubeb oil (ca. 250 μg) (Figure 4).

At most of the intensities compared, α-multistriatin elicited responses significantly larger in amplitude (Figure 2A) and significantly longer in recovery time (Figure 3A) than the other multistriatin isomers.

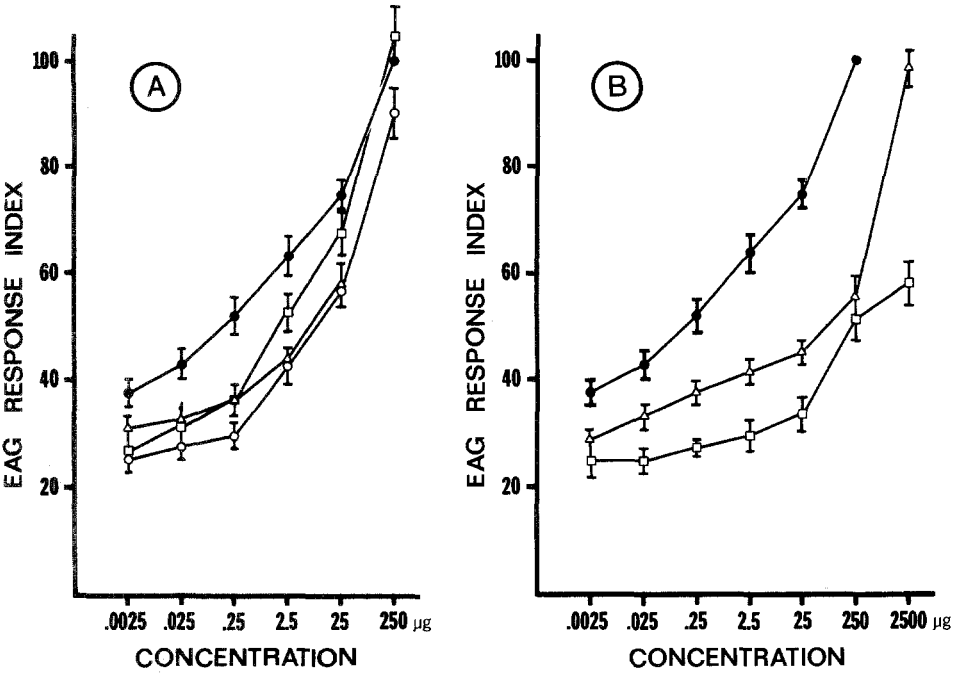

FIG. 2. Amplitude depolarization dose-response curves. (A) α-Multistriatin, closed circles; β-multistriatin, triangles; γ-multistriatin, squares; δ-multistriatin, open circles. (B) α-Multistriatin, closed circles; 4-methyl-3-heptanol, triangles; cubeb oil, squares. Due to limited supply, β-multistriatin was not tested at 250 μg. Vertical bars indicate one standard deviation. All values normalized to 250 μg α-multistriatin.

EAG amplitude for α-multistriatin exceeded response amplitudes for 4-methyl-3-heptanol and cubeb oil at comparable concentrations (Figure 2B). Recovery following stimulation with either of the latter two compounds was more rapid than was the recovery seen with α-multistriatin (Figure 3B).

The results of the differential adaption experiments are illustrated in Figure 5. Self-adaptation was evident for each of the stimulus compounds. Long-term exposure to 4-methyl-3-heptanol reduced subsequent responses to 4-methyl-3-heptanol, multistriatin, and cubeb oil (91.1–96.7%). However, adaptation to either multistriatin or cubeb oil did not obliterate a subsequent response to 4-methyl-3-heptanol or the remaining nonadapting compounds.

DISCUSSION

Differences in the amplitude of EAG responses presumably reflect differences in the numbers of individual nerve impulses generated by olfactory

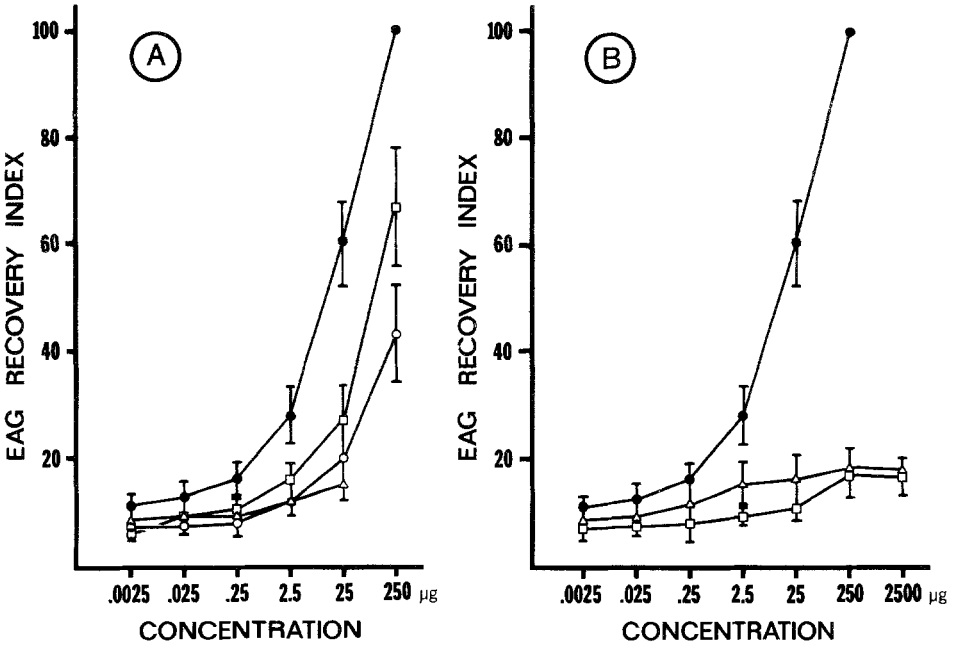

FIG. 3. Recovery rate dose-response curves. (A) α-Multistriatin, closed circles; β-multistriatin, triangles; γ-multistriatin, squares; δ-multistriatin, open circles. (B) α-multistriatin, closed circles; 4-methyl-3-heptanol, triangles; cubeb oil, squares. Due to the limited supply, β-multistriatin was not tested at 250 μg. Vertical bars indicate one standard deviation. All values normalized to 250 μg α-multistriatin.

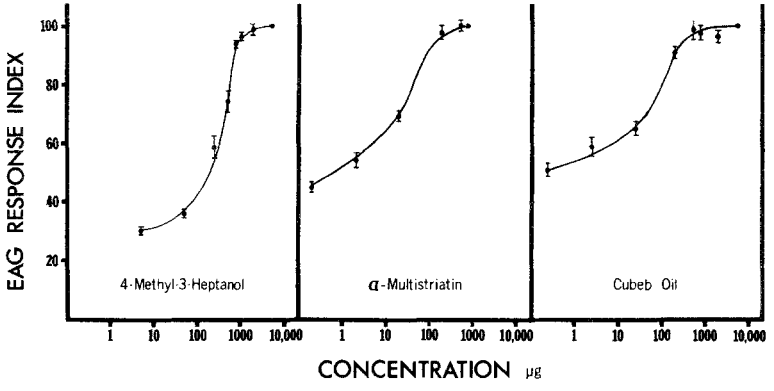

FIG. 4. Saturation response curves for the pheromone components.

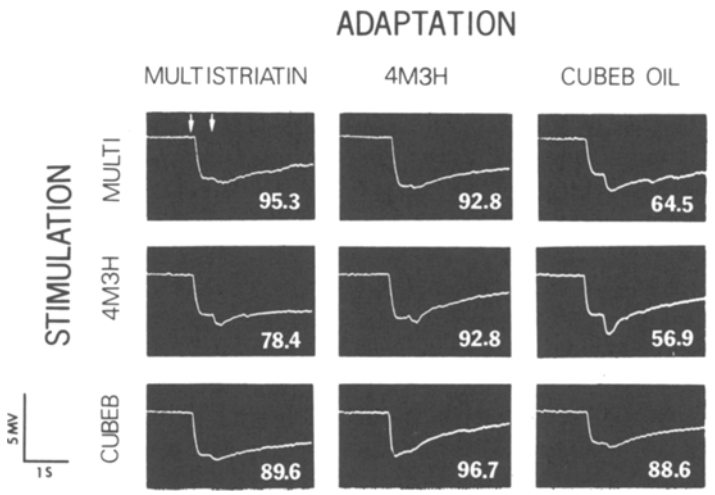

FIG. 5. Differential adaptation responses. Inset numbers are percent reduction of stimulation response following adaptation. In each case, the initial displacement represents the EAG for the adapting compound and the second displacement, if present, represents the response to the stimulating compound. Onset of adaptation and stimulation are indicated by arrows.

receptor neurons per unit time (Kaissling, 1971). From this assumption, it follows that each of the three pheromone components may affect different numbers of receptors at most of the concentrations tested.

The most striking difference in the EAG responses for the pheromone components is the long recovery rate following stimulation with multistriatin versus rapid recovery rate following stimulation with 4-methyl-3-heptanol or cubeb oil. Recovery indices increase sharply with concentration of multistriatin but only slightly with concentrations of 4-methyl-3-heptanol or cubeb oil (Figure 3B). Differences in EAG recovery rates have been hypothesized to be affected by differences in the amount of time that an individual odorant molecule spends bound to acceptors (Roelofs and Comeau, 1971) and by intrinsic differences in the time required for deactivation of the various odorant molecules (Kaissling, 1974). The relative contribution of these mechanisms to the recovery rate of the EAG is presently unclear because the molecular processes of transduction and deactivation of odorants are unknown. In either case, the net excitatory neural barrage entering the central nervous system must be enhanced for those compounds which elicit large EAGs with prolonged recovery rates.

By either measure of the EAG (amplitude or recovery time), α-multistriatin overshadowed the other isomers. Only at the highest (250 μg)

concentration was there a lack of significance in EAG amplitude between α and another isomer (in this case, γ-multistriatin). Furthermore, at these high concentrations, EAGs may reflect the presence of minute amounts of α-multistriatin, either as a residual impurity or by interconversion of the γ to the α isomer (Gore et al., 1975).

Data derived from the differential adaptation experiments are difficult to interpret. Adaptation to and subsequent stimulation by a second application of the same compound reduced the response to the second presentation 88–92%, rather than 100% predicted. Perhaps the apparent incomplete adaptation could be attributed to subtle differences between the two airstreams used for the serial presentation of the odorants. Most surprising was the nearly complete lack of stimulation by multistriatin and cubeb oil following adaptation with 4-methyl-3-heptanol. The rationale of Payne and Dickens (1976) for this phenomenon requires one to postulate that 4-methyl-3-heptanol adapts most of the receptor mechanisms that are used by multistriatin and cubeb oil. However, this seems inconsistent with electrophysiological experiments with single cells (Angst, 1982) that indicate there is little, if any, cross-responsiveness for these compounds in individual receptor neurons.

Perhaps the most interesting aspect of this work is how the EAG data correlate with behavioral response to the compounds. Prevalent interpretation of data from adaption experiments would hold that high concentrations of 4-methyl-3-heptanol could substitute for the presence of multistriatin and cubeb oil or may inhibit the response entirely. Field tests indicate that the converse is true; high dosage of multistriatin inhibited response to the tripartite mixture, while increasing 4-methyl-3-heptanol had no effect and increasing α-cubebene caused a gradual increase in response (Cuthbert and Peacock, 1978). Although results of adaptation experiments are anomalous, the low stimulation threshold and prolonged recovery rate for multistriatin are consistent with the behavioral importance of the compound.

The assertation by Gerken et al. (1978) that δ-rather than α-multistriatin is the isomer of paramount importance to *S. multistriatus* is not supported by our data. Explanations that the discrepancy in field tests in Germany and the United States (Lanier et al., 1977) might reflect geographic variations in the species are rendered unnecessary by subsequent experiments in Germany (Klimetzek et al., 1981) as well as field and laboratory experiments in Switzerland (Angst et al., 1982) which found that α-multistriatin was the only active isomer. We propose that the results of Gerken et al. (1978) could have been an artifact of very high release rates. At extremely low release rates, used by Lanier et al. (1977) (7 μg multistriatin dispensed over 4 days), *S. multistriatus* was attracted by α-multistriatin in combination with 4-methyl-3-heptanol and α-cubebene. At very high rates of release of α-multistriatin,

one might expect over-stimulation, prolonged recovery, and reduction of trap catch, as was observed by Cuthbert and Peacock (1978). Conversely, high concentrations of δ-multistriatin might be sufficient to cross-stimulate α-multistriatin receptor sites without being high enough to cause inhibition, as is articulated by Roelofs' (1978) response threshold hypothesis. Alternatively, a high concentration of δ-multistriatin may have contained sufficient α-isomer to evoke behavioral response. Unfortunately, Gerken et al. (1978) gave no indication of the release rates.

EAGs have been used to identify pheromone components of moths (Roelofs, 1976), but electrophysiological studies of bark beetles have been retrospective (i.e., testing compounds of known behavioral significance). Without having tested a large repertoire of compounds, we cannot state with any certainty that 4-methyl-3-heptanol, α-multistriatin, and α-cubebene could have been isolated by the EAG technique, but it is clear that α-multistriatin would have been predicted to be the multistriatin isomer of paramount importance. This study, in combination with those on the southern pine beetle *Dendroctonus frontalis* (Payne, 1970, 1975) and the pine engraver beetle *Ips pini* (Angst and Lanier, 1979), favors utilization of the technique in the isolation of compounds of behavioral significance to bark beetle species. Such an investigation should proceed in concert with behavioral tests with cognizance that high EAG activity may not necessarily be indicative of behavioral significance and, conversely, that behavioral significance is not necessarily reflected by high EAG activity. Compounds which elicit inhibitory responses in some receptor neurons should act to diminish the amplitude of the EAG irrespective of their significance for olfactory coding. Therefore, small EAGs do not necessarily indicate compounds of little behavioral significance; rather, the size of the EAG reflects the ratio and temporal congruity of excitatory and inhibitory influences in the antenna.

There are three principal motivations for electrophysiological investigations of insects: (1) they may aid in the isolation and identification of behavior-modifying chemicals; (2) they may help to explain the behavioral effects of certain chemicals; and (3) they may contribute to the understanding of the processes of olfaction. Studies that focus on any one of these objectives may produce data revelant to all three.

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RESPONSE OF *Scolytus multistriatus* (COLEOPTERA: SCOLYTIDAE) TO α- AND δ-MULTISTRIATIN IN SWITZERLAND

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Abstract—Laboratory bioassays and field tests demonstrated that a Swiss population of *S. multistriatus* responded much more strongly to α- than to δ-miltistriatin in combination with 4-methyl-3-heptanol and α-cubebene. High concentrations of brevicomin appeared to replace α-multistriatin in evoking a response by *Scolytus* species, but this effect can be explained by the fact that the brevicomin was contiminated with small amounts of α-multistriatin. Frontalin, another bicyclic ketal, showed no biological activity. Field tests indicated that *S. pygmaeus* aggregates to the same attractant mixture as *S. multistriatus*. *S. scolytus* also responded preferentially to this mixture, but the relative amounts of α-multistriatin to 4-methyl-3-heptanol do not appear to be as important as for *S. multistriatus*.

Key Words—Pheromone, multistriatin, 4-methyl-3-heptanol, brevicomin, frontalin, *Scolytus multistriatus*, *Scolytus scolytus*, *Scolytus pygmaeus*, Coleoptera, Scolytidae.

INTRODUCTION

Elm bark beetles of the genus *Scolytus* commonly breed in trees afflicted by Dutch elm disease (DED). Progeny emerging from diseased trees transmit the DED fungus, *Ceratocystis ulmi*, to healthy elms when they feed on the bark of twig crotches. Colonization of diseased wood by *S. multistriatus*

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(Marsham) is mediated by an aggregation pheromone produced when virgin females bore into elm wood.

North American populations of *S. multistriatus* aggregate best in response to a synergistic mixture of α-multistriatin, (−)-threo-4-methyl-3-heptanol, and (−)-α-cubebene (Pearce et al., 1975; Lanier et al., 1977). Field tests comparing the attractivenss of stereoisomers of α-multistriatin showed that only the (−)-α-enantiomer was active for an American (Elliott et al., 1979) as well as an English population (Blight et al., 1980b). Vité et al. (1976) reported that *S. multistriatus* in the upper Rhine Valley was attracted by multilure, a mixture of isomers of multistriation, 4-methyl-3-heptanol, and α-cubebene. Gerken at el. (1978) reported that the same population responded in greater numbers to mixtures containing (−)-δ-multistriatin than to those with the (−)-α-enantiomer. However, these results were contradicted by Klimetzek et al. (1981), who found that the (−)-α-enantiomer was most attractive to German *S. multistriatus*.

Field and laboratory assays were conducted to characterize the preference for isomers of multistriatin of a population of *S. multistriatus* from Aargau, Switzerland. Two analogous compounds, brevicomin and frontalin, were used as references of electrophysiological response because they are of the same class as multistriatin and they were available in purified form.

Scolytus scolytus (Fab.) aggregates in response to threo- and erythro-4-methyl-3-heptanol released by the male in combination with α-cubebene (Blight et al., 1978a,b) or limonene (Blight et al., 1980a) from the tree.

METHODS AND MATERIALS

The *Scolytus multistriatus* used were taken from colonies originating from *Ulmus glabra* (Huds.) at Aargau, Switzerland. The beetles were reared on *U. glabra* and were taken for laboratory tests just after emergence.

The substances were obtained from Chemical Samples Co., Columbus, Ohio, unless otherwise stated. GLC was used to determine the stated purities of the compounds. 4-Methyl-3-heptanol was used as a racemic mixture. The two isomers of multistriatin were separated by GLC at ESF.[4] The α-isomer contained about 0.5% of δ- and 1% of γ-multistriatin. δ-multistriatin contained ca. 1% of the α- and 1% of the β-isomer. Both *endo*- and *exo*-brevicomin contained less than 0.1% of multistriatin. The presence of multistriatin within the frontalin could not be detected. (−)-Limonene was purchased from Fluka, Switzerland, and was 97% pure.

Laboratory bioassays utilized the Moeck (1970) olfactometer, except that the arena on which the beetles were placed was open rather than glass-

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covered. Furthermore, two parallel airstreams 12 cm apart were used instead of one. Each delivered odorants in airstreams of 40 ml/min through 1-mm (ID) glass capillaries. Methods for conditioning of beetles prior to testing them and criteria for judging response have been outlined previously (Lanier et al., 1977). All beetles passed through both airstreams, but beetles that responded to the odorant in the first airstream were placed between the two airstreams so that they would then intersect the second airstream. For tests comparing α- and δ-multistriatin, the first airstream contained either δ-multistriatin, the blank, or the control (4-methyl-3-heptanol and α-cubebene), while the second contained α-multistriatin.

For comparison of α-multistriatin with brevicomin, the blank or the control was in the first airstream while the second airstream contained α-multistriatin or brevicomin in combination with 4-methyl-3-heptanol and α-cubebene. For comparison of the α- and δ-isomers of multistriatin, four replicates with 40 beetles of each sex were used. Bioassays for comparison of multistriatin and brevicomin used 25 beetles of each sex per replicate. Concentrations used were as follows: multistriatin, 2×10^{-9} g; brevicomin (1:1 mixture of *exo* and *endo*), 2×10^{-9} and 10^{-6} g; 4-methyl-3-heptanol, 2×10^{-8} g; α-cubebene, 10^{-7} g.

Field assays were conducted at Aargau, Schönenwerd, and Gösgen, Switzerland. The sticky traps consisted of polyethylene-coated white paper (65×80 cm) treated with Tanglefoot®. Traps were placed 10 m apart and 3 m above the ground around utility poles or nonhost trees. The traps were positioned 20–200 m outside the elm stands. Materials were dispensed from polyethylene snap-top vials. The release rates were ca. 1% per day for multistriatin; 1.5% per day for brevicomin, 4-methyl-3-heptanol, and (−)-limonene; and about 0.5% per day for α-cubebene. Baits were still active after termination of the experiments (50 days). For comparison of the isomers of multistriatin, 1 mg of the isomers was always tested in combination with 10 mg of 4-methyl-3-heptanol and 50 mg of α-cubebene (2 replicates with 100 mg). Comparisons of brevicomin and frontalin with α-multistriatin used mixtures containing 20 mg of 4-methyl-3-heptanol and 100 mg of (−)-limonene.

RESULTS AND DISCUSSION

Scolytus multistriatus

Isomers of Multistriatin. In laboratory bioassays *S. multistriatus* was clearly more attracted to α- than to δ-multistriatin. Response to the δ-isomer was only 15–20% of that to α-multistriatin where either was combined with 4-methyl-3-heptanol and α-cubebene (Table 1). No differences in response between the sexes were observed. These results correspond very well with

TABLE 1. INDICES OF RESPSONSE OF *S. multistriatus* IN THE LABORATORY TO ISOMERS OF MULTISTRIATIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND α-CUBEBENE

Material[b]	Index[a] of beetle response (mean ± standard error) Males	Females
α-M + H + α-C	100	100
δ-M + H + α-C	21 ± 10	15 ± 5.5

[a]Index of attraction = No. responding to test / No. responding to standard tripartite mixture. Standard is index 100. (63% and 68% of males and females respectively responded to standard); 4 replicates per sex, consisted of 40 beetles each.
[b]M = multistriatin, H = 4-methyl-3-heptanol, C = Cubebene; see text for explanation of dosages.

those reported by Lanier et al. (1977), who studied two American populations of *S. multistriatus*.

Field tests confirmed the findings obtained by laboratory assays (Table 3). Compared to the tripartite mixture of α-multistriatin, 4-methyl-3-heptanol, and α-cubebene, only 13% of the beetles responded to the mixture which contained δ-multistriatin in place of the α-isomer. Since the δ-multistriatin was contaminated with about 1% of the α-isomer, it must be assumed that the response to the δ-isomer was at least partly due to the α-multistriatin impurity. The results again demonstrate the correspondence with data from field tests conducted at Syracuse, New York (Lanier et al., 1977).

The importance of α-multistriatin as a component of the tripartite bouquet is demonstrated by the lack of difference between responses to the mixture of 4-methyl-3-heptanol and α-cubebene, and the blank.

Our results and those of tests conducted in Devon, England, by Blight et al. (1980b) are in conflict with reports of Gerken et al. (1978) that *S. multistriatus* in the upper Rhine Valley (ca. 200 km from Aargau) was attracted preferentially to the tripartite mixture containing (−)-δ-multistriatin and that (−)-α-multistriatin inhibited the response to the δ-isomer. Subsequent tests in the upper Rhine Valley, however, showed that mixtures containing α-multistriatin were more attractive than those containing the δ-isomer (Klimetzek et al., 1981). We therefore conclude that the populations of *S. multistriatus* of western Europe and the eastern United States do not differ in their preference for the α-isomer of multistriatin.

Comparison of α-Multistriatin, Brevicomin, and Frontalin. Laboratory bioassays comparing the attractiveness of α-multistriatin with the same concentration of brevicomin demonstrated a threefold lower response to

TABLE 2. INDICES OF RESPONSE OF *S. multistriatus* IN THE LABORATORY TO α-MULTISTRIATIN AND BREVICOMIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND α-CUBEBENE

Material[b]	Index[a] of beetle response (mean ± standard error) Males	Females
α-M^{-9} + H + α-C	100	100
B^{-6} + H + α-C	43 ± 1.1	37 ± 7
B^{-9} + H + α-C	30 ± 2.7	35 ± 1.6
H + α-C	12 ± 2.6	14 ± 3.7
Air	6 ± 3	5 ± 2

[a] Index of attraction = No. responding to test / No. responding to standard tripartite mixture. Standard is index 100. (88 and 81% of males and females, respectively, responded to standard); 4 replicates per sex, consisted of 25 beetles each.

[b] M = multistriatin, B = brevicomin, H = 4-methyl-3-heptanol, C = cubebene; see text for explanation of dosages, figures indicate grams.

mixtures containing brevicomin (Table 2). A 1000-fold higher dose of brevicomin resulted in only a slight increase in response. The reaction to 4-methyl-3-heptanol and α-cubebene alone was little better than to air only. The addition of either brevicomin or α-multistriatin to the latter mixture increased the attractiveness markedly.

Field tests confirmed the data obtained from the laboratory assays (Table 4). The control, 4-methyl-3-heptanol and (−)-limonene, again caught only a few beetles. Blanks were neglected in these tests since, in previous field experiments, traps baited with 4-methyl-3-heptanol and α-cubebene did not differ from the blank. The addition of 1 mg α-multistriatin or brevicomin to 4-methyl-3-heptanol and limonene resulted in higher number of beetles being caught, but α-multistriatin was very clearly the more active of the two compounds. The response to a 100-mg dose of brevicomin was about three times lower than the response to 1 mg of α-multistriatin. Subsequent field tests showed that 0.01–0.1 mg of α-multistriatin corresponded to the attractiveness of 100 mg brevicomin. Thus, there appears to be a 1000- to 10,000-fold difference in the effects of α-multistriatin and brevicomin. Electrophysiological recordings of single olfactory cells showed similar differences in sensitivity to (−)-α-multistriatin mixed isomers of brevicomin (Angst et al., 1981). Since there is an impurity of about 0.1% multistriatin within the brevicomin sample, it must be assumed that the attractiveness and electrophysiological response evoked by brevicomin is based on that impurity.

The addition of frontalin to the bipartite mixture, 4-methyl-3-heptanol and α-cubebene, did not increase the trap catches compared to the control.

TABLE 3. FIELD RESPONSE OF *S. multistriatus*, *S. scolytus*, AND *S. pygmaeus* TO ISOMERS OF MULTISTRIATIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND α-CUBEBENE, GÖSGEN, MAY 27–JUNE 18, JULY 1–30, 1980

	Response					
	S. multistriatus		*S. scolytus*		*S. pygmaeus*	
Blank	0.8	(0–2)	0.4	(0–1)	0.2	(0–1)
H + α-C	0.4	(0–2)	4.6	(1–15)	0	(0–0)
α-M + H + α-C	114.4	(94–134)	26.6	(10–50)	3.4	(0–8)
δ-M + H + α-C	15	(4–23)	24.2	(0–65)	0	(0–0)

[a] M = multistriatin, H = 4-methyl-3-heptanol, C = cubebene; 5 replicates per treatment; see text for explanation of dosages.

That the frontalin had no detectable contamination of α-multistriatin could explain its lack of biological activity.

Scolytus pygmaeus *and* Scolytus scolytus

S. pygmaeus were attracted best by α-multistriatin in combination with 4-methyl-3-heptanol and α-cubebene or (−)-limonene (Tables 3 and 4). The correspondence in the attractiveness of various baits for *S. pygmaeus* and *S. multistriatus* indicates that these two species might use the same pheromones for aggregation.

TABLE 4. FIELD RESPONSE OF *S. multistriatus*, *S. scolytus*, AND *S. pygmaeus* TO α-MULTISTRIATIN, BREVICOMIN AND FRONTALIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND (−)-LIMONENE, GÖSGEN, SCHÖNENWERD, AND AARAU, JULY 1–AUGUST 10, 1980

		Response					
		S. multistriatus		*S. scolytus*		*S. pygmaeus*	
Treatment[a]	Replicates	Mean	Range	Mean	Range	Mean	Range
H + L	4	8	(4–17)	3.5	(1–9)	0	(0–0)
1 α-M + H + L	5	371.4	(108–933)	10.6	(1–38)	25.2	(1–89)
1 B + H + L	5	22.4	(1–69)	11.6	(1–45)	0.4	(0–2)
100 B + H + L	5	115.4	(9–266)	11	(0–35)	9.6	(0–35)
100 F + H + L	3	3	(1–4)	3.6	(0–9)	0	(0–0)

[a] M = multistriatin, B = brevicomin, F = frontalin, H = 4-methyl-3-heptanol, L = (−)-limonene; see text for explanation of dosages, figures = μl.

TABLE 5. FIELD RESPONSE OF *S. multistriatus*, *S. scolytus*, AND *S. pygmaeus* TO α-MULTISTRIATIN AND BREVICOMIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND (−)-LIMONENE, GÖSGEN, AARAU, AND SCHÖNENWERD, AUGUST 1–SEPTEMBER 8, 1980

Treatment[a]	Replicates	Response *S. multistriatus* Mean	*S. multistriatus* Range	*S. scolytus* Mean	*S. scolytus* Range	*S. pygmaeus* Mean	*S. pygmaeus* Range
H + L	4	2.2	(1–4)	1.2	(0–4)	0	(0–0)
0.1 α-M + H + L	10	28.5	(1–89)	4.7	(0–17)	0.2	(0–2)
0.01 α-M + H + L	10	9.6	(0–30)	4.0	(0–12)	0	(0–0)
100 B + H + L	10	12.7	(1–33)	6.3	(0–17)	0.6	(0–3)

[a]M = multistriatin, B = brevicomin, H = 4-methyl-3-heptanol, L = (−)-limonene; see text for explanation of dosages, figures = μl.

S. scolytus showed no preference for either the α- or δ-isomers of multistriatin or the various concentrations of α-multistriatin and brevicomin in combination with 4-methyl-3-heptanol and α-cubebene or (−)-limonene (Tables 3-5). The addition of frontalin did not increase the number of beetles caught compared to traps baited with the bipartite mixture.

Since the experiments were laid out with the intention of trapping *S. multistriatus*, blanks are missing for the tests comparing the attractiveness of α-multistriatin, brevicomin, and frontalin. Again, it must be assumed that the α-multistriatin impurity within the sample caused the attraction to the traps baited with brevicomin. It can be concluded that for *S. scolytus* the proportion of α-multistriatin to 4-methyl-3-heptanol in the attractive mixture of odorants is not important for an optimal catch, whereas relative concentration is important for *S. multistriatus* (Cuthbert and Peacock, 1978). *S. scolytus* from England were attracted best to 4-methyl-3-heptanol and α-cubebene (Blight et al., 1980a); the addition of α-multistriatin reduced trap catches rather than increased them, as demonstrated in our tests. These differences may result from differences in release rate of the compounds or they may be artifacts of the rather low number of *S. scolytus* trapped in this study. Further field tests will be necessary to show whether the populations from England and Switzerland really differ in their responses to these compounds.

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PROTEIN-PRECIPITATING CAPACITY OF TANNINS IN *Shorea* (DIPTEROCARPACEAE) SEEDLING LEAVES

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Abstract—The protein-precipitating capacities of tanniferous extracts from immature and mature leaves of three *Shorea* spp. (Dipterocarpaceae) seedlings were measured by an adaptation of Goldstein and Swain's β-glucosidase precipitation assay. Protein precipitation by the extracts was not correlated with total phenolics (Folin-Denis assay) or proanthocyanidin content (BuOH—HCl assay) as measured in an earlier study. Extracts of *S. maxwelliana* mature leaves had much lower protein-precipitating capacity than those of *S. acuminata* and *S. leprosula*, but fewer insect species feed on and cause less damage to the foliage of *S. maxwelliana* compared with the other species' foliage. Immature leaf extracts of *S. leprosula* and *S. acuminata* had substantial protein-precipitating capacities which in the latter species exceeded that of its mature leaf extracts. Leaf extracts precipitated less protein when initial protein concentration was reduced, although not limiting, but no effect or the reverse effect occurred with quebracho tannin and tannic acid. Problems in the characterization of foliage astringency and the interpretation of its role as a potential antiherbivore defense are discussed.

Key Words—Tannins, *Shorea*, Dipterocarpaceae, insect herbivores, protein precipitation, β-glucosidase, phytochemical defenses.

INTRODUCTION

Tannins adversely affect the feeding, growth, or survival of certain insects (Bennett, 1965; Bernays, 1981; Bernays and Chamberlain, 1980; Bernays et al., 1980; Chan et al., 1978; Feeny, 1970; Fox and Macauley, 1977; Maxwell et al., 1967) and so may defend plants to some extent against herbivores (Feeny, 1976; Rhoades and Cates, 1976). An earlier study (Becker, 1981) found no correlation between insect attack and the concentrations of total phenolics

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(Folin-Denis assay) or proanthocyanidins (BuOH-HCl assay) in seedling leaves of *Shorea maxwelliana*, *S. acuminata*, and *S. leprosula* from a Malaysian rainforest. The assays employed in that study have serious shortcomings as indices of tannin content (Swain, 1979) and, moreover, equal amounts of different tannins may have quite different protein-precipitating capacities (Haslam, 1974). Since the potential defensive function of plant tannins against herbivores may depend on their capacity to form complexes with proteins (Feeny, 1970), we compared this property in the three *Shorea* species by adapting Goldstein and Swain's (1965) β-glucosidase precipitation assay to the analysis of leaf extracts. In this assay tannins in a leaf extract cause the precipitation of β-glucosidase from solution by forming a complex with the enzyme. After centrifugation, the amount of protein precipitated, as enzyme, is measured by the difference between the enzyme activities of the supernatant and an appropriate control.

Although leaves of the three *Shorea* spp. studied have similar concentrations of both total phenolics and proanthocyanidins (Becker, 1981), the potencies of their tannins, as measured by the β-glucosidase precipitation assay, differed markedly. Protein-precipitating capacity of the leaf extracts depended on the initial protein concentration, so we further investigated this effect using commercial tannins.

METHODS AND MATERIALS

Preparation of Extracts. Mature and immature leaves were collected from 0.1–0.4-m-tall seedlings of the 1976 mast crop growing in the shaded, primary forest understory of Pasoh Forest Reserve (Negeri Sembilan, W. Malaysia) during October and November 1979. (See Becker, 1981, for details of the collection protocol; insufficient *S. maxwelliana* immature leaves remained after previous phytochemical assays for use in this study.) Dried leaves were ground in a Culatti® microbeater and mortar to pass a 0.25-mm (No. 60) sieve and stored for 17 months at room temperature. Samples were oven-dried to constant weight at 70–72°C prior to extraction. All solutions with tannins were processed in glassware. All solutions less than 5 ml in volume were dispensed by adjustable Pipetmans®. Leaf powder (80.0 mg dry wt) was extracted twice for 8 min with 3.4 ml of boiling 50% (v/v) aqueous methanol in a marble-capped centrifuge tube at 90–95°C. The combined extracts were centrifuged (12,000 *g*, 15 min, 5°C), and the resulting pellet was resuspended in 2 ml of 50% methanol and centrifuged as before. The volume of the combined supernatants was brought to 10.0 ml with 50% methanol. Because methanol inhibits β-glucosidase, solvent was removed from 5.0 or 10.0 ml of this extract at 27–38°C under reduced pressure in a rotary concentrator. The slightly moist residue was redissolved in 5.0 ml of acetate

buffer (0.1 M, pH 4.8) by rotary agitation for 15 min. Protein-precipitating capacities of *S. leprosula* extracts assayed after 15 and 60 min resolubilization did not differ ($F[1,8] = 0.882$, $P > 0.25$). The stock extract in acetate buffer was centrifuged (800 *g*, 10 min), and its clear supernatant was diluted with acetate buffer to obtain extracts of various concentrations.

Ideally, leaves should be extracted and assayed for protein-precipitating capacity immediately after collection. When this is impossible, freshly collected leaves should be lyophilized and stored in a freezer. A lack of suitable equipment prevented following either of these protocols in this study. Drying and prolonged storage of *Shorea* leaves very likely altered their tannins. Provided that approximately parallel changes occurred in the different *Shorea* species, our inter- and intraspecific comparisons of protein-precipitating capacity would not be affected. Although there is an apparent loss in tannin content caused by drying, the rank correlation between analyses of comparable fresh and dry leaf samples is very high (Waterman et al., 1980). The rank order of proanthocyanidin concentrations in six oak species was unaltered after 12 months of storage of lyophilized leaf material, despite an apparent decrease in absolute values (Martin and Martin, 1982). Unfortunately, there is no comparable information about the effects of drying and storage of leaves on the protein-precipitating capacities of their extracts.

β-Glucosidase Precipitation Assay. Goldstein and Swain (1965) established the appropriate chemical and spectrophotometric parameters of this assay, so our study focused on its adaptation for the analysis of leaf extracts. The substrate used, esculin, does not form a complex with tannins (Goldstein and Swain, 1965). Haslam (1974), using natural and synthetic polyphenols, showed that loss of activity in the supernatant is due to precipitation and not to inhibition of β-glucosidase by residual soluble polyphenols.

Leaf extract in acetate buffer (0.30 ml) was added to 0.30 ml of a solution of β-glucosidase (0.50 or 1.0 mg of almond emulsin per ml, Sigma G-8625, Lot 4OF-4017, activity: 6 units/mg) in phosphate buffer (0.1 M, pH 7.0), swirled on a vortex mixer, and left at room temperature for 15 min. Simultaneously, four standards were prepared by adding 0.30 ml of acetate buffer to 0.30 ml of the enzyme solution. After centrifugation of the extract–enzyme preparations (12,000 *g*, 15 min, 5° C; the standards were only refrigerated), the supernatant was drawn off with a Pasteur pipet to avoid disturbing the precipitated enzyme. The activities of the enzyme in the supernatants and the standards were determined in triplicate by combining 20- to 50-μl aliquots with 3.0 ml of acetate buffer containing esculin hydrate (Sigma, 0.50 mM) and aluminum chloride (3.75 mM) at 25° C and determining the change in absorbance at 385 nm over 3 min (self-zeroing Zeiss M4 QII spectrophotometer with 1-cm quartz cuvettes zeroed against water).

The change in absorbance was linear over this time interval, and the initial reaction rate thereby measured was linearly related (with zero *y*

intercept) to enzyme concentration up to 1.0 mg emulsin/ml phosphate buffer as tested by polynomial regression analysis. By weighing dispensed enzyme solutions, we found that most of the variance among triplicate determinations of enzyme activity could be accounted for by pipetting error. Protein precipitation did not increase when enzyme–extract (*Quercus rubra*) mixtures were held for 50 instead of 10 min prior to centrifugation. Intensified centrifugation (30,000 *g*, 30 min, 5° C) of the enzyme–extract (*Q. rubra*) mixture did not increase sedimentation of complexed β-glucosidase (F [1,32] = 1.781, $P > 0.10$).

Extracts must be combined with the enzyme immediately after their preparation. A delay of only 4 hr resulted in reduced protein precipitation (*Q. bicolor* extracts, F [1,24] = 200.379, $P < 0.001$). Bate-Smith (1973, 1975) likewise reported that tannin solutions loose their astringency on standing.

The pH of the acetate buffer was unaffected by the presence of *S. acuminata* leaf extract. Since Goldstein and Swain (1965) found that precipitation of β-glucosidase was constant between pH 2.0 and 7.5, the assay is probably insensitive to normal differences in acidity of leaf extracts.

The proportions of β-glucosidase and the other component proteins vary among different lots of emulsin, and this probably affects the determination of binding capacity (see Discussion). Therefore, any comparative study should use a single enzyme lot.

Nontannin Inhibitors. To assess whether residual methanol or nontannin phytochemicals in the leaf extracts contributed to enzyme inhibition, tannin-free extracts were prepared by treatment with hide powder and then combined with β-glucosidase. Gustavson (1956) found that hide powder fixes ca. 30% of its weight of mimosa tannins and tannic acid at pH 4.8, so the quantity of hide powder used here was presumably sufficient to remove all tannins from the extracts.

Hide powder (100 mg, Calbiochem, fine grind), soaked in deionized water for ca. 3 hr, was centrifuged (800 *g*, 5 min), and excess water was discarded. This wetted hide powder was stirred magnetically with 1.0 ml of extract in acetate buffer, derived from 8.0 mg (*S. maxwelliana*) or 4.0 mg (remaining samples) of leaf powder. After centrifugation (800 *g*, 10 min), 0.3 ml of the cloudy supernatant was combined with 0.3 ml of emulsin solution (1.0 mg/ml, $N = 2$) and assayed as described above. Acetate buffer that was similarly treated with hide powder contained no leached substances that affected enzyme activity (Kruskal-Wallis test, $H[7] = 10.95$, $P > 0.10$).

Extractability of Tannins. Bate-Smith (1977) showed that considerable amounts of *Acer* leaf proanthocyanidins may exist in nonextractable forms that nonetheless show tannin activity, so we measured the residual protein-precipitating capacities of the methanol-extracted marcs. Leaf powder (5.0, 10.0, and 15.0 mg dry wt) was extracted with methanol and centrifuged following a procedure similar to the preparation of extracts. The marcs (pellets) were

dried under a stream of nitrogen for 10 min and then stored in an evacuated desiccator for 19–23 hr to remove residual methanol. The desiccated marcs were stirred magnetically for 35 min with 0.30 ml β-glucosidase in phosphate buffer (0.50 mg emulsin/ml) and 0.30 ml of acetate buffer, then centrifuged; the activity of the supernatant was determined as usual.

Tannic Acid and Quebracho. We further investigated the effect of initial protein concentration on protein-precipitating capacity of tannins using unpurified commercial preparations of tannic acid (Sigma T-0125) and bisulfited quebracho (Pilar River Plate Corp., Newark, New Jersey). Various concentrations of these tannins were made up in acetate buffer and assayed immediately for β-glucosidase precipitation at initial emulsin concentrations of 0.25, 0.50, and 1.0 mg/ml according to the same procedure used for leaf extracts. Moisture content was determined for separate samples of tannins dried to constant weight at 60–68° C in a vacuum oven. We made a second set of assays at some of the initial protein concentrations after discovering that the relationship between enzyme precipitation and tannin concentration was sometimes nonlinear.

Statistical Analyses. Each of the different leaf powders was extracted and assayed twice with emulsin at 1.0 mg/ml and once at 0.50 mg/ml. Although the emulsin used was 100% protein according to the manufacturer, the actual proportion of β-glucosidase was unknown, so it was impossible to calculate the absolute amount of protein precipitated by the extracts. Because of day-to-day variations in the activity of enzyme standards (not attributable to weighing errors), the results were standardized by calculating the percentage of activity lost from the supernatant of enzyme–extract mixtures (mean of $N = 3$ determinations) relative to the activity of the standards (mean of $N = 4 \times 3$ determinations).

Because a threshold concentration of extract was sometimes required for protein precipitation, regression analysis was necessary to overcome the problem of nonzero y intercepts when comparing the protein-precipitating capacities of different extracts. However, standardizing the data as described above introduced a covariance in the dependent variable (% enzyme activity lost) which invalidated an assumption of regression analysis. An appropriate transformation of the independent variable (extract concentration) in linear or nearly linear regions of the curves permitted the calculation of regression coefficients with corrected standard errors as described in the statistical note appended to this report. Since the usual multiple-comparisons tests were inappropriate, regression coefficients (slopes) were compared by Behrens-Fisher tests at $\alpha = 0.0083$ (0.05 divided by 6, the number of comparisons) to compensate for the lack of independence among multiple comparisons. For clarity the results in Figure 1 and Table 1 are expressed in terms of the untransformed data with suitably corrected standard errors.

Since the marcs were all assayed on the same day, these problems were

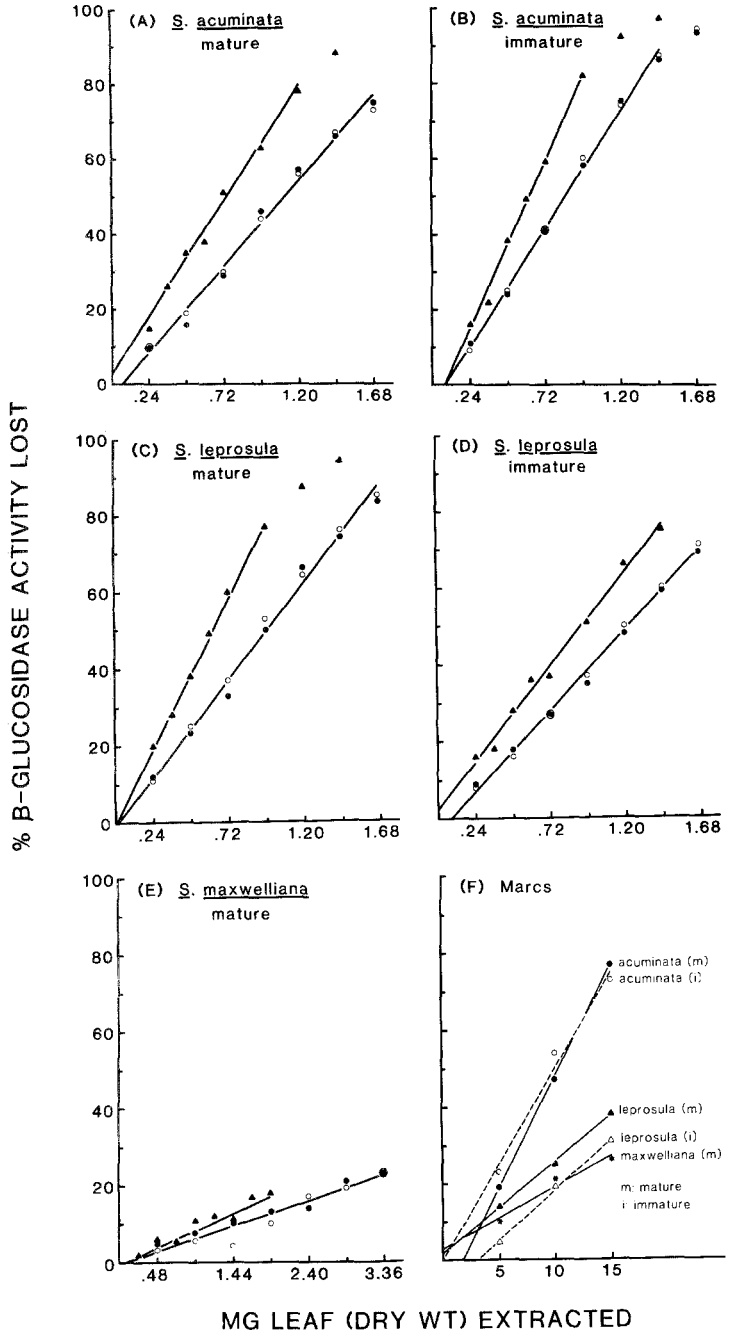

FIG. 1. Precipitation of β-glucosidase by tanniferous extracts (A–E) and marcs (F) of dipterocarp seedling leaves. The abscissa indicates the amount of leaf (per 0.3 ml acetate buffer) from which extractives or marcs were derived and combined with 0.3 ml phosphate buffer containing 0.15 mg (▲ of A–E and all runs in F) or 0.30 mg (○, ● of A–E) of emulsin. Lines represent linear, least-squares regressions.

TABLE 1. PROTEIN-PRECIPITATING CAPACITIES OF *SHOREA* SEEDLING LEAF EXTRACTS FOR TWO INITIAL PROTEIN CONCENTRATIONS

Initial emulsin conc. (mg/ml)	Leaf age	% β-glucosidase activity lost per mg leaf extracted (±SE)[a]		
		S. acuminata	*S. leprosula*	*S. maxwelliana*
1.0	Immature	65.5(±1.78)	43.4(±0.97) a	
	Mature	47.4(±2.04)A	52.3(±1.88)Aa	6.9(±0.54)
0.5	Immature	93.3(±3.43)	51.4(±1.99)	
	Mature	64.0(±3.07)	81.4(±2.07)	9.2(±1.09)

[a]Calculated as linear regression slopes from data in Figure 1A–E. Within lines or columns for a given initial emulsin concentration, values followed by the same letter did not differ ($P > 0.05$) by Behrens-Fisher tests actually made on transformed data (see Methods and Materials and Statistical Note). Y intercepts ± SE (df), lines 1–4 from left to right: −5.8±1.93(4), −3.4±1.26(5); −2.9±2.61(5), −0.7±2.37(5), −0.6±1.52(5); −7.8±2.16(4), 2.5±1.81(6); 2.7±2.33(5), −0.7±1.30(4), −0.6±1.52(6).

avoided by regressing the mean ($N = 3$) activity of the marc–enzyme mixtures on marc concentration. Regression coefficients (slopes) were compared by a simultaneous test procedure (Sokal and Rohlf, 1969). To facilitate comparison, the results in Figure 2 and Table 2 are expressed as for extracts.

Due to curvilinearity for some of the data, the protein-precipitating capacities of tannic acid and quebracho at different initial protein concentrations could not be compared by means of regression slopes. A given reduction of enzyme activity in a tannin–enzyme mixture, relative to its standard, corresponded to an unknown absolute amount of precipitated protein which varied directly with the standard's enzyme concentration. Therefore, to standardize the results relative to the highest protein concentration, the percentage of β-glucosidase activity lost was divided by 2 and 4 for the assays at 0.50 and 0.25 mg emulsin/ml, respectively, and expressed as arbitrary units of β-glucosidase precipitated. The resulting curves relating protein precipitation to tannin concentration were compared by profile analysis (Morrison, 1967) with appropriate adjustments for the covariance structure of the dependent variables.

RESULTS

Protein Precipitation by Extracts. Protein precipitation increased ($P < 0.001$) with increasing leaf extract concentration in all of the assays (Figure 1A-E). A threshold concentration of extract for β-glucosidase precipitation was apparently required for immature *S. acuminata* (0.50 and 1.0 mg

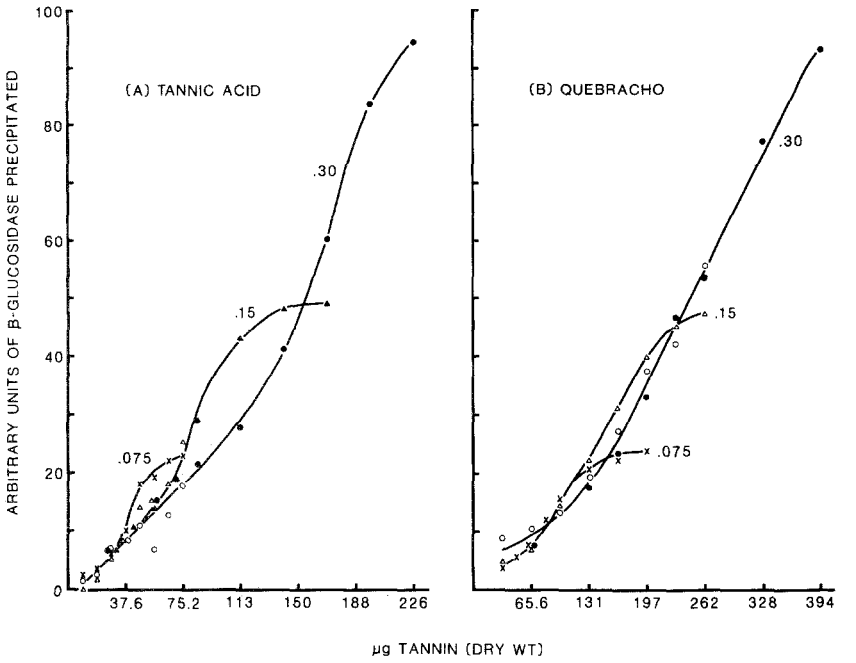

FIG. 2. Effect of initial protein concentration on precipitation of β-glucosidase by tannic acid (A) and bisulfited quebracho (B). The abscissa indicates the amount of tannin per 0.3 ml of acetate buffer combined with 0.3 ml of phosphate buffer containing 0.075 (×), 0.15 (△, ▲), or 0.30 (○, ●) mg of emulsin. Lines fitted by eye. Profile analysis compared protein-precipitation values corresponding to the following tannin concentrations (μg/0.3 ml, ×s and open symbols only)—tannic acid (0.25 vs. 1.0 mg emulsin/ml): 9.4–47.0; tannic acid (0.50 vs. 1.0 mg emulsin/ml): 9.4–65.8; quebracho (0.25 vs. 1.0 mg emulsin/ml): 32.8, 65.6, 98.4, and 131.2; quebracho (0.50 vs. 1.0 mg emulsin/ml): 32.8, 65.6, and 98.4–196.8.

TABLE 2. PROTEIN-PRECIPITATING CAPACITIES OF MARCS[a]

Leaf age	% β-glucosidase activity lost per mg marc[b]		
	S. acuminata	*S. leprosula*	*S. maxwelliana*
Immature	5.0 Aa	2.6 Ab	
Mature	5.8 a	2.4 Bb	1.6 B

[a] Initial emulsin concentration of 0.5 mg/ml.

[b] Calculated as linear regression slopes from data in Figure 1F. The slopes for immature *S. acuminata* and mature *S. maxwelliana* were not different ($P > 0.05$) from zero. Within lines or columns, values followed by the same letter did not differ ($P > 0.05$) by a simultaneous test procedure. Statistical tests were actually made on enzyme activity rather than the above derivative values (see Methods and Materials).

emulsin/ml) and immature *S. leprosula* (1.0 mg emulsin/ml only) since their regression lines had y intercepts (Table 1) different from zero (two-tailed t-tests, $P < 0.05$). Similar threshold effects have been noted in previous studies with leaf extracts (Bate-Smith, 1973; Martin and Martin, 1982) and tannins (Haslam, 1974; Schultz et al., 1981). The linearity of the non-asymptotic regions of the curves in Figure 1A-E permitted general comparisons of protein-precipitating capacity among extracts based on the regression slopes. However, the relative protein-precipitating capacities of the extracts at a particular initial protein concentration may be more similar or reversed from that suggested by such comparisons when protein-extract ratios are very high or low (Figure 1A-E).

The protein-precipitating capacity of *S. maxwelliana* extracts was much lower than that of the other two species (Table 1). Immature leaf extracts of both *S. acuminata* and *S. leprosula* had substantial protein-precipitating capacities which in the former species exceeded that of its mature leaf extracts. Differences among extracts were enhanced at the lower emulsin concentration; this affected the ranking of *S. leprosula* extracts only.

If tannins in the extracts bound and precipitated β-glucosidase in a stoichiometric manner, at least for the linear regions of the curves in Figure 1A–E, then halving the initial protein concentration should result in a doubling of the loss in β-glucosidase activity. This did not occur for any of the five extract types (Behrens-Fisher tests, $P < 0.05$), which had consistently lower than expected binding capacities at 0.50 mg emulsin/ml.

Nontannin Inhibitors. Significant residual inhibition of β-glucosidase activity by leaf extracts treated with hide powder to remove tannins occurred only in the cases of mature *S. leprosula* ($F[1,20] = 4.664$, $P < 0.05$) and *S. maxwelliana* ($F[1,20] = 18.041$, $P < 0.001$). Assuming that the inhibitory effect of the presumed nontannins (residual methanol or coextracted phytochemicals) was directly proportional to extract concentration, then they accounted for 25% of the observed (Table 1) reduction in enzyme activity by *S. maxwelliana* extracts but only 3.6% of that by mature *S. leprosula* extracts for assays at an initial emulsin concentration of 1.0 mg/ml. These results suggest that the protein-precipitating capacity of *S. maxwelliana* extracts relative to the other species was even lower than indicated by the data in Table 1.

Extractability of Tannins. Apparently there were no gross differences in tannin extractability to account for the basic differences in protein-precipitating capacity of leaf extracts. The cause of β-glucosidase inactivation by methanol-extracted marcs is uncertain since the enzyme may have been bound by tannins or other leaf constituents or otherwise inhibited, but the effect of the marcs was always small relative to that of the leaf extracts (Figure 1F, Tables 1 and 2). Absence of significant regressions for immature *S. acuminata* and mature *S. maxwelliana* was due to excessive residuals and

small sample sizes which probably also accounted for the statistical identity of the regression slopes for immature *S. acuminata* and *S. leprosula*.

Tannic Acid and Quebracho. Leaf extracts consistently precipitated less β-glucosidase at the lower initial protein concentration even though protein was still apparently in excess, as suggested by the positive linear relationship between enzyme precipitation and extract concentration. To determine whether this effect generally occurred with β-glucosidase, we studied its precipitation by commercial tannins. Solutions of these tannins treated with hide powder as described for leaf extracts had no residual inhibitory effect on β-glucosidase activity (tannic acid: $F[1,24] = 1.027$, $P > 0.25$; quebracho: $F[1,24] = 0.038$, $P > 0.75$).

For both tannic acid and bisulfited quebracho, the protein precipitation curves (Figure 2) were statistically indistinguishable (profile analysis, $P > 0.50$) over the compared regions, indicating no effect of initial protein concentration on protein-precipitating capacity of these tannins. For a given initial protein concentration, only protein precipitation values based on the same standards (to preserve balanced statistical design), occurring outside the asymptotic region, and corresponding to matching extract concentrations of the compared curve were tested. At tannic acid concentrations greater than those tested statistically and less than those at which protein precipitation had leveled off, however, there was a tendency for protein precipitation to increase as initial protein concentration was reduced. These results were contrary to those for leaf extracts.

Tannic acid precipitated about 1.7 times more β-glucosidase than an equal weight of quebracho. This is consistent with Haslam's (1974) findings for hydrolyzable and condensed tannins with this enzyme.

DISCUSSION

Tannin–Protein Chemistry. Our results underscore the need for suitable precautions when attempting to compare the protein-precipitating capacities of tannins. The robustness of differences in this property of tannins should be assessed by measurements made over a range of tannin and protein concentrations. Threshold and asymptotic effects of tannin concentration on protein precipitation limit the meaningfulness of measurements made at a single tannin concentration. While precipitation of β-glucosidase increased with increasing leaf extract or tannin concentration, the initial protein concentration affected the amount of protein precipitated in different ways, depending on the type and concentration of tannins.

Some insight into the complexities of tannin–protein interactions is provided by the finding of Van Buren and Robinson (1969) that gelatin and tannic acid form both soluble and insoluble complexes. They suggested that

the precipitability of tannin–protein complexes is affected by changes in their size and bonding configuration as the tannin–protein ratio varies. We suspect that the relative precipitability of a particular protein in a heterogeneous mixture may also be affected by interaction of tannins with the other proteins. This is suggested by our observation that *S. maxwelliana* leaf extract reduced β-glucosidase activity less than expected from the pellet's (tannin–protein precipitate) visible size.

Tannin Content and Astringency. For the three *Shorea* spp. studied here, Becker (1981) showed that seedling leaves of a particular age class have similar concentrations of both total phenolics and proanthocyanidins as measured by the Folin-Denis and BuOH-HCl assays, respectively. However, the protein-precipitating capacities of leaf constituents in these species, as measured here with β-glucosidase, differed markedly. The Folin-Denis assay includes all phenolic compounds, not just tannins, while the BuOH-HCl assay includes monomers of proanthocyanidins which do not precipitate proteins, so these results are not surprising (Rhoades and Cates, 1976; Swain, 1979). Other studies have also failed to find a correlation between the content and astringency of tannins in leaf extracts (Bate-Smith, 1977, 1981; Martin and Martin, 1982; Swain, 1979).

Given this discrepancy and the current beliefs about how tannins function defensively, we believe that the various assays for measuring tannin astringency (Bate-Smith, 1973; Martin and Martin, 1982) are more likely to be relevant in exploratory ecological studies than chemical, reactive group assays of tannin content (cf. Gartlan et al., 1980). There are at least three mechanisms by which tannins may defend plants from attack by herbivores: as feeding deterrents affecting palatability, as growth inhibitors affecting nutrient availability, and as direct toxic agents (Feeny, 1970; Bernays et al., 1980). All three mechanisms probably depend, at least partially, on the ability of tannins to form complexes with proteins.

Herbivory and Tannin Defense. Earlier attempts (Feeny, 1976; Rhoades and Cates, 1976; Cates, 1980) to generalize concerning the within-plant allocation of tannins between immature and mature leaves are questionable, as remarked previously by McKey (1979). Immature leaves of *S. acuminata* and *S. leprosula* contain substantial quantities of protein-precipitating phenolics; in the first species they appear to be more potent than those of mature leaves, contrary to the pattern predicted by the former investigators.

Extracts of *S. maxwelliana* leaves had much lower protein-precipitating capacity than those of *S. acuminata* and *S. leprosula* when measured by the β-glucosidase assay under favorable pH conditions, and differences in tannin extractability apparently did not account for this relationship. Superficially, these results suggest that the lower insect species richness and damage on *S. maxwelliana* foliage (Becker, 1981) are not due to its having more potent tannin defenses than the other *Shorea* species. However, the certainty of this

conclusion depends on whether the astringency of leaf extracts, as measured with β-glucosidase, adequately reflects the capacity of leaf tannins to bind with those other proteins (e.g., insect salivary proteins and digestive enzymes and leaf proteins) actually affecting the palatability, digestibility, and toxicity of *Shorea* foliage to insects. β-Glucosidase occurs in the guts of many insects (House, 1974), so its interaction with tannins has potential relevance to the digestibility of foliage. Although the degree of inhibition of different enzymes by a particular tannin or tanniferous extract can differ markedly (Griffiths, 1979; Mandels and Reese, 1963; Schneider and Hallier, 1970), it would still be possible to meaningfully rank foliage astringency if the component tannins vary similarly in their interaction with different proteins. There is a high correlation between the precipitability of β-glucosidase and bovine serum albumin (BSA) by tanniferous foliage extracts of six oak species (Martin and Martin, 1982), and extracts of the three species that have been tested rank identically with respect to their precipitation of BSA and ribulose 1,5-biphosphate carboxylase-oxygenase, the major soluble leaf protein (Martin and Martin, in press). A broader survey is required, but the results so far suggest that a consistent ranking of foliage astringency is possible.

Bernays (1981) has excellently reviewed available information concerning responses of insects to tannins and how some insects overcome the potential adverse effects of tannins and even benefit from their presence in food. She concluded that generalizations about the effectiveness of tannins as defensive compounds cannot yet be made, despite the earlier belief that this was possible (Feeny, 1976; Rhoades and Cates, 1976). We wish to emphasize that the utility of protein precipitation assays like the one described here lies in the identification of foliage in which tannins have a potential defensive role against herbivores. Confirmation or disproof of this role requires experimental study of the behavioral and physiological responses to tannins by potential and actual herbivores in the system. Nevertheless, correlative studies of insect feeding and foliage astringency importantly complement such experimental studies because of the difficulty of altering dietary tannin composition and concentration in a biologically relevant manner.

STATISTICAL NOTE

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For the comparison of protein-binding capacities of leaf extracts by regression analysis, we assumed that the basic measurements of enzyme activity in the standards (S_{ij}) and supernatants of the enzyme–extract mixtures

(M_{ij}) had the forms:

$$S_{ij} = \mu + \gamma_i + \epsilon_{ij} \tag{1.1}$$

and

$$M_{ij} = \mu'_i + \gamma'_i + \epsilon'_{ij} \tag{1.2}$$

where μ and μ'_i are the means, γ_i and γ'_i are random preparation effects, and ϵ_{ij} and ϵ'_{ij} are random measurement (within preparation) effects. We further assumed that $E(\gamma_i) = E(\gamma'_i) = E(\epsilon_{ij}) = E(\epsilon'_{ij}) = 0$, $\mathrm{Var}(\gamma_i) = \mathrm{Var}(\gamma'_i) = \sigma_p^2$, and $\mathrm{Var}(\epsilon_{ij}) = \mathrm{Var}(\epsilon'_{ij}) = \sigma_w^2$.

The dependent variable in the regressions was

$$Y_i = (\bar{\bar{S}} - \bar{M}_i)/\bar{\bar{S}} \tag{2}$$

where $\bar{\bar{S}} = (\sum_{i=1}^{4} \sum_{j=1}^{3} S_{ij})/12$ and $\bar{M}_i = (\sum_{j=1}^{3} M_{ij})/3$.

From (1.1) and (1.2) it follows that $\mathrm{Var}(\bar{\bar{S}}) = (\sigma_p^2/4) + (\sigma_w^2/12)$ and $\mathrm{Var}(\bar{M}_i) = \sigma_p^2 + (\sigma_w^2/12)$. Expanding (2) in a first-order Taylor series around the means of $\bar{M}_i$ and $\bar{\bar{S}}$ and using the sample values to estimate these means gives

$$\mathrm{Var}(Y_i) \approx (\bar{\bar{M}}^2/\bar{\bar{S}}^4)\mathrm{Var}(\bar{\bar{S}}) + (1/\bar{\bar{S}}^2)\mathrm{Var}(\bar{M}_i) = (\bar{\bar{M}}^2/\bar{\bar{S}}^4)\,[(\sigma_p^2/4) + (\sigma_w^2/12)] + (1/\bar{\bar{S}}^2)\,[\sigma_p^2 + (\sigma_w^2/3)] \tag{3.1}$$

and

$$\mathrm{Cov}(Y_i, Y_j) \approx (\bar{\bar{M}}^2/\bar{\bar{S}}^4)\,[(\sigma_p^2/4) + (\sigma_w^2/12)] \tag{3.2}$$

where $\bar{\bar{M}} = (\sum_{i=1}^{n} \bar{M}_i)/n$.

Consequently, we assumed a regression model of the form

$$Y_i = \alpha + \beta(X_i - \bar{X}) + \epsilon_i$$

where X_i is extract concentration and the ϵ_is have a multivariate normal distribution with means all equal to zero. $\mathrm{Var}(\epsilon_i) = a + b = \mathrm{Var}(Y_i)$, and $\mathrm{Cov}(\epsilon_i, \epsilon_j) = a = \mathrm{Cov}(Y_i, Y_j)$, where a and b are evident from (3.1) and (3.2).

By means of an orthogonal transformation (e.g., Arnold, 1979), the above regression model can be reformulated almost like the standard, homoscedastic, uncorrelated error case. Inference above β is exactly as in the standard case, i.e.,

$$\frac{\hat{\beta} - \beta}{[s^2/\sum_{i=1}^{n}(X_i - \bar{X})^2]^{1/2}} \tag{4}$$

has a t distribution on $n - 2$ degrees of freedom, where $\hat{\beta}$ is the usual least squares estimator of β and s^2 is the usual regression error mean square. Comparisons between different slopes were based on the Behrens-Fisher distribution (Box and Tiao, 1973; Fisher and Yates, 1963) with significance levels adjusted according to the Bonferroni inequality approach (Miller, 1966).

Inference about α was based on the fact that, given α, a, and b, $\overline{Y}$ is normally distributed with mean α and variance $a + (b/n)$. Here the usual regression mean square error has an expected value equal to b. An unbiased estimate of the variance of $\hat{\alpha}$ is

$$\widehat{\text{Var}}(\hat{\alpha}) = (\overline{\overline{M}}^2/12\overline{\overline{S}}^4)\Sigma_{i=1}^{4}(\overline{S}_i - \overline{\overline{S}})^2 + (s^2/n) \quad (5)$$

where s^2 is as in (4). Inference about α then depended on the approximation that $(\overline{Y} - \alpha)/[\widehat{\text{Var}}(\hat{\alpha})]^{1/2}$ has a t distribution on 3 degrees of freedom (the smaller of the two variance component estimators' degrees of freedom).

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VOLATILE MALE-SPECIFIC NATURAL PRODUCTS OF A COREID BUG (HEMIPTERA: HETEROPTERA)

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Abstract—The large coreid bug, *Pachylis laticornis* (Hemiptera: Coreidae), feeds on several mimosaceous trees in Guanacaste, Costa Rica. In addition to the presumably defensive metathoracic exocrine glands that occur in both sexes of this species, the adult males also possess a ventral abdominal gland, opening midventrally in the 7–8th abdominal intersegmental membrane, that releases volatile compounds. Two esters, (*E*)-2-hexenyl tiglate and (*E*)-2-hexenyl (*E*)-2-hexenoate, account for over 90% of the total volatiles in the ventral abdominal gland secretion of males. (*E*)-2-Octenyl tiglate and (*E*)-2-hexenyl benzoate are present at low concentrations, as are at least three other unidentified compounds. The biological role for this fragrant male-specific exudate is unknown.

Key Words—Pheromone, Hemiptera, Heteroptera, Coreidae, (*E*)-2-hexenyl tiglate, (*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-2-octenyl tiglate, (*E*)-2-hexenyl benzoate.

INTRODUCTION

In Guanacaste Province, Costa Rica, species of the legume trees *Pithecellobium* and *Enterolobium* (Mimosaceae) are the preferred host plants of a species of coreid bug in the genus *Pachylis*, probably *Pachylis laticornis* (Hemiptera: Coreidae). The nymphs form spectacular feeding aggregations on twigs of the host plant and seem to be able to complete development on a diet of phloem alone (Aldrich and Blum, 1978). Adults are nonaposematic but large (ca. 3 cm in length), migratory (Aldrich, unpublished observation; Torre-Bueno, 1945), and have been observed feeding on *P. saman* seeds and twigs both singly and in copulo (Kinsman, personal communication). The hind femora are much larger in adult males than in adult females, an adaptation which is believed to aid in the defense of territories and the

maintenance of harems by males of other coreid species (Mitchell, 1980; Fujisaki, 1980). Field observations suggest that this species also mates in groups consisting of one male with one or more females. The males mate successively with females in the group, often remaining at one site for several days, while females move more frequently between groups (Kinsman, personal communication 1982).

Although the Hemiptera are notorious for the production of noxious defensive secretions, it is probably not generally appreciated that adult males of many hemipterans, including the Coreidae, release blends of volatile compounds from an ectodermal abdominal gland opening midventrally in the 7–8th intersegmental membrane (Figure 1) (Thouvenin, 1965). The ventral abdominal gland secretions of the coreid species analyzed to date are species-specific and contain predominantly aromatic alcohols such as benzyl alcohol (odor of cherries) and 2-phenylethanol (odor or roses) (Aldrich et al., 1976, 1979). Of the insect species whose exocrine secretions have been analyzed, these hemipteran scents are most reminiscent of the volatile compounds released during courtship by male Lepidoptera (Aplin and Birch, 1970; Petty et al., 1977) which serve as aphrodisiacs (Jacobson et al., 1976; Weatherston and Percy, 1977).

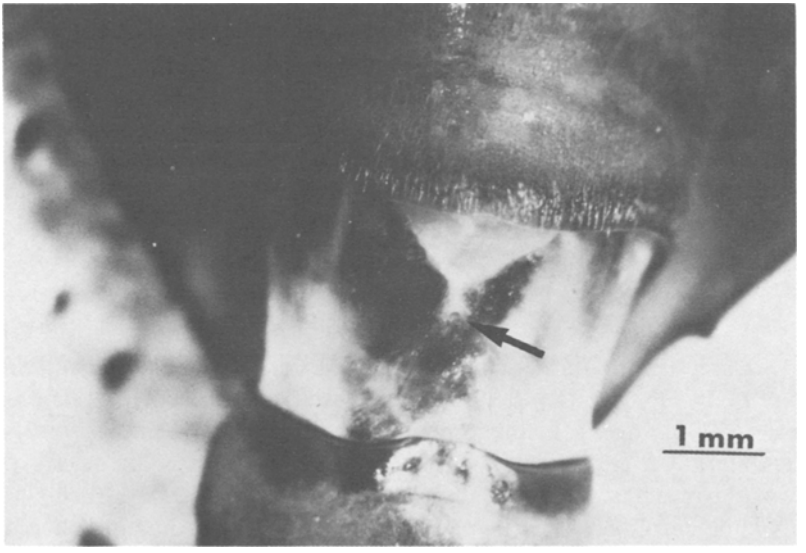

FIG. 1. The posterior sternum of a *Pachylis laticornis* adult male showing the opening (arrow) of the ventral abdominal gland in the intersegmental membrane between the last unmodified abdominal segment (segment 7) and the first genital segment (segment 8).

We report here the chemical identification of the volatile constituents from the 7–8th ventral abdominal gland of adult male *P. laticornis*.

METHODS AND MATERIALS

Late fifth instars of the coreids were collected during the dry season near Palo Verde field station in Guanacaste Province, Costa Rica, at the same site described for an investigation of the aposematic aggregations of the nymphs of this species (Aldrich and Blum, 1978). Originally, the species was determined to be in the genus *Thasus*, probably *Thasus acutangulus* (Aldrich and Blum, 1978). However, the publication of taxonomic revisionary work for coreid species placed in the tribe Mictini (O'Shea and Schaefer, 1978; O'Shea, 1980) prompted us to have the species redetermined. Specimens used in the present study were sent to Dr. Carl Schaefer (University of Connecticut) and redetermined to be in the sister genus of *Thasus*, the genus *Pachylis*, probably the species *Pachylis laticornis* (Fabricius). The uncertainty of the specific determination is due to the fact that this neotropical genus has never been revised (Schaefer, personal communications). Voucher specimens of adult *Pachylis laticornis* have been placed in the Cornell University Insect Collection under Lot. No. 1112.

A permit to import nymphs of *Pachylis laticornis* into the United States from Costa Rica was granted by the Animal and Plant Health Inspection Service (APHIS, USDA). Approximately 1 week after emergence, the 7–8th ventral abdominal glands were dissected from freshly sacrificed males and extracted in CS_2 as previously described (Aldrich et al., 1979).

Preliminary gas chromatography (GC) was performed on a Packard 7400 GC equipped with a flame ionization detector using a 6-ft column of 3% OV-101, temperature programed from 50° C to 250° C at 10°/min. Gas chromatographic retention times of unknowns were compared to those of authentic standards on a 3% SE-30 column and a 3% OV-275 column programed from 60° C to 250° C at 10°/min. The relative proportions of the identified components were calculated by cutting out the peaks from a GC chromatogram and weighing the pieces of paper.

Gas chromatographic–mass spectrometric analyses were conducted on two different systems: an LKB-9000 mass spectrometer using a 6-ft GC column of 0.75% SE-30 operated isothermally at 130° C, and a Finnigan 4000 mass spectrometer using a 6-ft GC column of 1% OV-17 operated isothermally at 170° C. Electron impact (EI) spectra were collected at 70 eV on both instruments. Chemical ionization (CI) spectra were also obtained with the Finnigan system using isobutane as the reagent gas.

Authentic standards of (*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-2-hexenyl benzoate, and (*E*)-2-octenyl tiglate [(*E*)-2-octenyl (*E*)-2-methyl-2-butenoate]

were prepared by the acid chloride method. The acids (Aldrich Chemical Company) were treated with excess thionyl chloride in the presence of a few drops of dimethylformamide. The vacuum-distilled acid chloride (20 mm Hg), in ether, was added to a stirred, ice-cooled solution of the alcohol and pyridine in ether, followed by refluxing for 1/2 hr. (*E*)-2-Hexenyl tiglate was prepared from the crude acid chloride (acid + PCl_3) and (*E*)-2-hexenol or, alternatively, from tiglic acid–hexenol in benzene with *p*-toluenesulfonic acid as catalyst (Dean-Stark trap). Both methods gave equivalent products by GC, so they were combined for distillation. After washing with water, dilute acid, water, and bicarbonate, each ester was dried and vacuum-distilled. The boiling points of the esters were: (*E*)-2-hexenyl tiglate, 102–112° C (mostly 108–112° C)/13 mm; (*E*)-2-octenyl tiglate, 125–138° C (almost all 136–138° C)/10 mm; (*E*)-2-hexenyl (*E*)-2-hexenoate, 122–126° C/15 mm; and (*E*)-2-hexenyl benzoate, 148–152° C/13 mm.

RESULTS

Each ventral abdominal gland consists of two main secretory tubules, arising from the external orifice, which subdivide to form several branches. The secretory tubules are similar in appearance to Malpighian tubules except the gland tubules are bright red in color. Due to their bright color, the branches of the gland are easily traced in the abdomen; they are located ventrally and extend anteriorly nearly to the thorax.

Preliminary gas chromatography showed that the adult male ventral abdominal gland secretion contains high concentrations of two volatile compounds plus relatively low concentrations of at least five compounds (Figure 2).

Components 1–3. Unidentified.

Component 4. This compound is the most abundant component of the secretion, accounting for 73% of the identified volatiles. The EI mass spectrum showed a small molecular ion (1.5%) at *m/e* 182, which was confirmed by the CI mass spectrum [base peak at *m/e* 183, $(M+H)^+$], and characteristic ions at *m/e* 167, 153, 137, 125, 83 (base peak), 67, 55, and 41. The spectrum was not in the computer file of mass spectra (Heller 1972), nor could a matching published spectrum be found. However, the spectrum was similar to the published mass spectrum of the allyl ester of tiglic acid; therefore, it was suspected that the compound could be a hexenyl ester of tiglic acid. The spectrum of the unknown is identical to the mass spectrum of the synthesized standard of (*E*)-2-hexenyl tiglate, and the GC retention times of the unknown and the authentic standard are identical on SE-30 and OV-275 columns.

Component 5. This compound accounts for 19% of the identified volatile compounds in the secretion. The EI mass spectrum had a small molecular ion (1.5%) at *m/e* 196, confirmed as the molecular ion by the CI spectrum, with

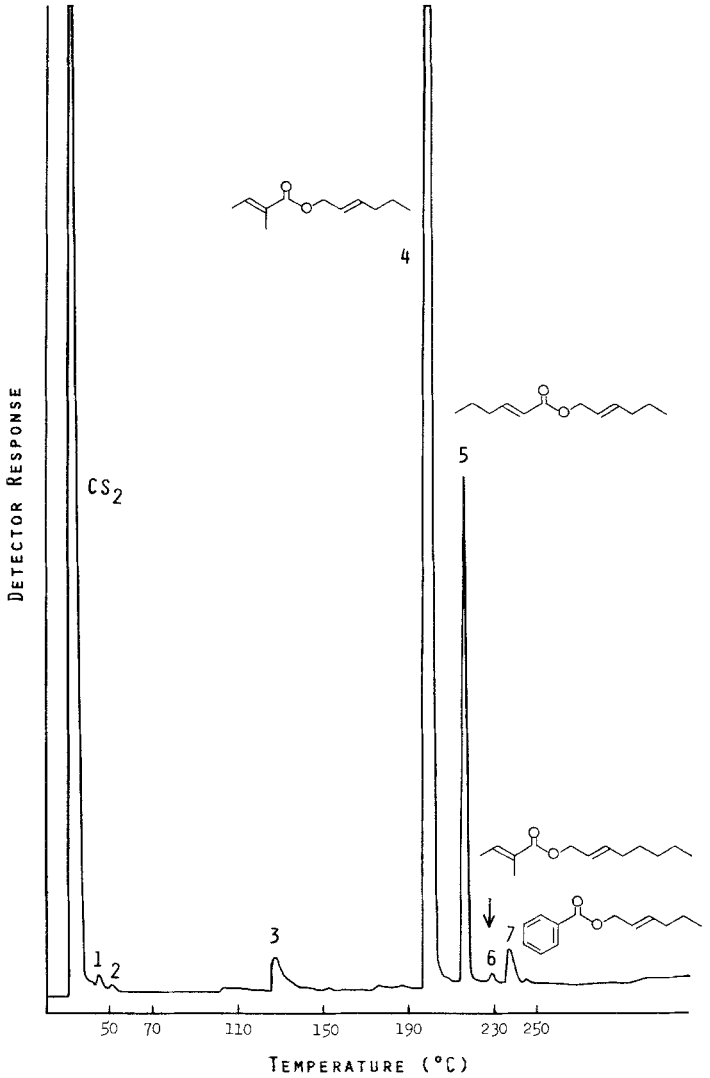

FIG. 2. Gas chromatogram (3% OV-101 column programed from 50° to 250° C at 10° C/min) of a carbon disulfide extract of the ventral abdominal glands from *Pachylis laticornis* adult males.

characteristic ions at *m/e* 167, 153, 139, 125, 111, 97 (base peak), 82, 67, 55, and 41. The EI mass spectrum matches the spectrum of the synthetic standard of (*E*)-2-hexenyl (*E*)-2-hexenoate and the spectrum of this compound in the computer file (Heller, 1972). The GC retention times of the unknown matched the retention times of authentic (*E*)-2-hexenyl (*E*)-2-hexenoate on SE-30 and OV-275 columns.

Component 6. This minor component (2%) had an EI mass spectrum very similar to that of peak 4, but with a small molecular ion (1.0%) at *m/e* 210 instead of 182. Other characteristic ions occurred at *m/e* 181, 165, 125, 95, 83 (base peak), 69, 55, and 41. The EI mass spectrum matches the spectrum of the synthesized standard of (*E*)-2-octenyl tiglate, and the retention times of the unknown and standard matched on SE-30 and OV-275 columns.

Component 7. The EI mass spectrum of this minor component (6%) exhibited a base peak at *m/e* 105, suggesting a benzoate moiety, and a small molecular ion (1.3%) at *m/e* 204 which was confirmed by the CI mass spectrum. Other characteristic ions were present in the EI spectrum at *m/e* 123, 82, 77, 67, 55, 51, and 41. The EI mass spectrum of the unknown matches the spectrum of authentic (*E*)-2-hexenyl benzoate, and the retention times matched on SE-30 and OV-275 columns.

DISCUSSION

Two esters, (*E*)-2-hexenyl tiglate and (*E*)-2-hexenyl (*E*)-2-hexenoate, account for over 90% of the total volatiles in the 7–8th ventral abdominal gland secretion from adult males of the coreid bug, *P. laticornis*. (*E*)-2-Octenyl tiglate and (*E*)-2-hexenyl benzoate are also present in the secretion at low concentrations, as are at least three other unidentified compounds. Tiglate esters have not, to our knowledge, been previously found in arthropods.

Tiglate esters are known to occur naturally in plants and are used for perfumes and as flavoring agents. Geranyl tiglate, for example, is a major constituent of the oil of geranium (Geraniaceae) and butyl tiglate is present in the oil of the Roman chamomile, *Anthemis nobilis* (Compositae) (Windholz, 1976). Tiglic acid [(*E*)-2-methyl-2-butenoic acid] and methacrylic acid (2-methylpropenoic acid) are synthesized and used defensively as chemical irritants by many species of carabid beetles (Schildknecht, 1970). Of the many hemipterans whose metathoracic gland secretions have been analyzed, only the broad-headed bug, *Megalotomus quinquespinosus* (Alydidae), has been found to produce methyl-branched esters in this secretion (Aldrich and Yonke, 1975). Interestingly, this is a species in which the 7–8th ventral abdominal gland is absent.

Esters were the only class of compounds identified in the ventral abdominal gland secretion of the young *P. laticornis* males examined here. Although it is conceivable that compositional changes occur with age in a male's exudate (Aldrich et al., 1978), it appears that the chemical theme expressed in the ventral abdominal glands of *P. laticornis* is distinct from the aromatic alcohol-dominated secretions of other coreid bugs (Aldrich et al., 1979). As such, this secretion from *Pachylis* males more closely resembles the

blends of acetate esters liberated as sex pheromones by many female moths than the aphrodisiacs produced by male moths (Blum, 1977).

At this point we can only speculate that the esters produced in the ventral abdominal glands of adult male *P. laticornis* constitute some type of pheromone. In at least one species of Pentatomidae (the southern green stink bug, *Nezara viridula*), males are responsible for the production of an aggregation pheromone (Harris and Todd, 1980). Perhaps the male-specific secretion of *P. laticornis* functions as an attractant pheromone. Alternatively or concurrently, the secretion could serve as a territorial marker or as a mating stimulant during courtship. These seem to be the most likely functions; other functions may exist.

Our identification of the distinctive array of esters from *P. laticornis* males and the relative ease with which the compounds can be synthesized should provide impetus for future research on the biology of this interesting coreid species.

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APHID ALARM PHEROMONE DERIVATIVES AFFECTING SETTLING AND TRANSMISSION OF PLANT VIRUSES

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Abstract—1,4-Cycloaddition reactions between (*E*)-β-farnesene (the aphid alarm pheromone) and dienophiles, such as SO_2 and $RO{\cdot}CO{\cdot}C \equiv CCO{\cdot}OR$, gave products more stable and less volatile than the parent compound. In laboratory experiments, the numbers of *Myzus persicae* (Sulz.) settling and nymphs deposited on plants were decreased by some of the reaction products. Acquisition, and thereby transmission, of the semi-persistent beet yellows virus and the nonpersistent potato virus Y, was decreased.

Key Words—1,4-Cycloaddition, Diels-Alder reaction, alarm pheromone, *Myzus persicae* (Sulz.), Homoptera, Aphididae, sugar beet yellows virus, potato virus Y.

INTRODUCTION

(*E*)-β-Farnesene, the main component of the alarm pheromone of many species of aphid, substantially enhanced effectiveness of contact pesticides in laboratory tests (Griffiths and Pickett, 1980). However, it is not persistent enough for long-term protection against aphid colonization and has not prevented virus transmission either in laboratory tests (Yang and Zettler, 1975) or in crops (Hille Ris Lambers and Schepers, 1978), probably because it is easily oxidized and is highly volatile.

The terminal conjugated diene group in (*E*)-β-farnesene is susceptible to rapid aerial oxidation and was therefore the target for chemical modification to give products with (1) less unsaturation and therefore greater stability in air

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and (2) higher molecular weight and consequently lower volatility. An attractive method for the desired modifications was to use the Diels-Alder (1,4-cycloaddition) reaction (Hamer, 1967) of dienophiles with the suitably oriented 1,3-diene system in (*E*)-β-farnesene. With various dienophiles, products could be obtained with different stabilities, volatilities, and polarities, so that factors influencing activity could be recognized. Reversible reactions in which the pheromone would be readily regenerated were considered first.

METHODS AND MATERIALS

Crude (*E*)-β-farnesene (47%) prepared from commercial nerolidol (*E* and *Z* mixture, 2:1) was used without further purification (Dawson et al., 1982). Dienophiles were purchased or prepared by standard methods. Yields, refractive indices, nuclear magnetic resonance (NMR) spectroscopy (Jeol: PMX-60, CCl_4, Me_4Si as standard, $\delta = 0.00$), and mass spectrometry (MS) (VG Micromass: MM 70-70F + Data System 2035, electron impact at 70 eV, probe tip samples 200° C) of new compounds are summarized in Table 1.

1,4-Cycloaddition Reactions

3-(4,8-Dimethyl-3,7-nonadienyl)sulfolene (*I*). Crude (*E*)-β-farnesene (17 g) and liquid sulfur dioxide (20g) were sealed in a glass ampoule and stored at ambient temperature for 18 hr. The ampoule was opened after cooling and excess SO_2 allowed to evaporate. The residue was then chromatographed on Florisil using hexane, an ether–hexane mixture of increasing concentration in ether, and finally ether, the effluent being monitored by NMR. Removal of the solvent under vacuum from appropriate fractions gave 3-(4,8-dimethyl-3,7-nonadienyl)sulfolene as a straw-colored liquid (9 g, 58%) (found: C, 66.9; H, 8.9; S, 11.7. $C_{15}H_{24}O_2S$ requires C, 67.2; H, 8.9; S, 11.9%).

On heating at 180° C at 1 torr, the sulfolene regenerated the (*E*)- and (*Z*)-β-farnesene mixture, characterized by coinjection on GC and by GC-MS (Dawson et al., 1981).

1,2-Bis(ethoxycarbonyl)-4-(4,8-dimethyl-3,7-nonadienyl)-1,2,3,6-tetrahydropyridazine (*II*). A solution of crude (*E*)-β-farnesene (14.5 g) in ether (20 ml) was cooled to −20° C and diethyl azodicarboxylate (8.7 g) slowly added with stirring. The mixture was stored at −20° C overnight and then at 4° C, the reaction being shown by NMR to be complete after 8 hr at 4° C. The mixture was then fractionally distilled to give 1,2-bis(ethoxycarbonyl)-4-(4,8-dimethyl-3,7-nonadienyl)-1,2,3,6-tetrahydropyridazine as a yellow oil (8 g, 43%); bp 180–185° C/0.3 torr.

1,2-Bis (ethoxycarbonyl)-4-(4,8-dimethyl-3,7-nonadienyl) cyclohex-4-

ene (*III*). Crude (*E*)-β-farnesene (10 g) and diethyl maleate (5.0 g) were heated together at 180°C for 2 hr. The mixture was then distilled under reduced pressure to give 1,2-bis(ethoxycarbonyl)-4-(4,8-dimethyl-3,7-nonadienyl) cyclohex-4-ene as a pale yellow oil (8.5 g, 75%); bp 175–180°C/0.3 torr.

Preparation of Adducts between (E)-β-Farnesene and Other Dienophiles

Crude (*E*)-β-farnesene was reacted with maleic anhydride, diethyl acetylenedicarboxylate, acrolein, methyl ethenyl ketone, didecyl maleate or didecyl acetylenedicarboxylate as for diethyl maleate but at 25°C, 80°C, 100°C, 150°C, 180°C, and 80°C, respectively, the first two reactions being in carbon tetrachloride, to give the compounds IV, V, VI, VII, VIII, and IX, respectively.

Derivatives from 1,4-Cycloaddition Reaction Products

2-Ethoxycarbonyl-4-(4, 8-dimethyl-3, 7-nonadienyl)-1,2,3,6-tetrahydropyridazine (*X*). Compound II (4.0 g) was hydrolyzed using potassium hydroxide (5.0 g), water (5.0 g), and ethanol (25.0 ml) for 4 days at room temperature. The resulting solution was partitioned with water (25 ml) and light petroleum (60/80°, 25 ml). The aqueous phase was then acidified with acetic acid, causing loss of CO_2, and extracted with light petroleum (60/80°, 25 ml) which was dried (Mg_2SO_4), concentrated, and distilled to give 2-ethoxycarbonyl-4-(4, 8-dimethyl-3, 7-nonadienyl)-1, 2, 3, 6-tetrahydropyridazine as a yellow oil (2.5 g, 77%); bp 160–168°/0.4 torr.

1, 2-Dimethyl-4-(4, 8-dimethyl-3, 7-nonadienyl)-1, 2, 3, 6-tetrahydropyridazine (*XI*). Compound II (5.0 g) was added slowly to a stirred suspension of lithium aluminium hydride (1.5 g) in dry ether (100 ml) and the mixture heated under reflux for 0.5 hr. The excess hydride was destroyed by adding ethyl acetate and a granular precipitate formed by adding, in turn, water (1.5 ml), 2 M NaOH (1.5 ml), and water (4.5 ml). The precipitate was filtered off and the filtrate concentrated and distilled to give 1,2-dimethyl-4-(4,8-dimethyl-3,7-nonadienyl)-1,2,3,6-tetrahydropyridazine as a yellow oil (3.1 g, 77%) bp 130–135°C/0.3 torr.

Benzylgeranyl (*XII*). Magnesium turnings (2.8 g) were covered with diethyl ether (25 ml), then treated with ethyl iodide (one drop), warmed to initiate the reaction, and a solution of benzyl chloride (2.95 g) in diethyl ether (75 ml) added at a rate that maintained reflux which continued for 1.0 hr. After cooling, the solution was decanted into cooled geranyl bromide (5.0 g) and heated under reflux for 1.0 hr. The reaction mixture was poured onto saturated ammonium chloride solution (50 ml), shaken, and partitioned. The organic phase was washed with water, saturated brine, and dried (Mg_2SO_4).

TABLE 1. (*E*)-β-FARNESENE DERIVATIVES and RELATED COMPOUNDS

Compound	Yield (pure compound % theoretical)	Refractive index	MS: molecular ion (m/z, % of base peak)	NMR: ^{1}H (unless specified) (ppm)
I	58	1.5080	268 (0.02)	1.70 (m, 9H, 3xCH_3—C=) 2.08 (m, 8H, 4xCH_2—C=) 3.70 (m, 4H, ring) 5.16 (m, 2H, 2x=CH) 5.78 (br t, 1H, =CH ring)
II	43	1.4938	378 (2.9)	1.30 (t, 6H, 2xCH_3 ester) 1.70 (m, 9H, 3xCH_3—C=) 2.08 (m, 8H, 4xCH_2—C=) 3.60–4.40 (m, 4H, 2xCH_2—C=ring) 4.26 (q, 4H, 2xCH_2 ester) 5.20 (m, 2H, 2x=CH) 5.60 (br t, 1H, =CH ring)
III	75	1.4894	376 (6.6)	1.24 (t, 6H, 2xCH_3 ester) 1.70 (m, 9H, 3xCH_3—C=) 2.08 (m, 8H, 4xCH_2—C=) 2.36 (m, 4H, 2xCH_2—C=ring) 2.80 (m, 2H, 2xCH—CO ring) 4.14 (q, 4H, 2xCH_2 ester) 5.20 (m, 2H, 2x=CH) 5.40 (br t, 1H, =CH ring)
IV	70	1.5097	302 (2.3)	1.70 (m, 9H, 3xCH_3—C=) 2.08 (m, 8H, 4xCH_2—C=) 2.20–2.70 (m, 4H, 2xCH_2—C=ring) 3.36 (m, 2H, bridgehead) 5.16 (m, 2H, 2x=CH) 5.70 (br t, 1H =CH ring)
V	60	1.5012	374 (1.6)	1.32 (t, 6H, 2xCH_3 ester) 1.70 (m, 9H, 3xCH_3—C=) 2.08 (m, 8H, 4xCH_2—C=) 3.00 (m, 4H, 2x=C—CH_2—C=ring) 4.28 (q, 4H, 2xCH_2 ester) 5.20 (m, 2H, 2x=CH) 5.60 (br t, 1H, =CH ring)
VI	50	1.5033	260 (3.6)	1.70 (m, 9H, 3xCH_3—C=) 1.90–2.30 (m, 15H, 7xCH_2, CH) 5.16 (m, 2H, 2x=CH) 5.50 (br t, 1H, =CH ring) 9.84 (br s, 1H, CHO)
VII	50	1.4998	274 (3.0)	1.70 (m, 9H, 3xCH_3—C=) 2.00–2.80 (m, 15H, 7xCH_2, CH) 2.22 (s, 3H, CH_3—CO) 5.20 (m, 2H, 2x=CH) 5.50 (br t, 1H, =CH ring)

VIII	46	1.4800	600 (6.0)	0.91 (t, 6H, 2xCH_3 ester) 1.28 (m, 32H, 16xCH_2 ester) 1.70 (m, 9H, 3xCH_3—C=) 2.08 (m, 8H, 4xCH_2—C=) 2.50 (m, 4H, 2xCH_2—C ring) 3.06 (m, 2H, 2xCH ring) 4.27 (q, 4H, 2xCH_2—O ester) 5.20 (m, 2H, 2x=CH) 5.50 (br t, 1H, =CH ring)
IX	62	1.4877	598 (3.0)	0.92 (t, 6H, 2xCH_3 ester) 1.30 (m, 32H, 16xCH_2 ester) 1.70 (m, 9H, 3xCH_3—C=) 2.08 (m, 8H, 4xCH_2—C=) 3.00 (m, 4H, 2xCH_2—C=ring) 4.23 (q, 4H, 2xCH_2—O ester) 5.20 (m, 2H, 2x=CH) 5.53 (br t, 1H, =CH ring)
X	77	1.5022	306 (38.6)	1.30 (t, 3H, CH_3 ester) 1.70 (m, 9H, 3xCH_3—C=) 2.08 (m, 8H, 4xCH_2—C=) 3.40 (m, 2H, 3—CH_2 ring) 3.96 (br s, 2H, 6—CH_2 ring) 4.22 (q, 2H, CH_2—O ester) 4.30 (br t, 1H, —NH) 5.20 (m, 2H, 2x=CH) 5.60 (br t, 1H, =CH ring)
XI	77	1.5034	262 (100)	1.70 (m, 9H, 3xCH_3—C=) 2.08 (m, 8H, 4xCH_2—C=) 2.34 (s, 6H, 2xCH_3—N) 3.10 (m, 4H, 2xCH_2—C=ring) 5.20 (m, 2H, 2x=CH) 5.47 (br t, 1H, =CH ring)
XII	57	1.5123	228 (1.3)	1.70 (m, 9H, 3xCH_3—C=) 2.00–2.70 (m, 8H, 4xCH_2) 5.20 (m, 2H, 2x=CH) 7.27 (m, 5H, ring)

The residue (5.5 g) was chromatographed on Florisil (100–200 mesh, 60 g) eluting with hexane (100 ml) to yield benzylgeranyl (3.3 g, 57%).

Bioassays

Alarm response Test. Air (20 ml) saturated with test compound (10 mg) was blown over colonies of feeding *Myzus persicae* (Sulz.) (Dawson et al., 1982). The numbers of aphids that moved from the feeding sites in response to the pheromone were recorded 1 min after pheromone discharge had ceased.

For application to plants, compounds were emulsified with water using Ethylan BV, a nonionic surfactant, at 10% active ingredient (w/w).

Aphid Settling Test. The upper (adaxial) surfaces of large Chinese cabbage (*Brassica pekinensis* Rupr.) leaves were painted (*ca.* 0.01 ml/cm^2) with test compounds to one side of the midrib only. Groups of apterous aphids were confined on the leaves under glass Petri dish lids, positioned centrally so that aphids had approximately equal areas of treated and untreated leaf on which to settle. The tests were done in dim light with leaves orientated randomly. Settled aphids on each side of the midrib were counted after 24 hr: wandering aphids and those on the midrib were not counted.

Tests with plant viruses. Gibson et al. (1982a) give details of S (insecticide susceptible) and R1 and R2 (insecticide resistant) clones of *M. persicae*; of the nonpersistent potato virus Y (PVY) and the semipersistent beet yellows virus (BYV); and of the laboratory test for assessing effects of compounds on acquisition of each virus by apterae. By this method, aphids were confined on treated or control half-leaves for 4 hr for BYV acquisition and 2.5 min for PVY. In BYV tests, numbers of aphids settled and nymphs produced were recorded. To assess acquisition, aphids were transferred to indicator seedlings and numbers of plants infected by test and control aphids were compared.

RESULTS AND DISCUSSION

Sulfur dioxide reacts with many dienes to give sulfolenes (Elvidge and Smith, 1968), and Nesbitt et al. (1973) used the reversible nature of this reaction to purify lepidopteran sex pheromones with conjugated double bonds. Reaction between (*E*)-β-farnesene and sulfur dioxide was investigated first, and the sulfolene (I, Figure 1) was readily obtained as an odorless oil. Other 1,4-cycloaddition products (II–VII) were prepared using standard and relatively low-molecular-weight dienophiles. The much less volatile didecyloxycarbonyl compounds VIII (*M* 600) and IX (*M* 598), the higher homologs of the diethoxycarbonyl compounds III and V, were also prepared (Figure 1). All subsequent compounds were more stable than the sulfolene (I), as reflected in the intensities of the MS ion at *m/z* 204 [the molecular ion for

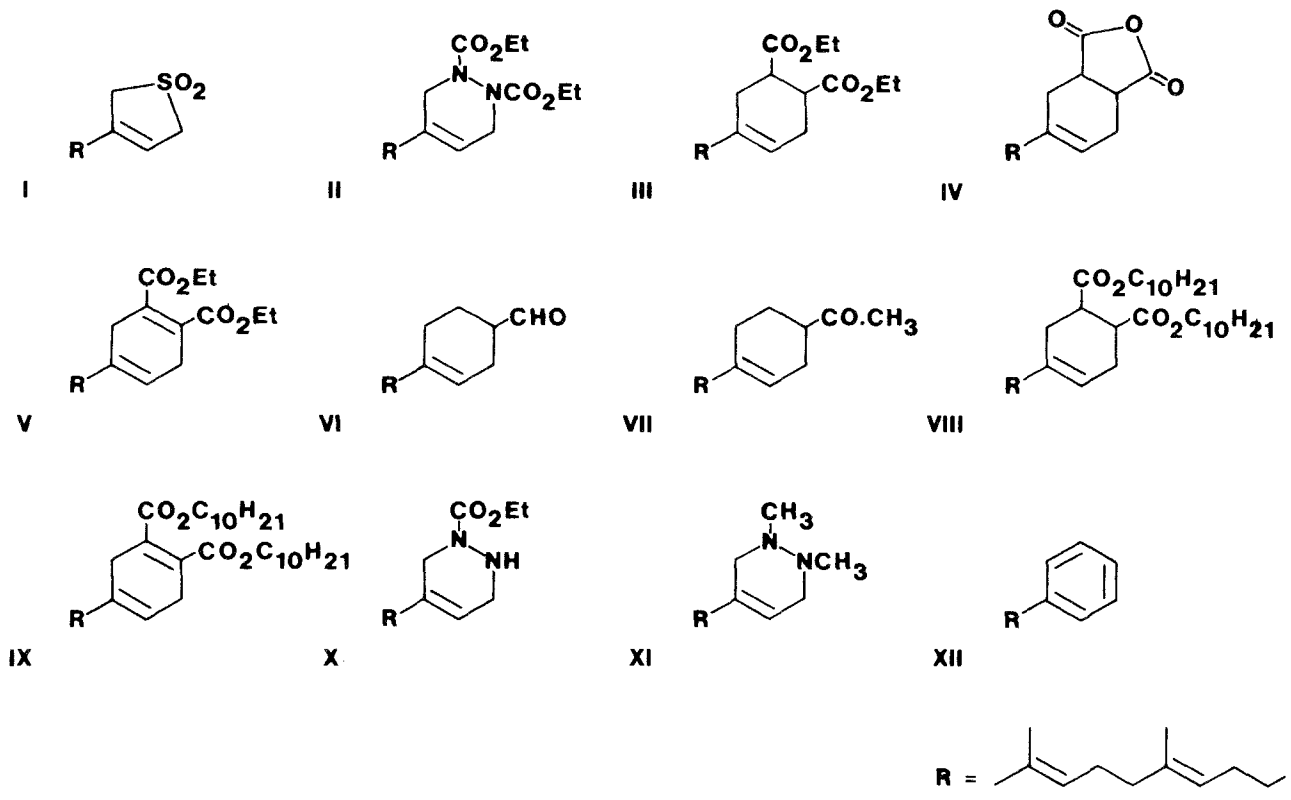

FIG. 1. Structure of 1,4-cycloaddition reaction products and related compounds.

(*E*)-β-farnesene] which were less than 1.0% of the base ion compared with 36.5% for the sulfolene (I). Attempts were made to convert the derivatives into compounds that decomposed to (*E*)-β-farnesene somewhat more easily. However, tetrahydropyridazine (XIII), which would have given the dihydropyridazine (XIV) by aerial oxidation and then (*E*)-β-farnesene, could not be prepared because on hydrolysis and decarboxylation either (*E*)-β-farnesene was formed immediately or under less hydrolytic conditions the *N*-monoethoxycarbonyl compound X (*m*/*z* 204, 7.9%) was the major product (Figure 2). By reduction of the *N*,*N*′-diethoxycarbonyl compound II, the *N*,*N*′-dimethyl compound XI was obtained which showed a greater tendency to give (*E*)-β-farnesene (*m*/*z* 204, 13.5%) than did II. Although the six-membered rings of 1,4-cycloaddition compounds can be oxidized to the aromatic system, benzylgeranyl (XII) was best obtained by formation of the side chain on an aromatic starting material.

All the new compounds described here (I–XII) were characterized by NMR and MS (Table 1). However, each contained different isomeric forms, and only structures for the major isomeric components are shown (Figure 1). Compounds I–XI each contained ca. 60% of the isomer with the designated (*E*) structure, ca. 30% of the corresponding (*Z*) isomer [arising from (*Z*)-β-farnesene in the starting material] and a small amount (ca. 10%) of isomers with terminal methylene groups. The aldehyde (VI), the methylcarbonyl compound (VII), and the *N*-monoethoxycarbonyl compound (X) may exist with substitution at the alternative position in the ring; structures most likely from mechanistic and spectral considerations are given. Benzylgeranyl (XII), although formed by a different route, also comprises a ca. 2:1 mixture of (*E*) and (*Z*) isomers.

Although sulfolene (I) could regenerate (*E*)-β-farnesene, it did not cause

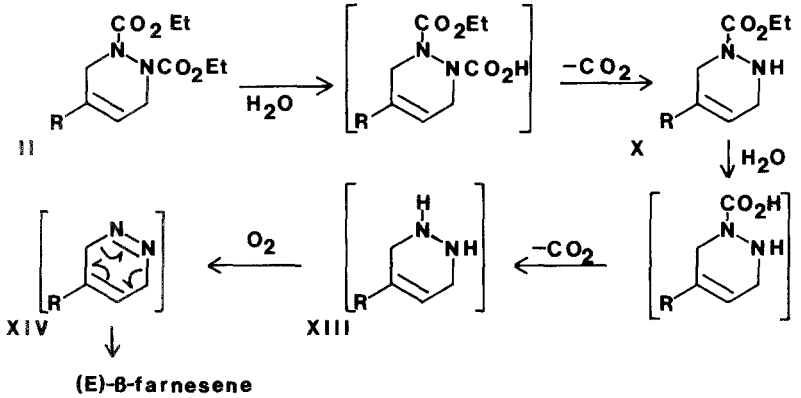

Fig. 2. Derivatives from the 1,4-cycloaddition reaction product, II.

TABLE 2. PERCENTAGE OF *Myzus persicae* SHOWING ALARM RESPONSE TO SYNTHETIC COMPOUNDS

Compound	Response (% ± standard error)
I	18 ± 9.6
(E)-β-Farnesene (thermal decomposition of I)	95 ± 1.8
(E)-β-Farnesene (Dawson *et al.*, 1981)	99 ± 0.6
XII	8 ± 6.8

settled aphids to move from feeding sites (Table 2). However, (*E*)-β-farnesene regenerated from sulfolene (I) by thermolysis caused an intense response similar to that from the original (*E*)-β-farnesene. As benzylgeranyl (XII) had a vapor pressure similar to that of (*E*)-β-farnesene, this was also tested for alarm activity but had none.

In the settling test (Table 3), the numbers of aphids on treated leaves were lowered by all compounds tested at 1% active ingredient except for (*E*)-β-farnesene where there was slight aggregation. Sulfolene (I) caused marked leaf damage, presumably by releasing sulfur dioxide. At 0.5% active ingredient sulfolene (I), the *N*,*N*′-diethoxycarbonyl compound (II), the diethoxycarbonyl compound (V), and benzylgeranyl (XII) did not decrease settling; at 0.1% only the high-molecular-weight didecyloxycarbonyl compound (IX) gave a highly significant effect. Thus it appeared that, although the pheromone derivatives decreased aphid settling, there was no correlation between release of (*E*)-β-farnesene and effectiveness. A similar pattern of activity was observed when selected compounds were tested in virus transmission bioassays (Tables 4 and 5). In addition, aphids resistant to organophosphorus insecticides showed no cross-resistance to compounds in the tests. When plants were treated with test compound and a period was allowed to elapse before applying aphids, the higher-molecular-weight compounds VIII and IX generally gave good response (Tables 3–5) whereas the lower-molecular-weight compounds often performed poorly. Volatility therefore appears to be a significant factor in determining the practical performance of this series of compounds. Summarizing all results in Tables 3–5, compound IX, the 1,4-cycloaddition product from (*E*)-β-farnesene and didecyl acetylenedicarboxylate, appeared the most active. This compound persisted for at least 1 week and showed no phytotoxicity.

The structural requirements for alarm activity as determined by Bowers et al. (1977) are present in the compounds tested here, but their effect is to interfere with settling and virus transmission rather than to disturb settled aphids. The nature of the association between behavioral response to (*E*)-β-

TABLE 3. MEAN NUMBERS OF *Myzus persicae* SETTLING ON *Brassica pekinensis* LEAVES TREATED WITH ALARM PHEROMONE DERIVATIVES

Compound	Number of aphids settled		Statistical significance of differences (P)
	control	treated	
1%			
I	15.5	2.5	<0.001
II	11.4	3.6	<0.001
III	11.3	1.8	<0.01
IV	13.4	1.6	<0.001
V	14.5	3.9	<0.001
VI	9.6	5.6	<0.01
VII	14.4	1.9	<0.001
VIII	16.1	2.6	<0.001
IX	14.1	3.0	<0.001
X	15.9	2.8	<0.001
XI	9.4	2.3	<0.01
XII	13.2	5.0	<0.01
(*E*)-β-Farnesene	7.0	10.2	<0.05 !
0.5%			
I	9.7	8.3	None
II	9.8	8.6	None
III	12.7	6.0	<0.01
IV	13.6	3.3	<0.001
V	8.3	9.0	None
VI	10.4	5.1	<0.01
VII	13.2	4.2	<0.01
VIII	12.2	5.4	<0.01
IX	13.4	3.8	<0.001
X	10.7	6.5	<0.05
XI	12.2	4.6	<0.01
XII	9.3	9.4	None
0.1%			
III	9.6	8.2	None
IV	9.4	8.4	None
V	9.1	9.4	None
VIII	9.6	6.8	None
IX	13.0	6.0	<0.001
1.0% on plant 3 days before test			
III	11.0	6.7	<0.05
V	12.5	5.2	<0.01
VIII	14.9	3.2	<0.001
IX	11.2	2.6	<0.001

TABLE 4. SETTLING, NYMPH PRODUCTION, AND ACQUISITION OF BVY BY *Myzus persicae* RESISTANT (R1) STRAIN FROM LEAVES TREATED WITH ALARM PHEROMONE DERIVATIVES

Compound	Difference from control (%)		
	Settling	Nymph production	Virus-infected plants
1%			
I	−68	−89	−75
II	−23	−81	−58
III	−10	−33	−22
IV	−38	−73	−32
V	−15	−45	−10
X	−31	−62	−71
1% on plant 24 hr before test			
II	−22	−82	−6
III	−29	−76	−23
IV	−2	−36	+12
V	−53	−68	−52
VI	−11	−51	−2
VIII	−41	−64	−26
IX	−47	−96	−60
X	−2	−23	−4
XI	leaves destroyed by test compound		
XII	+2	−11	−2

TABLE 5. ACQUISITION OF PVY BY *Myzus persicae* SUSCEPTIBLE (S) AND RESISTANT (R1 AND R2) STRAINS FROM LEAVES TREATED WITH ALARM PHEROMONE DERIVATIVES

Compound	Virus infection, % difference from control (formulation only)			Statistical significance of difference for combined result (P)
	S	R1	R2	
1%				
IV	−57	−77	−96	<0.001
V	−20	−65	−42	<0.001
IX	−100	−97	−96	<0.001
XI	−56	−75	−68	<0.001
IX on plant for 7 days before test	−95	−92	−78	<0.001
Tests run concurrently				
IX (1%)	−95	−93	−92	<0.001
IX (0.1%)	−39	−42	−19	<0.05
IX (0.01%)	+12	+12	+13	None

farnesene derivatives and virus transmission is unclear, but other compounds have similar effects. For example, polygodial inhibits settling, nymph production, and virus transmission (Gibson et al., 1982b). However, dodecanoic acid, while also inhibiting settling (Greenway et al., 1978; Sherwood et al. 1981) and nymph production, actually enhances acquisition of PVY (Gibson et al., 1982b). In contrast, mineral oils appear to have no behavioral effect but can diminish virus transmission (Bradley et al., 1962; Wijs, 1980), perhaps by chemical rather than physical mechanisms.

The discovery of compounds that inhibit aphid settling and virus transmission even by pesticide-resistant aphids and show reasonable persistence in laboratory tests is a promising lead for development of a new type of crop-protection agent. Further compounds related to the (*E*)-β-farnesene derivatives will be assayed for this form of activity in parallel with studies into mode of action and use in field crops.

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A COMPARATIVE STUDY OF THE EXOCRINE PRODUCTS OF CLEPTOPARASITIC BEES (*Holcopasites*) AND THEIR HOSTS (*Calliopsis*) (HYMENOPTERA: ANTHOPHORIDAE, ANDRENIDAE)

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Abstract—The cephalic secretion of females of the cleptoparasitic bee *Holcopasites calliopsidis* contains two main volatiles, 6-methyl-5-hepten-2-one and geranyl acetone. The mandibular gland secretion of its host, *Calliopsis andreniformis*, on the other hand, contains the two isomeric forms of citral (neral and geranial). Neral and geranial are also produced by mandibular glands of two other species of *Calliopsis* and one species in the related genus *Nomadopsis*. The Dufour's gland of *C. andreniformis* contains an oily secretion composed of hydrocarbons that are deposited on the brood cell walls and pollen balls providing a waterproof lining for both.

Key Words—*Holcopasites*, *Calliopsis*, *Nomadopsis*, Hymenoptera, Anthophoridae, Andrenidae, cleptoparasite, mandibular glands, Dufour's gland, cell lining, 6-methyl-5-hepten-2-one, geranyl acetone, hydrocarbons, neral, geranial.

INTRODUCTION

The genus *Holcopasites* contains 15 species of small (2.5–8 mm) wasplike bees with black heads and thoraces, red abdomens, and patches of white

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pubescence. Species of this genus are cleptoparasites parasitizing panurgine bees in the genera *Calliopsis*, *Hypomacrotera*, and *Pseudopanurgus* (Hurd and Linsley, 1972). As is typical of nomadine bees, the hosts are not closely related to the cleptoparasites, so the latter could not have evolved from their host genera or tribes.

Holcopasites calliopsidis occurs throughout most of the United States; the more northern nominate subspecies are known to parasitize *Calliopsis andreniformis*. While each soil nest of *C. andreniformis* is occupied by a single female, the nests are typically aggregated in level or slightly sloping areas of bare or sparsely vegetated soil. Both males and females overwinter, and mating typically occurs at nest sites where the males patrol and defend territories. Nests consist of simple burrows with cells constructed at the end of lateral tunnels that are filled with soil after oviposition. The cells are lined with a glandular waxlike secretion that is soluble in ethyl ether, presumably originating from the well developed Dufour's gland (Shinn, 1967).

Female *Calliopsis*, when roughly handled, secrete a liquid from their mouthparts that smells like lemongrass. The soil mound (tumulus) surrounding the nest entrance also has this odor. Shinn (1967) speculated that this nest odor influences the bees to build their nests in aggregations and may help a bee locate its own nest, although his experiments in transferring tumuli were inconclusive.

Holcopasites calliopsidis is frequently encountered at nest sites of *C. andreniformis*, as reported by Ainslie (1937) and Shinn (1967) and as observed in Ithaca, where all but one of the aggregations of the host bee also contain *Holcopasites*. Adult males and females emerge at the same time as or shortly after the emergence of their hosts, and mating occurs at the nest site. Female cleptoparasites spend much time walking or flying around the host nest site, stopping frequently to examine tumuli and potential nest entrances. *Holcopasites* dig through the soil plugs to enter host burrows and may remain inside for up to half an hour. The cleptoparasites seem to have little regard for female *Calliopsis* and are not chased by them, even when the host female enters a burrow in which a *Holcopasites* is already inside. Male *Calliopsis*, however, will chase *Holcopasites* (Shinn, 1967).

Chemical mediation of some behavioral interactions between andrenid hosts and nomadine cleptoparasites has been demonstrated for *Andrena* and *Nomada* by Tengö and Bergström (1976a, 1977). In this paper we describe the chemistry of exocrine gland secretions of female *H. calliopsidis* and of both sexes of three species of *Calliqpsis*, and we discuss the possible behavioral roles of these chemicals.

METHODS AND MATERIALS

Insects. Adult *Holcopasites* were netted at a *C. andreniformis* nesting aggregation in Ithaca, New York, and brought live to the laboratory where

they were briefly examined under a stereomicroscope to determine species and sex. The bees were then chilled on ice and the heads of the females removed and extracted in methylene chloride. Males and females *C. andreniformis* were also collected at Ithaca and in Kansas. Heads of males and females were extracted in methylene chloride, while Dufour's glands were dissected out of chilled females and extracted in the same solvent. In addition methylene chloride extracts of *C. coloradensis* and *C. nebraskensis* collected near Logan, Utah, and excised mandibular glands of *Nomadopsis scutelaris* (a species in a genus closely related to *Calliopsis*) were analyzed. Analysis of dissected mandibular glands of *Calliopsis* species demonstrated that these organs are the source of the secretion.

Chemical Analyses. The *Calliopsis* and *Nomadopsis* extracts were initially analyzed on a Microtek gas chromatograph operated isothermally at 125° C utilizing a 1.8-m column of 10% Carbowax 20 M. Final identifications were achieved by analyses on an LKB 9000 combined gas chromatograph-mass spectrometer with a 1.8-m SE-30 column. Analyses of the Dufour's gland secretion of *C. andreniformis* and of the cephalic secretions of *C. andreniformis* and *H. calliopsidis* were performed on an LKB 2091 combined GC-MS utilzing a SE-30 capillary column. In all cases, identities of the compounds were confirmed by comparing their mass spectra and isothermal retention times with those of authentic standards. Comparative analyses of Dufour's glands, pollen balls, and cell linings of *C. andreniformis* were performed on a Ribermag R-10-10 combined GC-MS fitted with a Girdel GC and a Ros splitless injector (needle type), using a 30-m, wide-bore, fused-silica glass SE-30 capillary column (J & W Scientific). Injection was followed by a 2-min hold, a 150–300° C temperature program at 5°/min, and a 10-min isothermal hold. Spectra were collected with a SADR GC-MS data system. Retention times reproducibility between runs for the same chemical was ± 0.5% (<1° C, <10 sec).

Behavioral observations. In June 1980, two set of three small velvet squares, each treated with pentane solutions containing citral, geranyl acetone, or just solvent were set out in a nest aggregation of *C. andreniformis*, following the method of Cane and Tengö (1981). Each set of three squares was observed simultaneously by two observers who recorded the numbers of hosts and cleptoparasites approaching and landing on the square.

RESULTS

The cephalic secretion of females of *H. calliopsidis* contains two main volatile constitutents (Figure 1). The first compound, identified as 6-methyl-5-hepten-2-one by its mass spectrum, elutes at 140° C, and its identity was confirmed by comparing its mass spectrum and retention time to those of an authentic standard. The second component (peak 2 in Figure 1) is less volatile, elutes at 200° C, and also exhibits a mass spectrum characteristic of a ketone

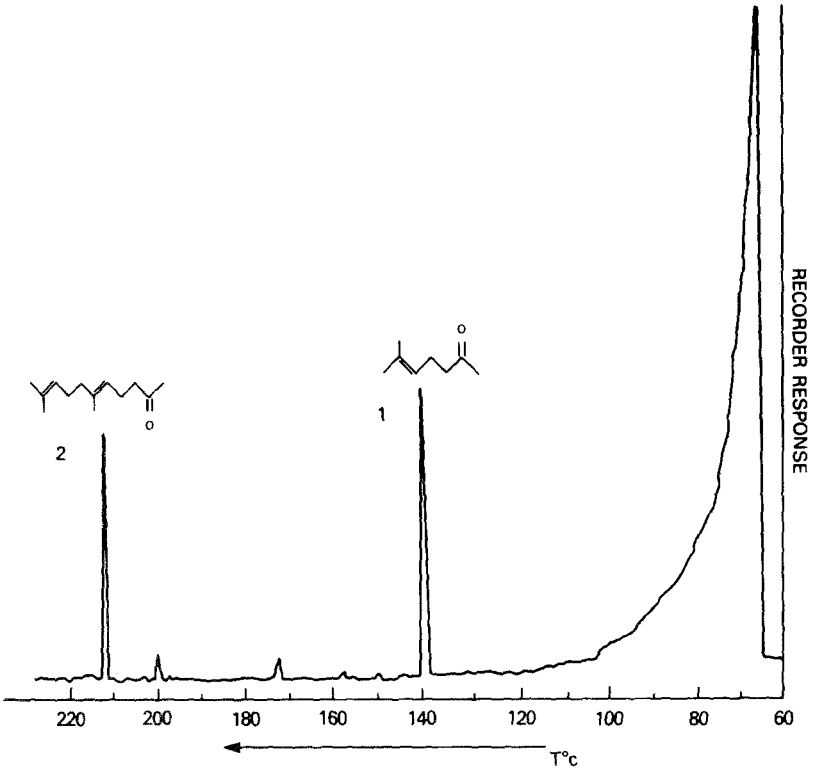

FIG. 1. Gas chromatogram of the cephalic secretion of females of *H. calliopsidis*. The major components were identified as (1) 6-methyl-5-hepten-2-one and (2) geranyl acetone. Samples were run on a 50-m 3% SE-30 capillary column programed from 60°C to 220°C at 10°C/min.

(Figure 2). It has a molecular ion at m/z 194 and a base peak at m/z 43, probably due to the ion CH_3CO^+. The second most intense ion at m/z 69 can be attributed to an isoprene fragment ($C_5H_9^+$), and the ion at m/z 125 corresponds to the loss of isoprene group (M-69). The loss of acetone (M-58) is responsible for the ion at m/z 136 and is also indicative of a ketone, suggesting that the compound is a terpenoid ketone. The compound was identified as (*E*)-6,10-dimethyl-5,9-undecadien-2-one, commonly called geranyl acetone. This identification was corroborated by comparing the cephalic secretion of the bee with an authentic sample of geranyl acetone (K & K Laboratories).

The isomers of citral, on the other hand, are the dominant terpenes in the head extract of females of the host bee, *C. andreniformis*. Both isomers of citral also occurred in *C. coloradensis* and *C. nebraskensis*. Analyses of

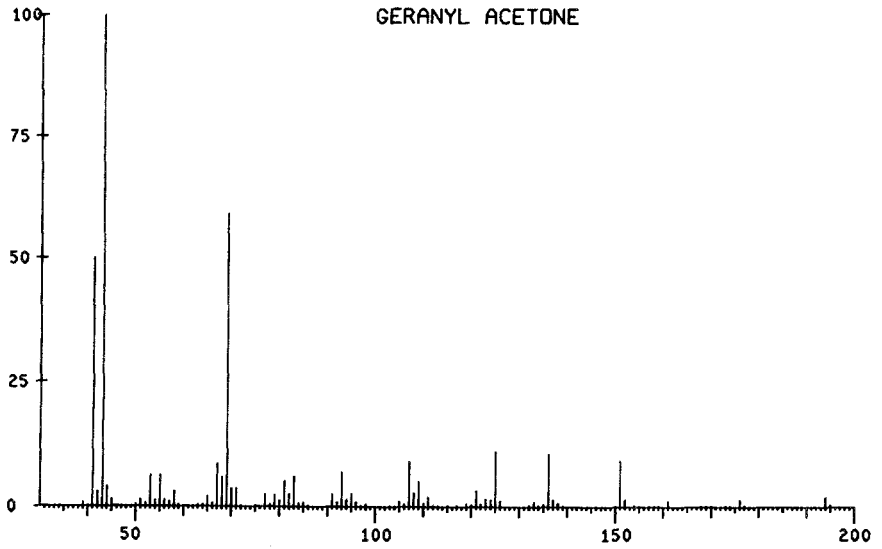

FIG. 2. Mass spectrum of geranyl acetone.

dissected mandibular glands demonstrated that these organs were the source of citral in the cephalic extracts of *Calliopsis* species. In all the *Calliopsis* species investigated, the males possessed only trace amounts of mandibular gland volatiles in comparison with females. The possible presence of trace amounts of citral in the mandibular glands of *Calliopsis* males was suggested by GC retention times but not by GC-MS, and the evidence for its presence is at best suggestive. The isomers of citral are also present in the mandibular glands of females.

The oily Dufour's gland secretion of *C. andreniformis* comprises homologous series of hydrocarbons, the major components of which were also detected in the pollen-ball and cell-lining extracts (Table 1). The alkanes and alkenes were checked where possible with Stenhagen's Registry of Mass Spectra, which they matched peak for peak without exception. Their relative retention times within homologous series were as predicted. The methyl groups were deduced from the parent ion masses and the usual odd number of carbons found in insect systems. Their spectra were virtually indistinguishable from straight-chain alkanes, as predicted for these molecular sizes. The alkadienes were determined from parent ion masses and the likelihood of an alkadiene as compared to an alkyne. The alkadiene spectra were otherwise identical with the alkene spectra.

The velvet squares bearing citral and geranyl acetone did not elicit different frequencies of approach or pouncing from flying *C. andreniformis*

TABLE 1. CHEMICAL DETECTED IN DUFOUR'S GLAND SECRETION, CELL LININGS, AND POLLEN-BALL EXTRACTS OF *Calliopsis andreniformis*[a]

Chemical	Relative abundance		Presence in pollen balls
	In Dufour's gland	In cell linings	
Tricosane	7	5	+
Pentacosene	6	9	+
Pentacosane	5	1	+
Methylpentacosane	10	9	−
Heptacosene	3	8	+
Heptacosane	4	2	+
Methylheptacosene	9	—	−
Methylheptacosane	9	9	−
Nonacosadiene	9	—	−
Nonacosene	1	4	+
Nonacosane	2	4	+
Methylnonacosene	8	—	−
Hentriacontadiene	2	8	+
Hentriacontene	2	3	+
Hentriacontane	2	6	+
Methylhentriacontane	8	—	−
Tritriacontadiene	2	8	−
Tritriacontene	1	8	−
Tritriacontane	2	7	−
Pentatriacontadiene	10	—	−
Pentatriacontene	8	—	−
Pentatriacontane	9	—	−

[a]For experimental methods see text. Relative abundance was calculated from integrated peak areas and are ranked 1 = most; 10 = least.

and *H. calliopsidis* of either sex when compared to the control squares. Under our rather artificial experimental conditions of unblended, high-concentration chemicals, we found no evidence that these chemicals function as attractant pheromones for either nesting host females or their cleptoparasites.

DISCUSSION

The cephalic secretions of the cleptoparasite *Holcopasites calliopsidis* and of species in its host genus *Calliopsis* and the related genus *Nomadopsis* are sources of terpenoid compounds. While in females of *Calliopsis* and *Nomadopsis* both isomers of citral, (neral and geranial) predominate, the secretion of the cuckoo bee contains two compounds, 6-methyl-5-hepten-2-one and geranyl acetone, in approximately equal amounts. Both citral and

6-methyl-5-hepten-2-one are relatively common exocrine products of hymenopterous insects. Citral is present in ants (Blum and Hermann, 1978, and references therein), stingless bees (Blum, 1966; Blum et al., 1970), honeybees (Shearer and Boch, 1966), solitary bees of the colletid genera *Hylaeus* (Blum and Bohart, 1972; Bergström and Tengö, 1973; Duffield et al., 1980), and *Colletes* (Bergström and Tengö, 1978; Hefetz et al., 1979a). In *Colletes* it is accompanied by linalool and this blend promotes aggregation behavior.

6-Methyl-5-hepten-2-one is also produced by a wide variety of ants (Blum and Hermann, 1978), for which it mainly functions as an alarm pheromone. This ketone is also a mandibular gland product of several species of *Andrena* bees (Tengö and Bergström, 1976a) and is accompanied by citral.

In *H. calliopsidis* 6-methyl-5-hepten-2-one is accompanied by geranyl acetone, a compound that has not been previously identified in arthropod secretions. Interestingly, these two compounds were isolated, among other volatiles, from the urine of the red fox, *Vulpus vulpus*, where they are believed to possess a communicative function (Jorgensen et al., 1978).

In preliminary behavioral studies we were not able to support Shinn's (1967) hypothesis that the mandibular gland secretion is deposited in the nest tumulus and serves a communicative function, perhaps inducing other bees to nest nearby. We also found no evidence that the cleptoparasitic bee *H. calliopsidis* used host mandibular gland secretions as kairomones to locate host nests. However, our experiments are not definitive because we tested only synthetic citral, not the actual secretions. Citral may also have a defensive function for *Calliopsis* and *Nomadopsis*, as it is released when the bees are roughly handled.

The lack of aggressive response of female *Calliopsis* to *Holcopasites*, even when encountered in a nest, may have a chemical basis. A female *Holcopasites* might deter host aggression by releasing mandibular gland allomones. Velvet ants' unrelated nest parasites produce several ketones in their mandibular glands, and an allomonal function has been hypothesized for them (Schmidt and Blum, 1977, Fales et al., 1980).

Tengö and Bergström (1976a,b, 1977) have shown striking similarities between male cephalic secretions of some species of *Nomada* and Dufour's gland secretions of their host species of *Andrena* and *Melitta*. We did not have male *H. calliopsidis* available for chemical analysis, so we could not determine if a similar correspondence occurs between the species in our study.

The Dufour's glands extract of *C. andreniformis* is composed of various hydrocarbons. As seen in Table 1 the major constituents are also found in the brood cell lining and the pollen ball, providing a waterproof lining for both. In this respect the Dufour's gland function is similar to that of *Colletes thoracicus* (Hefetz et al., 1979b) or *Anthophora abrupta* (Norden et al., 1980). In contrast to the above-mentioned cases, in *C. andreniformis* the chemicals

are not modified after deposition, exhibiting a third method for achieving waterproof shield for the brood and its provision.

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FRONTALIN IN THE MALE MOUNTAIN PINE BEETLE[1]

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Abstract—Frontalin and *exo*-brevicomin were identified by GC-MS in air drawn over male *Dendroctonus ponderosae* Hopk. (MPB) from Oregon that had joined females for 1–2 days in the bark of lodgepole and ponderosa pine logs. Unfed males released *exo*- and *endo*-brevicomin but not frontalin. These three compounds were not detected in either unfed or fed females. Arrestment of males by *trans*-verbenol and terpenes in an olfactory walkway was reduced by the addition of racemic frontalin; production of attractant chirps also diminished. Racemic frontalin also strongly reduced the aggregation of MPB in lodgepole and ponderosa pine stands to sticky traps baited with the aggregation pheromone *trans*-verbenol and host terpenes; however, the function of the natural enantiomer of frontalin in MPB is unknown.

Key Words—Coleoptera, Scolytidae, *Dendroctonus ponderosae*, pheromone, frontalin, *exo*-brevicomin, *Pinus contorta*, *Pinus ponderosa*.

INTRODUCTION

The pheromone frontalin, 1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane, was first isolated and identified from male western pine beetles, *Dendroctonus brevicomis* LeC. (Coleoptera: Scolytidae) (Kinzer et al., 1969) and shown to function as an aggregation pheromone (Vité and Pitman, 1969; Bedard et al., 1970). Frontalin was also shown to function as an aggregation pheromone for *D. frontalis* Zimm. (Kinzer et al., 1969; Renwick and Vité, 1969) and *D. pseudotsugae* Hopkins (Pitman and Vite, 1970). The mountain pine beetle, *D.*

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ponderosae Hopkins (referred to here as MPB), aggregates in response to another pheromone, *trans*-verbenol (Pitman et al., 1968; Pitman and Vité, 1969). The antiaggregation pheromone 3-methycyclohex-2-en-1-one is released by *D. pseudotsugae* when the male and female pair (Furniss et al., 1972; Rudinsky et al., 1972). Pheromones that interrupt aggregation are also known for other species of *Dendroctonus*, e.g., verbenone and *trans*-verbenol for *D. brevicomis* (Bedard et al., 1980a,b), *endo*-brevicomin and verbenone for *D. frontalis* (Vité and Renwick, 1971; Payne et al., 1978), and *exo*- and *endo*-brevicomin for MPB (Rudinsky et al., 1974; Pitman et al., 1978; Ryker and Rudinsky, 1982). Rudinsky et al.(1974) identified frontalin from vapors of unfed pairs of MPB but did not determine which sex produced it nor did they bioassay it for function. However, in another study, frontalin was not detected from extractions of hindguts of either sex of unfed and fed MPB (Pitman et al., 1969). The purpose of the present study was to determine the presence or absence of frontalin in air passed over male and female MPB that emerged from infested lodgepole pine, *Pinus contorta* Dougl. var. *murrayana*, and from ponderosa pine, *P. ponderosa* Dougl. *ex*. Laws., both with and without prior feeding in fresh bolts cut from the same hosts. Behavioral response of the beetles to racemic frontalin was tested in both the laboratory and field.

METHODS AND MATERIALS

Collection and Identification of Pheromones. Volatile compounds released by MPB confined in two's (Rudinsky et al., 1974) in small, open-ended screened glass vials were collected onto Porapak-Q® (Rudinsky et al., 1973). Preparation, elution, and analysis of samples by coupled GC-MS was described previously (Ryker et al., 1979), except that the sonic transducer closing the collection chamber was replaced by a glass plate. Volatile substances were trapped for 5 hr from 20–50 beetles/sample. Beetles emerging from lodgepole and ponderosa pine were sampled separately. Two samples each of both males and females unfed, fed 24 hr, and fed 48 hr were collected from beetles from each host pine. Beetles were fed only in the host species from which they emerged. For feeding, females were introduced into holes drilled into the bark of freshly cut bolts paraffined on the ends. Males to be fed were each placed in a gallery with a female already established for one day. The males of this species typically mate and begin moving frass within 30 min of introduction (Ryker and Rudinsky, 1976). However, male feeding was not verified in this study. One or two days after introduction, the bark was stripped from the log and the males or females were recovered for pheromone trapping. Mated females were not tested. Areas of GC peaks of frontalin, *exo*-, and *endo*-brevicomin were measured, and the quantities of pheromones released per male were estimated by comparison with measured amounts of

ethyl ester standards, particularly ethyl pentanoate, injected onto the Porapak-Q just before elution of each sample into the GC column. Peak areas of marker solutions of ethyl esters processed through the Porapak-Q system were essentially the same (±3%) as areas for the same quantities of frontalin and *exo*-brevicomin (Ryker et al., 1979).

Laboratory Bioassay. A laboratory bioassay of walking male beetles was used to screen frontalin as a candidate pheromone of MPB prior to the onset of the flight period in July. Olfactory behavior of walking beetles is useful because it can indicate the volatile compounds for which an insect has sensory receptors. However, a positive walking response to a compound does not indicate that it is an aggregation pheromone nor does a negative response indicate antiaggregative function, although walking and flying responses have been correlated in many species of bark beetles (Borden et al., 1975).

Walking male MPB were tested for their response to a control attractant mixture of 0.1% *trans*-verbenol, 0.5% (+)-alpha-pinene, and 0.5% myrcene vs. the same mixture plus 0.001%, 0.01%, and 0.1% frontalin. The compounds were diluted in dibutyl phthalate (Rudinsky and Ryker, 1980). The attractant solution was selected on the basis of preliminary tests in which these concentrations elicited the greatest arrestment. The two terpenes were selected on the basis of known attractancy (Billings et al., 1976; McKnight, 1979; Pitman, 1971). The olfactory walkway simulated the male approach to a natural female gallery except that the odorous stimuli came from a chemical solution rather than female frass (Jantz and Rudinsky, 1965). Male beetles walked toward a light source up an alcohol-extracted, dried piece of white pine bark held at a 45° slope. Two Plexiglas railings guided the male over a hole drilled in the bark. The hole was screened underneath to prevent the beetle from dropping into the vial containing 130 mg of test solution. This walkway was modified from that used with the Douglas-fir beetle (Rudinsky and Ryker, 1977) as follows. The walkway was inclined to 45° because this species tends to climb upward and would not stay in a horizontal walkway. Bark was used as the walking surface instead of Plexiglass, which was too slippery at this slope. Also, the hole was provided in the bark because most males passed over a flat screen above the test vial without pausing. Beetles were scored positive if they were arrested at the hole more than 20 sec, either with their head down in the hole or walking around and turning back toward the hole, and if they produced an interrupted chirp typical of naturally attracted males (Ryker and Rudinsky, 1976). Tests were run at 22–25° C and 30–40% relative humidity in a room darkened except for a microscope light source beyond the end of the walkway. Beetles were warmed to room temperature several minutes before testing. Instrumentation used to monitor beetle sound production was described previously (Rudinsky and Ryker, 1976). Three samples of 20 males were observed for each treatment.

Attractant control tests were run as every fourth test, but only the first three control samples were used for statistical comparison. Two-way ANOVA and Scheffé's test were used for comparisons of means (Snedecor and Cochran, 1967). Beetles tested emerged from infested logs in a greenhouse usually 2–4 days and no more than 7 days prior to bioassay. They were stored at 3° C in moist paper toweling until use. Male sex was determined by the presence of a stridulatory plectrum on the 7th abdominal tergite (Michael and Rudinsky, 1972).

Field Bioassay. Frontalin was tested with the attractant, *trans*-verbenol, (+)-alpha-pinene, and myrcene, using sticky traps (Ryker and Rudinsky, 1982). The assays were in stands of mature lodgepole pine near Paulina Peak and ponderosa pine averaging 60 years old on Lookout Mountain, Deschutes National Forest near LaPine, Oregon, from July 5 to August 5, 1981.

Test compounds were evaporated individually from open 2-ml glass vials 1 cm diameter × 3 cm deep with a 4.5-mm opening (0.5 dram) placed inside perforated aluminum film cans (Furniss and Schmitz, 1971). Evaporation rates of compounds were estimated by monitoring the loss of measured quantities of each compound in the field (Table 3, footnote *a*). An evaporation rate for frontalin of 8 mg/day was selected because this concentration was known to attract flying *D. brevicomis* (Ryker and Rudinsky, unpublished data) and *D. pseudotsugae* (Rudinsky et al., 1972; Rudinsky and Ryker, 1980) to traps. This evaporation rate also seemed reasonable because the quantities of frontalin released by fed male MPB (see Table 1) and fed female *D. pseudotsugae* (Ryker et al., 1979) were similar. Odor dispensers were wired to the northwest edge of the north facing of two 0.25 × 1-m sticky screens made from 6.4 mm (0.25-in.) mesh hardware cloth coated with Stikem Special® (after Bedard et al., 1969) and stapled at breast height to cardboard cylinders 32 cm in diameter and 2.44 m tall. The cylinders were painted dark brown to mimic the visual silhouettes of tree boles. Beetles collected from the north- and south-facing screens on each cylinder were combined each day as a single sample in the lodgepole stand. Beetles caught per cylinder were combined over two days as a single sample in the ponderosa pine stand, as the beetle population there was sparse and trap catches were small. MPB were collected from traps, sexed, and counted daily.

Two sets of traps were placed in rows 20 m apart in both stands. One trap for each treatment (see Table 3) in each set was provided with test compounds each day in lodgepole or every two days in ponderosa pine stands. The test was run for 18 days (lodgepole) and 36 days (ponderosa). Test compounds were reused on different traps for several time intervals and replenished weekly. Treatments were assigned at these time intervals to particular traps by a random numbers table. Traps were spaced at least 20 m apart at least 3 m from the nearest unattacked tree, and care was taken to avoid infested trees,

although there were infested trees between the rows of traps in one of the two sets in the lodgepole pine stand.

Treatments were compared using a nonparametric test, Wilcoxon's matched-pairs, signed-ranks test, due to high variance in control trap catches on different days. We tested the hypotheses that: (1) the catch at the attractant + frontalin was equal to the catch at the attractant alone against the (one-sided) alternative that the catch at the attractant + frontalin was less than the catch at the attractant alone (interruption); and (2) the catch at the terpenes + frontalin was equal to the catch at the terpene control against the (one-sided) alternative that catch at the terpenes + frontalin was greater than the catch at the control (attraction).

Racemic frontalin utilized in laboratory and field tests was of 99% purity and racemic *trans*-verbenol of 99.7% purity; both were obtained from Albany International, Columbus, Ohio. Terpenes used were a 1:1 mixture of 95% (+)-alpha-pinene (optical purity unknown) from K & K Laboratories, Inc., Plainview, New York, and 90% myrcene donated by SCM Terpene Products Group, Jacksonville, Florida. The impurities in mycrene were monoterpene hydrocarbons.

RESULTS AND DISCUSSION

No pheromones were identified from air passed over unfed female MPB. After feeding for one or two days, females from both lodgepole and ponderosa pine released numerous volatile terpenes, including *trans*-verbenol, but frontalin, *exo*-, and *endo*-brevicomin were not detected. Pairs of unfed males released *exo*- and *endo*-brevicomin but no detectable amount of frontalin (Table 1). Males that had occupied galleries of females for one or two days produced substantial amounts of frontalin (Figure 1), which were in every case more than the *exo*-brevicomin produced (Table 1).

These data are in disagreement with Rudinsky et al. (1974), who identified frontalin from unfed males and females of MPB confined together in glass vials. Rudinsky et al. (1974) used more beetles (70 pairs) and trapped from them for 35 hr, as compared to our 5-hr samples. Amounts of pheromones produced by MPB vary from sample to sample and may be strongly influenced by the condition of the host tree. We feel fairly certain that male MPB from Oregon have little or no frontalin before joining the female in her gallery. The data also disagree with the study of Pitman et al. (1969), in which frontalin was not detected from hindguts of fed and unfed male and female MPB. Male *Dendroctonus* introduced into the bark of cut logs do not remain in the phloem unless a female is present. They usually bore under the retaining screen at the bark surface, and many escape (unpublished data). In the study by Pitman et al., male MPB were introduced into logs without

TABLE 1. PHEROMONE RELEASE BY MALE *Dendroctonus ponderosae* CONFINED IN VIALS, EITHER UNFED OR FOLLOWING PAIRING WITH FEMALES IN FRESHLY CUT LOGS FOR 1–2 DAYS (ANALYSIS BY GM-MS)

Treatment		GC peak area estimate (ng/male)[a]		
Host tree[b]	Hours in host	Frontalin	*exo*-Brevicomin	*endo*-Brevicomin
Lodgepole	0	ND	2.52	0.20
	0	ND	4.12	ND
Ponderosa	0	ND	4.85	0.50
	0	ND	1.67	ND
Lodgepole	24	9.44	1.35	ND
	24	7.48	2.14	ND
Ponderosa	24	3.40	1.04	ND
	24	6.31	1.02	ND
Lodgepole	48	10.02	1.50	ND
	48	11.70	1.01	trace
Ponderosa	48	1.55	ND	ND
	48	6.34	5.79	ND

[a]Peak areas compared to 60 ng peak of ethyl pentanoate injected onto the Porapak-Q before elution of the beetle compounds. ND = not detected.
[b]Beetles emerged from a host pine were fed in bolts from the same species. Data are presented from two samples for each treatment.

females (personal communication from G.B. Pitman). The design of the present study did not allow us to determine whether the production of frontalin by males depends on their being in the terpene-rich air of the female gallery, feeding, courting, or mating with a female, or some combination of factors.

exo-Brevicomin was identified from male MPB both before and after joining the female in the bark phloem, but was not detected in females (Table 1). Pitman et al. (1969) found *exo*-brevicomin not only in "fed" and unfed males, but in fed females as well. This difference may be due to variation in populations of MPB.

endo-Brevicomin was released by male MPB in small amounts, usually less than 10% of the quantity of *exo*-brevicomin released (Table 1) (Figure 1 of Rudinsky et al., 1974). In the present study, the quantities of *endo*-brevicomin found were at the limit of resolution of our GC-MS system (about 1 ng), and smaller amounts may have been present but undetected in the "fed" males (Table 1).

Quantities of pheromones estimated by collections from confined males do not relate directly to the quantities released by males interacting with

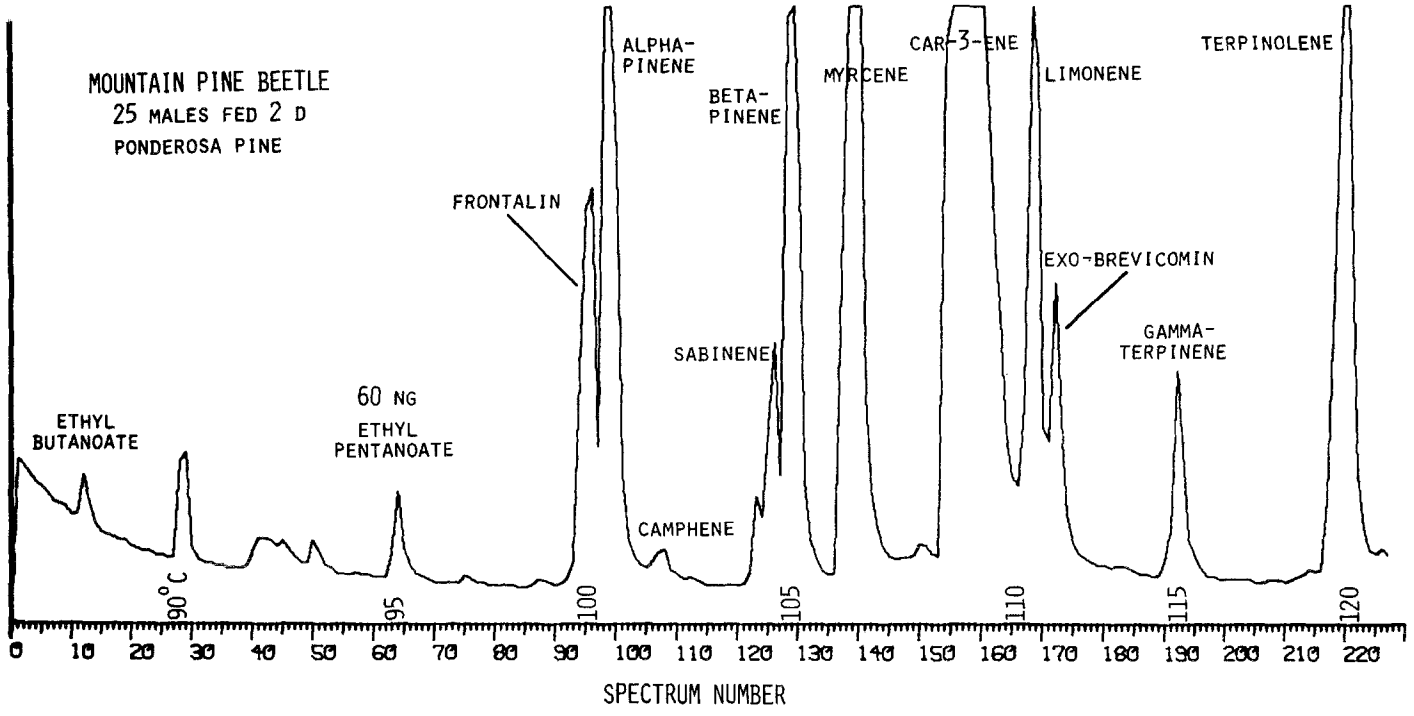

FIG. 1. Gas chromatogram of volatiles from air drawn over 25 male *Dendroctonus ponderosae* fed two days in ponderosa pine and confined in glass vials. Analysis by GC-MS; ethyl butanoate and ethyl pentanoate were standards.

females in natural galleries. Males confined in vials fought, and in 10–20% of the vials one of the two males was killed and its abdomen, which is the site of pheromone storage, was destroyed. We could have avoided this complication by pairing each male with a female, a more amicable arrangement that results in little or no mortality. However, by excluding females, we were certain that any frontalin detected originated in the males.

About 60–70% of the male MPB were arrested in the olfactory walkway by a solution of 0.1% *trans*-verbenol and 1% terpenes. The addition of 0.01% frontalin reduced male arrestment to about 30% for beetles reared from both species of pine (Table 2). A tenfold lower concentration of frontalin did not significantly alter the response of males to the attractant, and a tenfold greater concentration did not further reduce arrestment. The acoustic response of males to the attractant solution, a distinctive interrupted chirp, also occurred least frequently in the presence of 0.01% frontalin.

The addition of frontalin to the attractant *trans*-verbenol and terpenes significantly reduced the number of beetles caught on sticky traps in both lodgepole and ponderosa pine stands (Table 3). Frontalin added to terpenes was not attractive to MPB. In the lodgepole stand the male-to-female ratio of MPB responding to traps baited with attractant + frontalin was 1.00, not significantly different from the 1.20 sex ratio (t-test, $N = 10$) from traps baited only with attractant. Corresponding values from the ponderosa pine stand

TABLE 2. RESPONSE OF UNFED MALE *Dendroctonus ponderosae* TO A SOLUTION OF *trans*-VERBENOL AND TERPENES WITH SEVERAL CONCENTRATIONS OF FRONTALIN IN AN OLFACTORY WALKWAY

	Mean ± SE of 20 males, 3 replications	
Treatment	Arrested	Interrupted chirps
From lodgepole pine		
*t*V + terpenes[a]	14.3 ± 0.33	12.3 ± 0.67
+ 0.001% frontalin	11.7 ± 0.67	6.7 ± 0.33
+ 0.01% frontalin	6.0 ± 1.00*[b]	5.3 ± 2.33*
+ 0.10% frontalin	7.3 ± 0.33*	7.7 ± 0.33
From ponderosa pine		
*t*V + terpenes	13.3 ± 0.67	13.0 ± 1.00
+ 0.001% frontalin	12.3 ± 0.33	9.7 ± 0.88
+ 0.01% frontalin	6.7 ± 0.67**	4.3 ± 1.20**
+ 0.10% frontalin	7.7 ± 0.67**	8.0 ± 0.58*

[a] *t*V = 0.1% *trans*-verbenol; terpenes = 1% mix of myrcene and (+)-alpha-pinene diluted in dibutyl phthalate.

[b] Significantly different from response to the attractant, *t*V + terpenes, at * $\alpha = 0.05$ and at ** $\alpha = 0.01$ with ANOVA and Scheffé's test for multiple comparisons of means.

TABLE 3. CATCH OF *Dendroctonus ponderosae* ON STICKY TRAPS WITH VERTICAL SILHOUETTES AND BAITED WITH ATTRACTANTS WITH AND WITHOUT FRONTALIN IN LODGEPOLE AND PONDEROSA PINE STANDS, JULY–AUGUST 1981 DESCHUTES NATIONAL FOREST, OREGON

Treatment	Mean ± SE of catch/trap/replication	
	In lodgepole	In ponderosa pine
*t*V + terp	12.2 ± 1.78	4.2 ± 0.91
*t*V + terp + frontalin[a]	1.7 ± 0.50 **[b]	1.7 ± 0.48*
Terp + frontalin	0.9 ± 0.30 NS	0.0 ± 0.00 NS
Terpenes	1.6 ± 0.34	0.2 ± 1.01
Blank trap	0.6 ± 0.16	0.2 ± 1.37

[a] *t*V = *trans*-verbenol evaporated at 0.5 mg/day; terp = 1:1 mixture of (+)-alpha-pinene and myrcene at 10 mg/day; frontalin at 8 mg/day.
[b] Significantly different at the * $\alpha = 0.05$ level and ** $\alpha = 0.01$ level, Wilcoxon's matched-pairs, signed-ranks test, one-tailed, $N = 18$; NS = not significantly different.

were 0.67 with and 0.88 without frontalin; a greater proportion of females responded in the stand of ponderosa pine but the addition of frontalin did not significantly affect the sex ratio.

Taken together, these data suggest that male MPB affect the colonization process by releasing the pheromones *exo*-brevicomin, *endo*-brevicomin, and later frontalin. Not knowing the optical rotations of the natural pheromones makes the assignment of antiaggregative functions to the male pheromones premature, based on the results of our partially defined, synthetic chemical system, even though racemic frontalin, *exo*-, and *endo*-brevicomin all dramatically reduced trap catches (Table 3) (Ryker and Rudinsky, 1982). Bioassay of purified enantiomers of these compounds should clarify whether they function as attractants or interruptants in the natural system.

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DEFENSIVE SECRETIONS OF THREE OXYTELINAE ROVE BEETLES (COLEOPTERA: STAPHYLINIDAE)

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Abstract—The secretions of the abdominal glands of *Bledius spectabilis* Kraatz, *Platystethus arenarius* Fourcr., and *Oxytelus piceus* L. have been shown to contain four 1-alkenes; toluquinone; toluhydroquinone; C_{10}-, C_{11}-, C_{12}-, and C_{14}-γ-lactones; C_{12}-δ-lactone; citral; and decyl-, undecyl-, and dodecyl acetates. Quantitative results indicate that 1-alkenes are formed probably from present lactones by decarboxylation. According to the known life histories of the beetles, it is suggested that the gland material is not used as an algal growth regulator but represents a unique defensive blend characteristic for the whole subfamily.

Key Words—Coleoptera, Staphylinidae, Oxytelinae, defensive secretion, glands, acetates, lactones, alkenes, quinones, citral.

INTRODUCTION

In the sequence of investigations dealing with the taxonomic and ecological influences on the chemical composition of defensive secretions of water beetles (Dettner, 1979; Dettner and Schwinger, 1980), we continued the comparative chemical studies with abdominal glands of the rove beetle subfamily Oxytelinae. Since Wheeler et al. (1972) found γ-dodecalactone as main component within the gland system of algae-cultivating *Bledius* species, it was not clear whether one or more components of this blend might act as an algal growth regulator as was suggested by the authors.

In addition to new chemical results which were obtained from *Bledius spectabilis* glands, we now report a chemical analysis of the species *Oxytelus piceus* and *Platystethus arenarius*. These two Oxytelinae rove beetles share a

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similar coprophagous nutrition biology and a common dung habitate, distinctly apart from the algae-feeding *Bledius* species of the seashore.

When molested, all Oxytelinae specimens bend their abdominal tip dorsally and emit a sweetish smelling gland material. The secretion is produced within an oval gland, passes an efferent duct, and is stored in a reservoir which opens between the paired 9th and unpaired 10th tergite (Figure 1) (Araujo, 1973; Happ and Happ, 1973).

METHODS AND MATERIALS

Specimens of *Bledius spectabilis* Kraatz were collected within the sand flat area of the Königshafen (Isle of Sylt, North Sea). *Oxytelus piceus* L. and *Platystethus arenarius* Fourcr. were collected from horse excrement near Aachen. From the frozen beetles, gland reservoirs were excised in an ice bath and deposited into the tiny groove of a cooled wire plunger which is movable within the needle (Mini Injector, Precision Sampling Corporation). This method allowed us to analyze glands of single specimens by splitless or split (1:10) capillary gas chromatography. Silylation was performed by using MSTFA (N-methyl-N-trimethylsilyl = trifluoracetamide).

Gland material was separated by using a Carlo Erba Fractovap 2900

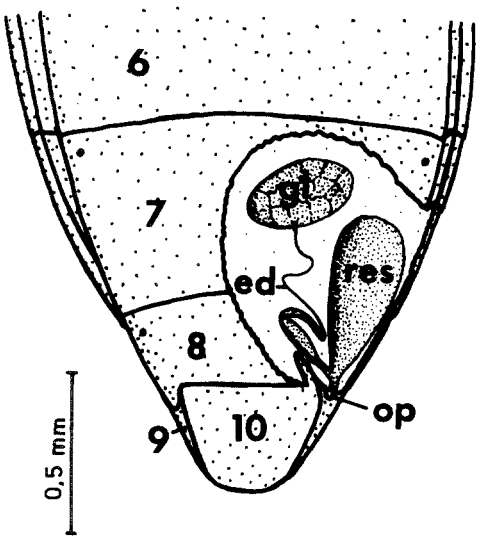

FIG. 1. Dorsal view of the opened abdominal tip of *Bledius spectabilis* with one defensive gland system (gl: gland; res: reservoir; ed: efferent duct; op: opening; numbers indicate tergites).

capillary gas chromatograph equipped with a FID detector (carrier gas: helium; 1 ml/min) and a Spectra Physics computer integrator (System I). The following glass capillary columns and temperature programs were used: 8 m CW 20 M (45° C: 2 min isothermal; 45–100° C: 10° C/min; 100–225° C: 5° C/min), 16 m WG 11 (65° C: 2 min isoth; 65–165° C: 10° C/min; 165–225° C: 5° C/min); 16 m OV-101 (65° C: 2 min isoth; 65–225° C: 10° C/min).

Capillary gas chromatography–mass spectrometry was performed on a Varian 3700 capillary gas chromatograph coupled to a MAT 44 quadrupole mass spectrometer which operated at 80 eV and was connected with a Varian SS 200 computer system. Cl-MS data were obtained with the same system using isobutane as reactant gas (130 eV). For GC-MS, a 8-cm CW 20 M glass capillary column was used, temperature programed from 65° C (1 min isoth) to 210° C with 12° C/min. Whole-gland reservoirs were splitless injected without solvent as described above.

RESULTS

Chemical Analysis of Secretions (Figures 2 and 3)

Alkenes. According to EI-MS data, components 1–4 showed typical alkene fragmentation patterns with molecular masses 126 (1), 140 (2), 154 (3), and 182 (4). Quasimolecular ions at m/z 155 (3, M+1) and m/z 183 (4, M+1) were obtained for compounds 3 and 4 by CI-MS but not for the more volatile minor components 1 and 2. In addition to 1-undecene (3) in two *Bledius* species (Wheeler et al., 1972), the alkenes were 1-nonene (1), 1-decene (2), and 1-tridecene (4), as confirmed by comparison of their EI mass spectra with authentic samples. Retention times of 1–4 from gland material matched those of the above-mentioned 1-alkenes, both on polar (8 m CW 20 M; 16 m WG 11) and apolar (16 m OV-101) capillary columns.

p-Toluquinone. Peak 5 showed a molecular mass of 122 (CI-MS: 123) and represents the main component of the defensive secretion of the three Oxytelinae species. EI mass spectral data are indistinguishable from authentic *p*-toluquinone which was additionally confirmed by coinjection with gland material on the three capillary columns and by thin-layer chromatography.

Citral. CI-MS data for compounds 6 and 7 showed quasimolecular ions at m/z 153 (M+1). EI fragmentation patterns were identical to authentic cis and trans isomers of citral (neral, geranial) and were further confirmed by coinjection of the gland material from *Bledius spectabilis* and *Platystethus arenarius* with freshly distillated citral.

Lactones. EI mass spectral data for compounds 8–10 and 12 altogether showed a base peak at m/z 85 accompanied by fragments at m/z 41, 43, 55, 56, 57, 69, and 128. The periodic succession of retention times of the four gland

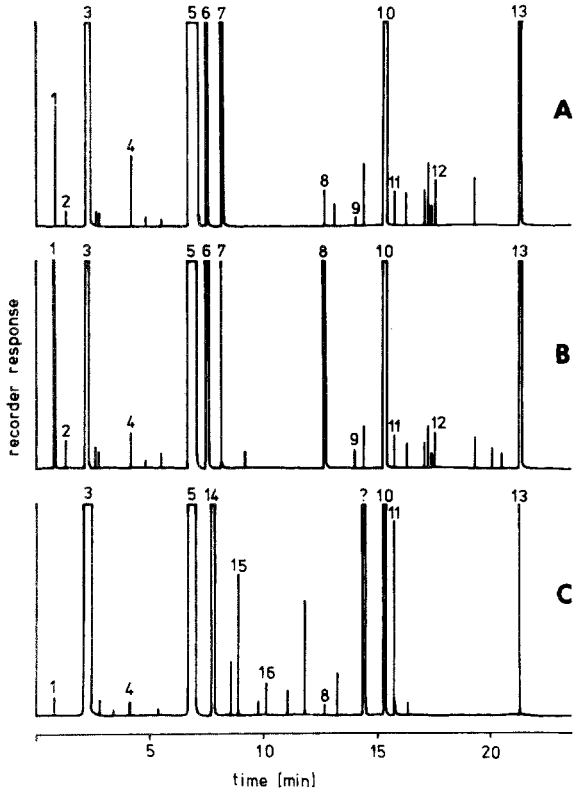

FIG. 2. Capillary gas chromatograms (copies of originals; 8 m CW 20 M; see Methods and Materials) obtained by injections of single gland reservoirs of *Bledius spectabilis* (A), *Platystethus arenarius* (B), and *Oxytelus piceus* (C).

components on a 8-m CW 20 M and a 16-m WG 11 capillary column were in favor of a homologous series of γ-lactones which exhibit a characteristic *m/z* 85 fragment, due to the pentacyclic lactone ring. This assumption is supported by the finding of dodecane-4-olide in two *Bledius* species (Wheeler et al., 1972). By CI-MS the following quasimolecular ions were obtained from the gland material of *Bledius spectabilis*: 171 (M+1 of compound 8), 185 (M+1 of compound 9), 199 (M+1 of compound 10), and 227 (M+1 of compound 12). EI mass spectra of authentic, straight-chain γ-lactones with carbon numbers 10, 11, 12, and 14 were indistinguishable from *Bledius* components 8–10, and 12, which were therefore found to be decane-4-olide (8), undecane-4-olide (9), dodecane-4-olide (10), and tetradecane-4-olide (12). Besides base peaks at *m/z* 85, compounds 8–10 and 12 exhibit small M^+ peaks (1%) and distinct $M-H_2O$ (2%) and $M-2H_2O$ (2%) peaks in their EI mass spectra. All four

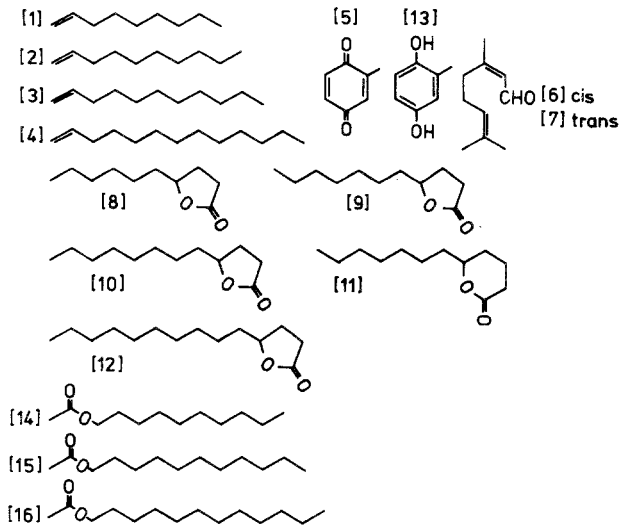

FIG. 3. Defensive components of three Oxytelinae rove beetle species.

authentic γ-lactones showed identical retention times on a 8-m CW 20 M and a 16-m WG 11 capillary column when compared with *Bledius* gland components 8, 9, 10, and 12.

Sufficient amounts of compound 11 were only present in *Oytelus piceus* defensive glands for recording an EI mass spectrum. This spectrum was characterized by a base peak at m/z 99, a weak M^+ peak at m/z 198 (0.8%) and distinct $M-H_2O$ and $M-2H_2O$ peaks at m/z 180 (2%) and m/z 162 (1.5%). Further fragments were found at m/z 41, 42, 43, 55, 70, 71, 84, and 114. δ-Lactones exhibit characteristic m/z 99 peaks due to the hexacyclic lactone ring; compound 11 was therefore identified as dodecane-5-olide. This was confirmed by the EI mass spectrum of authentic dodecane-5-olide which was identical to the EI-MS of compound 11 from *Oxytelus piceus* glands. From the GC-separated gland constituents (16 m WG 11; 8 m CW 20 M) of *Oxytelus piceus*, compound 11 was matched with authentic dodecane-5-olide. As a minor component, the same δ-lactone could also be detected in the gland reservoirs of *Bledius spectabilis* and *Platystethus arenarius*.

p-Toluhydroquinone. In the three species investigated, compound 13 was only present as a distinct peak when short capillary columns and drastic heating programs were used. EI mass spectral data revealed a M^+ peak at m/z 124 which was supported by a quasimolecular ion at m/z 125 (CI-MS). Due to the EI-MS fragments at m/z 77, 95, 105, 106, 107, and 123, compound 13 was tentatively identified as a hydroxybenzyl alcohol. Silylated 2-, 3-, and 4-hydroxybenzyl alcohols, however, did not match with the silylated compound

13 from the gland contents of *Bledius spectabilis*. (Retention times of TMS ethers on a 16-m OV-101 capillary column: 2-hydroxybenzyl alcohol 615 sec, 3-hydroxybenzyl alcohol 659 sec, 4-hydroxybenzyl alcohol 693 sec, compound 13 648 sec.) Based on biogenetic considerations, compound 13 was finally identified as *p*-toluhydroquinone which was confirmed by identical EI-MS data of the natural and authentic material and by TLC separation of the gland contents. On the above-mentioned apolar capillary column, the coinjected TMS-ether of authentic *p*-toluhydroquinone could not be separated from the TMS ether of compound 13 of the three Oxytelinae species.

Acetates. Compounds 14–16 could only be found in the defensive secretion of *Oxytelus piceus*. EI mass spectra were consistent with acetates, having base peaks at *m/z* 43 and diagnostic peaks at *m/z* 61 from protonated acetic acid. Compound 14 gave a M−60 peak at *m/z* 140, compound 15 gave a M−60 peak at *m/z* 154, while compound 16 exhibited this fragment ion at *m/z* 168. EI-MS data of compounds 14–16 of *Oxytelus piceus* were therefore found to be identical to decyl acetate (14), undecyl acetate (15), and dodecyl acetate (16) and were confirmed by comparison of R_t values of authentic and naturally occurring compounds on both polar (8 m CW 10 M, 16 m WG 11) and apolar (16 m OV-101) capillary columns.

Several minor and one main component (*Oxytelus piceus*, R_t 14.2 min) have not been identified conclusively.

Biological and Quantitative Observations

The qualitative analysis of the defensive gland secretion was always species specific and constant. Considerable quantitative variations within one species were found to be mainly dependent on duration and temperature during excision of the gland reservoirs. Volatile alkenes like 1-nonene and 1-decene were absent when beetles were stored in the refrigerator for several months. In *Bledius spectabilis*, the highest amounts of gland material could be only detected in specimens with mature gonads. As a rule male specimens store more gland material for they are characterized by a greater gland and body volume. Males and females with undifferentiated gonads store only small amounts of defensive secretion.

Peak area values showed distinct correlations when single 1-alkenes were compared with single γ-lactones with an additional carbon atom. For example, the following ratios of peak areas were obtained from three Oxytelinae specimens: *Platystethus arenarius*, specimen 1, 1-nonene:decane-4-olide = 0.4:2.1, 1-decene:undecane-4-olide = 0.06:1, 1-undecene:dodecane-4-olide = 4.07:21.4, 1-tridecene:tetradecane-4-olide = 0.02:0.2; *Platystethus arenarius*, specimen 2, 1-nonene:decane-4-olide = 0.72:0.86, 1-decene:undecane-4-olide = 0.08:0.05, 1-undecene:dodecane-4-olide = 14.3:8.3, 1-tridecene:tetradecane-4-olide = 0.1:0.07 *Bledius spectabilis*,

specimen 1, 1-nonene:decane-4-olide = 0.22:0.11, 1-decene:undecane-4-olide = 0.004:0.002, 1-undecene:dodecane-4-olide = 26.13:13.12, 1-tridecene:tetradecane-4-olide = 0.21:0.13. Compared with *Bledius spectabilis*, *Platystethus arenarius* secretes distinctly more decane-4-olide and consequently more 1-nonene (Figure 2).

These quantitative results were only observed in freshly killed beetles whose glands were immediately excised in an ice bath. These findings support the biogenetic suggestion of Wheeler et al. (1972) that 1-alkenes in *Bledius* species might be formed by decarboxylation of γ-lactones.

DISCUSSION

Apart from dodecane-4-olide, 1-undecene, citral, and *p*-toluquinone (Wheeler et al., 1972), other defensive components were found for the first time within Oxytelinae defensive glands. Decane-4-olide, undecane-4-olide, tetradecane-4-olide, and dodecane-5-olide are not known from other insect glands where similar lactones with isolated penta- and hexacyclic rings have a very limited distribution. γ-Lactones are known as pheromones from *Trogoderma glabrum* (hexane-4-olide; Yarger et al., 1975), *Popillia japonica* (5-tetradecene-4-olide; Tumlinson, 1979), *Lasius flavus* (9-octadecene-4-olide, 9-hexadecene-4-olide, function unknown; Bergström and Löfquist, 1970), and *Eldana saccharina* (3,7-dimethyl-6-octene-4-olide; Kunesch et al., 1981). δ-Lactones were found in the mandibular gland secretions of *Xylocopa hirsutissima* (2-methyl-hexane-5-olide; Wheeler et al., 1976) and two *Camponotus* species (2-decene-5-olide; Cavill et al., 1968). From head extracts of the queens of *Vespa orientalis*, hexadecane-5-olide has been isolated (Ikan et al., 1969).

Acetates are present in many insect glands, especially as mono- and polyunsaturated esters which act as pheromones (Bestmann and Vostrowsky, 1981). While octyl acetate represents the ester with maximal carbon-chain length in defensive secretions of Heteroptera (Weatherston and Percy, 1978), esters from nonyl acetate to hexadecyl acetate are known from hymenopteran glands (Blum and Hermann, 1978). Decyl and dodecyl acetate furthermore could be found in lepidopteran glands (Dazzini and Finzi, 1974; Weatherston et al., 1979).

Apart from the three acetates (14–16) which are recorded from *Oxytelus piceus*, acetates were found in tergal glands of the chrysomelid larvae of *Gastrophysa atroceana* [octadecyl acetate, (*Z*)-11-eicosenyl acetate; Sugawara et al., 1978] and in pygidial defensive glands of 11 carabid beetle species. Within this family there were recorded nonyl acetate (Helluoninae; Eisner et at., 1968; Moore and Wallbank, 1978), decyl acetate (Dryptinae; Moore, 1979), dodecyl acetate (Psydrinae; Moore, 1979), tetradecyl acetate

(Psydrinae, Panagaeinae; Moore, 1979), and hexadecyl acetate (Panagaeinae; Moore, 1979). Further esters which have been recently found by milking other rove beetles of the subfamily Aleocharinae are ethyl and methyl decanoate, ethyl octanoate, and ethyl dodecanoate (Gnanasunderam et al., 1981). Although this subfamily is characterized by several abdominal gland systems (Pasteels, 1968), it may be supposed that these esters are produced in the aleocharine tergal gland systems which are not homologous to the defensive glands of the Oxytelinae species investigated. The remaining recorded compounds (1–7 and 13) are widespread within insect glands.

The components of the abdominal gland systems found in the Oxytelinae are probably used as defensive substances. When there is a slight irritation (pinching or handling), all Oxytelinae species immediately bent their abdominal tips dorsally and released gland secretion which is perceived by the lactonic odor. Preliminary chemotaxonomic results within other members of the Oxytelinae show that the gland chemistry of the whole subfamily is characterized by lactones, 1-alkenes, *p*-toluquinone, and *p*-toluhydroquinone, although these species investigated show different life cycles and feeding habits. Based on these chemotaxonomic indications, it seems unlikely that one or more components of this gland secretion might regulate the growth of algae, which flourish within the burrows of *Bledius* and upon which adults and larvae feed (Wheeler et al., 1972). Moreover there are no indications that any of the sequestered gland compounds might act as plant growth regulators.

Platystethus arenarius, which shows nearly the same gland composition as *Bledius*, exclusively feeds on dung although it too shows a subsocial behavior (Hinton, 1944). Females construct a brood chamber in dung and defend eggs and first-stage larvae against intruders in the chamber (Hinton, 1944). During this defense, females attack intruders by jabbing them with their abdominal tips and by biting them with their mandibles. Furthermore Hinton (1944) noticed that females protect their eggs by preventing fungi from growing in the egg chamber. This special function too might be ascribed to this gland secretion, for normally most defensive secretions possess bactericide and fungicide properties (Duffey, 1976). Although the life cycle of *Oxytelus piceus* is not known, it is suggested that this species too is coprophagous, for chitin fragments were never found within the proventriculus of this dung-inhabiting species. The gland chemistry of *Oxytelus piceus* differs from *Bledius* and *Platystethus* in that chiral is replaced by the three acetates (14–16).

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CHEMICAL PROTECTION OF A FISH (*Abudefduf leucogaster* Bleeker) BY A SOFT CORAL (*Litophyton viridis* May).[1]

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Abstract—Around Laing Island (Bismarck Sea), juveniles of the fish *Abudefduf leucogaster* are associated with the alcyonarian *Litophyton viridis* which, on mechanical stimulation, can release a potent ichthyotoxin and appears to be accordingly disliked by large predators. The fish has no special immunity to high levels of toxin but is affected more slowly than others. This has been interpreted in terms of an original strategy whereby *A. leucogaster* plays its long reaction time against the diffusion of the toxin, allowing the fish to remain around the soft coral and benefit from indirect chemical protection. Possible biological implications of the toxin release are discussed.

Key words—*Abudefduf leucogaster*, *Litophyton viridis*, fish, soft coral, alcyonarian, toxin, chemical defense.

INTRODUCTION

Many gregarious species of tropical reef fish live in close proximity to branching hermatypic corals; when frightened they dart and wedge themselves into the branches. It is well known by divers that if one then lifts the coral clear out of the water, the fish will often stay in it, attesting to the strength of their link with the host. In contrast, it is of common knowledge that the fish population associated with soft corals is very much smaller, both as to numbers of species and numbers of fish, although many species of alcyonarians are branching and could conceivably offer good shelter to small

[1]Laing Island Biological Station contribution No. 39.

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fish. It might be argued that hard corals are preferred for the better mechanical protection they can offer or, more convincingly, that fish are deterred by the frequent occurrence of potent ichthyotoxins in soft corals (Ne'eman, Fishelson and Kashman et al., 1974; Tursch et al., 1978; Bakus, 1981). Soft corals are, indeed, practically immune from fish predators with the exception of certain Chaetodontidae (Reese, 1973; Hobson, 1974; Anderson et al., 1981).

The following observations were made from August to November 1980, during a study of soft corals (Verseveldt and Tursch, 1979) on a 600-m^2 quadrat on the leeward shore of Laing Island, In Hansa Bay, on the north coast of Papua, New Guinea. The single largest contributor to the biomass of the quadrat (Tursch and Tursch, 1982) is the alcyonarian *Litophyton viridis*. This is a supple, branching soft coral up to 45 cm in height, forming dense monospecific carpets reaching a diameter of over 2 m and offering many potential hiding places for small fish under their thick, gently undulating fronds. Over 120 hrs of underwater observation (including dusk and night dives), using SCUBA gear, established that, when compared to the neighboring colonies of scleractinians, the stands of *L. viridis* harbor much fewer species of fish and that these, with one notable exception, are always found in very small numbers.

The exception is constituted by aggregations of juvenile *Abudefduf leucogaster* which are regularly seen hovering just above the canopy of large *L. viridis* around which they retreat when frightened. It was estimated that about 1000–3000 juveniles were present on the quadrat. They were not seen more than a couple of feet away from *L. viridis*—less frequently from a substitute host (less than 10% of the sightings)—and generally keep much closer. The groups vary in size from a dozen to several hundred individuals and characteristically contain fish of various sizes, thus suggesting that egg-laying extends over a long period of time or that the members of the population of one *L. viridis* colony are the offspring of different parents. The distribution of juvenile *A. leucogaster* is rather patchy, the fish being found only around certain colonies (preferably large ones) and not around others. The same colonies were seen to be occupied by fish for periods of over 6 weeks, although temporary migration to neighboring sites was sometimes observed. No juveniles were seen during night dives, and their nocturnal whereabouts are not known: they are probably well hidden within the reef. When the fish reach a size of about 4 cm, they become less sedentary and adults are commonly observed in a wide variety of reef environments. Neither juveniles nor adults were ever seen feeding on the soft coral. The juvenile troops exhibit the typical behavior of plankton feeders (Reese, 1978).

When kept in an aquarium together with a small colony of *L. viridis*, a variety of small reef fish show no obvious sign of discomfort over prolonged periods of time as long as the soft coral is left undisturbed. If the alcyonarian is

then gently prodded with the fingers, the water immediately becomes cloudy and all the fish (including *A. leucogaster*) are very rapidly disabled and die within minutes.

The vicinity of the fronds of *L. viridis* can thus be very unsafe for fish and the question naturally arose as to whether *A. leucogaster* is endowed with some special adaptation(s) to cope with such a potentially hazardous environment. Some species of fish have developed an immunity to alcyonarian toxins when taken orally. For instance on the observation quadrat *L. viridis* was found to be the preferred food of adult *Chaetodon ocellicaudus* and an occasional food of *C. uliatensis*. The simplest hypothesis was that *A. leucogaster* would be specially resistant to high levels of *L. viridis* toxin. Thie could easily be tested by comparing the resistance of *A. leucogaster* with that of juveniles of other gregarious percoid fish living in the observation quadrat.

METHODS AND MATERIALS

All the fish tested were juveniles measuring 20–25 mm. *Chromis ternatensis*, *Pomacentrus violascens*, *Dascyllus aruanus*, *D. melanurus* all live gregariously around scleractinians and are very common in the vicinity of *L. viridis*. Together with *A. leucogaster* they were all collected by hand netting in the study quadrat. In order to test another member of the genus *Abudefduf* toxicity tests were also effected on the gregarious juveniles of *A. curacao*. This fish lives in a different environment and was never seen in the proximity of *L. viridis*; it was collected around the wooden pilings of the pier.

When immediately examined under the microscope, the cloudy, highly toxic solution obtained by handling *L. viridis* in seawater was found to contain finely dispersed oily droplets. No nematocysts were detected. Centrifugation followed by paper filtration resulted in a nearly total loss of activity, thus indicating that the toxin is not hydrosoluble.[2] Thin-layer chromatographic comparisons showed that a methylene chloride extract of the toxic solution possessed all the spots exhibited by a direct methylene chloride–acetone extract of whole fresh colonies, although with some variations in the relative intensities. It was thus deemed reasonable to effect the toxicity tests by using the readily available and quantifiable methylene chloride–acetone extract of whole colonies of *L. viridis*.

Instead of measuring the usual lethal doses, it was considered more significant to establish active doses, since fish *in situ* would probably take evasive action well before being exposed to lethal levels. The active dose (AD_{50}) was defined as the concentration that disables half the test fish within

[2]Although the observed effects might be due to a mixture of compounds, the term "toxin" will be used here for simplification.

30 min. A fish was deemed disabled when it rolled over on its side and stayed at the bottom of the experimental vessel for a count of 10 sec or when it did not react any more to gentle prodding with a spatula.

Fish were tested in 200 ml seawater containing 1 ml EtOH solution of the extract, using a procedure very similar to that of Ne'eman et al. (1974). A minimum of three specimens of each species was tested for each of 2, 5, 10, 25, and 50 mg/liter concentrations. AD_{50} values were obtained by interpolation of the experimental curves. Blank controls were used in every case.

During these assays, it was noted that for a given concentration of toxin some species of fish were consistently affected much faster than others. The time required to disable the fish at an arbitrarily fixed concentration of 50 mg/liter was thus recorded (to the nearest minute) under the above experimental conditions.

RESULTS

The observed AD_{50}s were found to vary little from species to species (from 12.5 to 20 mg/liter), and individual measurements showed considerable overlap. Although the value recorded for *A. leucogaster* was the highest, the observed spread (±25% around a median value of 16 mg/liter) was considered too small to be interpreted as indicating a significant adaptation of *A. leucogaster*.

By contrast, the time required to disable the fish at a 50 mg/liter concentration of extract was found to vary from 2 to 12 min for all the quadrat species examined. These results are summarized in the histogram of Figure 1.

It can be seen that *A. leucogaster* reacts more slowly and indeed 90% of the other fish are disabled before the first *A. leucogaster* is seriously affected. Application of the Wald-Wolfowitz runs test[3] shows that the difference observed between *A. leucogaster* and every other species is significant at the 0.05 level.

The histogram of Figure 2 shows that the reaction time of *A. curacao* (which lives in a different environment) is much closer to that of *A. leucogaster* than that of any other fish tested.

At a concentration of 50 mg/liter of extract, all the test fish will eventually die if left in the experimental vessel. If the stunned fish are immediately removed and placed into fresh seawater, nearly all of them do recover, thus indicating the reversibility of the intoxication in its first phase. Most of the test fish could be released *in situ* after use.

[3] Modified for ties according to S. Siegel, Nonparametric Statistics for the Behavioural Sciences, McGraw-Hill, New York, 1956.

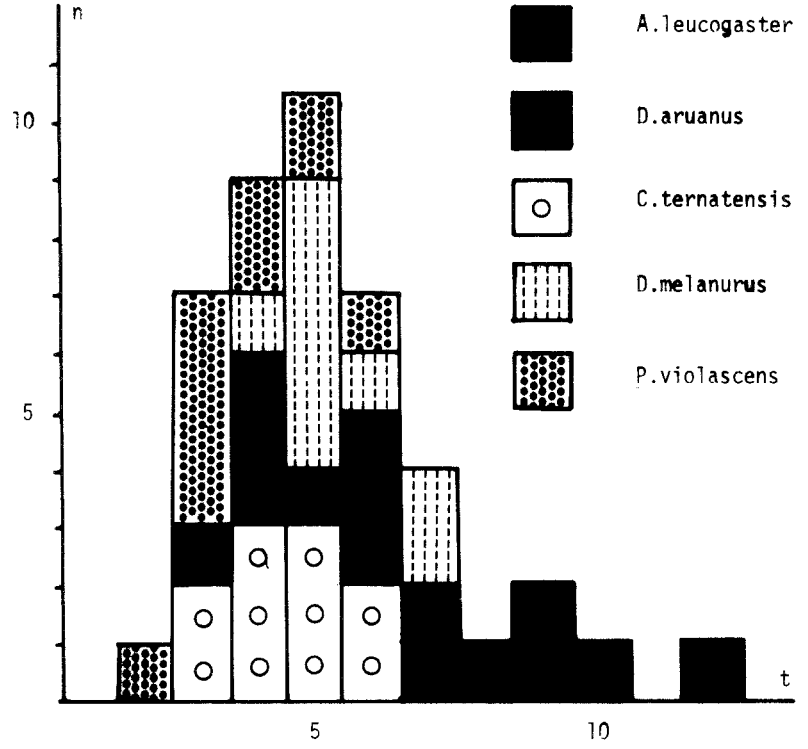

FIG. 1. Reponse of various reef fish to a 50 mg/liter concentration of *L. viridis* extract; n = number of fish disabled; t = time in minutes.

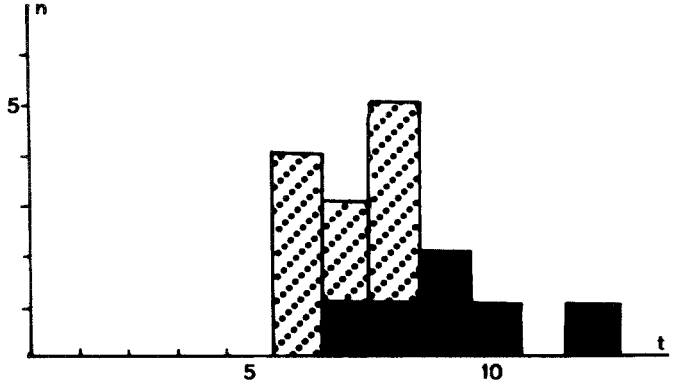

FIG. 2. Response of *A. curacao* (dotted) and *A. leucogaster* (black) to a 50 mg/liter concentration of *L. viridis* extract; n = number of fish disabled; t = time in minutes.

DISCUSSION

While active doses comparisons were inconclusive, measurements of time of reaction established that *A. leucogaster* reacts particularly slowly to the toxin. The adaptation that allows juvenile *A. leucogaster* to live in close proximity to *L. viridis* appears thus to be of an essentially kinetic nature. Should the soft coral release its toxin under the influence of an external stimulus, the fish would have to take evasive action much later than other species. It would gain great advantage by the ratio of the diffusion rate of the toxin in the water to the rate of intoxication. The effect of diffusion is indicated by the repeated observation that if *L. viridis* colonies harboring *A. leucogaster* are disturbed, the fish will momentarily abandon their host if the water is very still but will generally stay if the water is in motion.

It is not very likely that the association is based upon constant release of small amounts of toxin by the soft coral. Indeed various fish in aquariums have been observed to coexist with *L. viridis* for prolonged periods as long as the soft coral is left undisturbed.

That *L. viridis* can offer effective protection to specially adapted small fish (such as *A. leucogaster*) is indicated by the observation that it is largely avoided by ambush-hunters such as the common *Epinephelus merra* and the various species of *Pterois*. Fast pack-hunters such as the small schools of *Caranx* sp. that regularly feed in the quadrat were also observed to carefully avoid close contact with the soft coral. The action of the toxin of *L. viridis* was shown to be reversible in its first phase and avoidance of strong contact with the soft coral could thus be directly learned by fish.

While *A. leucogaster* is certainly at an advantage there is no reason to believe that its protection is completely effective. In addition to indirect chemical protection from large predators, *A. leucogaster* would also benefit from the exclusion of competitors from its feeding grounds.

The threshold of stimulation necessary to release toxin emission has not been measured, but all observations point towards a small magnitude. It is tempting to speculate that this (in conjunction with other factors such as feeding and territorial space) could explain the loosening of the bond between *A. leucogaster* and the soft coral with increasing fish size. While lighter and slower juveniles would be relatively safe, adults could have enough mechanical momentum to trigger toxin release on accidental contact and would thus tend to stay in other habitats.

Toxin release could also explain the peculiar, dainty behavior of *Chaetodon ocellicaudus* when feeding upon *L. viridis*. Instead of systematically plucking the available polyps of a given colony, the fish generally take one or two delicate bites at one specimen then proceed to another one, thus covering relatively large distances for their food. Prevention of polyp retraction can hardly justify this behavior since *L. viridis* is one of the few

nephtheids whose polyps are devoid of a supporting bundle of sclerites and *C. ocellicaudus* were occasionally seen to feed on retracted colonies. It seems more likely that the fish is sensitive to toxin intake through the gills and that its feeding mode is geared to minimize toxin release in the water. The result is that grazing is distributed over a large number of colonies and its effects are barely noticeable to the observer.

The reaction time of *A. curacao* (a species that was not seen in the vicinity of *L. viridis*) is much closer to that of *A. leucogaster* than that of any other fish tested. One might speculate that members of the genus *Abudefduf* could be preadapted to cope with nephtheid toxins and, indeed, another species, *A. melanopus*, although much less frequent than *A. leucogaster*, is regularly seen around the stands of *L. viridis* where it establishes fiercely defended territories. This hypothesis is further supported by the observation that in the Red Sea *A. sulfureus* (Fishelson, 1970) and *A. melas* (Fishelson, *in litt.*) do inhabit *Litophyton arboreum*.

Although ichthyotoxins are widespread among alcyonarians, their release in the environment has been so far described only in one other case, that of *Cespitularia aff. subviridis* (from the Seychelles) where the main toxic agent has been shown to be the sesquiterpene palustrol (Tursch et al., 1978). It is suspected that further observation will greatly increase the number of examples.

Previous investigations of the chemical content of *L. viridis* (collected at Leti Island, South Moluccas) have established the presence of the diterpenes nephtenol and 2-hydroxynephtenol (Tursch, et al., 1975) and the sterol derivatives 24-methylene cholest-5-ene-3β, 7β, 19-triol (Bortolotto et al., 1976) and 4α-methyl-3β,8β-dihydroxy-5α-ergost-24(28) en-23-one (Bortolotto et al., 1977). None of these compounds seems responsible for the observed activity. Preliminary data indicate that the toxicity reported here is associated with a complex mixture of sesquiterpenes, presently being investigated in this laboratory.

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EFFECTS OF COTTON CONDENSED TANNIN, MAYSIN (CORN) AND PINITOL (SOYBEANS) ON *Heliothis zea* GROWTH AND DEVELOPMENT[1,2]

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Abstract—Maysin, a flavone glycoside from corn silks, inhibits ingestion, and thus growth, of *Heliothis zea* (Boddie) larvae. Pinitol from soybeans inhibited *H. zea* growth by the same mechanism. Despite the widely held assumption that tannins inhibit growth by inhibiting assimilation, cotton condensed tannin inhibited *H. zea* growth by reducing ingestion; no evidence was found for a reduction in assimilation. Neonate larvae are shown to be much more sensitive to allelochemics than larvae that have fed on control diet before being transferred to diet containing plant allelochemics.

Key Words—Lepidoptera, Noctuidae, *Heliothis zea*, bioassay, host plant resistance mechanisms, allelochemicals.

INTRODUCTION

Recent investigations of the phytochemical nature of host plant resistance to insects have shown the existence of growth-inhibiting compounds in each of several hosts for *Heliothis zea* (Boddie). Maysin is a flavone glycoside from

[1]Reference to a company and/or product named by the department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

[2]Published with the approval of the director of the Delaware Agricultural Experiment Station as Miscellaneous Paper No. 968, Contribution No. 514 of the Department of Entomology and Applied Ecology, University of Delaware, Newark, Delaware 19711.

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corn silks. When incorporated into an artificial diet, it inhibits the growth of *H. zea*. It occurs at higher concentrations in such field corn lines as Zapalote Chico and US-13 (relatively resistant *H. zea*), than in sweet corn lines such as Stowell Evergreen and 471-U6 × 81-1 (relatively susceptible to *H. zea*) (Waiss et al., 1979). Pinitol is a growth-inhibiting compound from soybeans (Dreyer et al., 1979). *H. zea* larval growth is also reduced by condensed tannin isolated from cotton (Chan et al., 1978a). We report here the mechanisms by which maysin, pinitol, and cotton condensed tannin inhibit growth in *H. zea* larvae.

METHODS AND MATERIALS

H. zea larvae were hatched from eggs that were shipped to the Western Regional Research Center from the USDA Southern Grain Insects Research Laboratory, Tifton, Georgia.

Materials were incorporated into an artificial diet as outlined by Chan et al. (1978b).

Nutritional index experiments were performed using techniques modified from Reese and Beck (1976a), as described below. Twenty larvae (neonate or 1 day old, as specified in each experiment) were weighed and placed individually on weighed pieces of experimental diet (standard diet plus experimental compound) in 2-oz plastic catsup cups from Thunderbird Co., allowed to feed ad libitum for the duration of the experiment, weighed, and dried to constant weight. Twenty larvae were also placed on fresh standard diet and treated in a similar manner. At the end of the experimental period, feces were carefully separated from uneaten diet, weighed, and dried to constant weight. The uneaten diet was dried to constant weight. All weights were taken to the nearest 0.1 mg on a Mettler PI-1200 analytical balance.

Nutritional indices (AD, ECD, and ECI) were calculated on dry weight basis (Waldbauer, 1968). AD (approximate digestibility) measures the assimilation of food. ECD (efficiency of conversion of digested food) measures the efficiency with which assimilated food is converted into insect tissue. This index will decrease as the proportion of assimilated food metabolized for energy increases (Waldbauer, 1968). ECI (efficiency of conversion of ingested food) measures the overall ability of the insect to convert ingested food into tissue. Indices were calculated with a Fortran program modified from the calculator program originally developed by Reese and Beck (1976a).

RESULTS

Maysin. At 0.15% maysin did not reduce growth significantly (Table 1), even though the ED_{50} (that concentration of material in the diet which caused a 50% reduction in the measured response, weight gain in this case, when

TABLE 1. EFFECTS OF 0.15% MAYSIN ON NUTRITIONAL INDICES AND RELATED PARAMETERS IN *Heliothis zea* LARVAE [a]

	Control (±SE)	0.15% maysin (±SE)	% of control
Dry weight eaten (mg)	111.1 ± 8.4	99.7 ± 7.4	89.7
Initial fresh weight of larvae (mg)	0.4 ± 0.0	0.4 ± 0.0	100.0
Dry matter of larvae (%)	13.0 ± 0.4	13.3 ± 0.3	102.3
Dry weight gain (mg)	25.4 ± 2.4	23.2 ± 2.1	91.3
Dry weight of feces (mg)	74.5 ± 5.4	67.9 ± 5.0	91.1
Dry matter of feces (%)	27.0 ± 0.6	28.2 ± 0.8	104.4
AD (%)	32.9 ± 1.3	32.0 ± 1.2	97.3
ECD (%)	68.7 ± 3.0	73.1 ± 3.0	106.4
ECI (%)	22.3 ± 1.0	23.0 ± 0.9	103.1

[a] Neonate larvae were placed on control diet for 24 hr, then transferred to fresh experimental or control diet and allowed to feed for 10 days.

compared to the controls) is about 0.13% (Waiss et al., 1979). In the experiments in which the ED_{50} was obtained, neonate larvae were placed directly on control or treated diet instead of letting larvae feed 24 hr on control diet before putting them on control or treated diet, as was the case for the nutritional index experiments. Therefore, for the nutritional index experiments, it was necessary either to increase the concentration to 0.25% (Table 2) or to start the experiment with neonate larvae. As shown in Table 2, using

TABLE 2. EFFECTS OF 0.25% MAYSIN ON NUTRITIONAL INDICES AND RELATED PARAMETERS IN *Heliothis zea* LARVAE [a]

	Control (±SE)	0.25% maysin (±SE)	% of control
Dry weight eaten (mg)	120.8 ± 6.4	50.8 ± 6.9	42.1[b]
Initial fresh weight of larvae (mg)	0.5 ± 0.0	0.5 ± 0.0	100.0
Dry matter of larvae (%)	13.2 ± 0.2	11.8 ± 0.5	94.9[c]
Dry weight gain (mg)	23.4 ± 1.7	11.7 ± 1.7	50.0[b]
Dry weight of feces (mg)	84.6 ± 4.7	34.0 ± 4.5	40.2[b]
Dry matter of feces (%)	22.3 ± 0.9	24.9 ± 1.1	111.7
AD (%)	29.9 ± 1.3	32.8 ± 2.1	109.7
ECD (%)	65.7 ± 2.6	72.8 ± 6.8	110.8
ECI (%)	19.3 ± 0.8	22.5 ± 1.3	116.6

[a] Neonate larvae were placed on control diet for 24 hr, then transferred to fresh experimental or control diet and allowed to feed for 10 days.
[b] Significant at $P < 0.01$ level.
[c] Significant at $P < 0.05$ level.

TABLE 3. EFFECTS OF 0.7% PINITOL ON NUTRITIONAL INDICES AND RELATED PARAMETERS IN *Heliothis zea* LARVAE[a]

	Control (±SE)	0.7% pinitol (±SE)	% of control
Dry weight eaten (mg)	127.0 ± 7.2	116.0 ± 5.7	92.0
Initial fresh weight of larvae (mg)	0.5 ± 0.0	0.5 ± 0.0	100.0
Dry matter of larvae (%)	13.0 ± 0.3	13.4 ± 0.3	103.1
Dry weight gain (mg)	31.5 ± 2.3	29.5 ± 2.0	93.7
Dry weight of feces (mg)	82.8 ± 4.5	73.8 ± 3.4	89.1
Dry matter of feces (%)	29.5 ± 1.7	31.1 ± 1.4	105.4
AD (%)	34.4 ± 1.4	36.2 ± 1.5	105.2
ECD (%)	72.4 ± 1.7	69.9 ± 2.2	96.5
ECI (%)	24.7 ± 0.8	25.0 ± 0.9	101.2

[a]Neonate larvae were placed on control diet for 24 hr, then transferred to fresh experimental or control diet and allowed to feed for 10 days.

24-hr-old larvae required a 67% increase (over that used in Table 1) in the maysin concentration to achieve the ED_{50} level in terms of growth inhibition.

The mechanism by which maysin inhibited growth is also illustrated in Table 2. Assimilation, efficiency of conversion of assimilated diet, and efficiency of conversion of all diet ingested were all similar for the treatments and the controls. However, the amount ingested on maysin diet was reduced to only 42.1% of the controls, showing that growth inhibition is due to reduced ingestion.

TABLE 4. EFFECTS OF 0.75% PINITOL ON NUTRITIONAL INDICES AND RELATED PARAMETERS IN *Heliothis zea* LARVAE[a]

	Control (±SE)	0.7 pinitol (±SE)	% of control
Dry weight eaten (mg)	312.5 ± 25.0	139.1 ± 6.8	44.5[b]
Initial fresh weight of larvae (mg)	0.2 ± 0.0	0.2 ± 0.0	100.0
Dry matter of larvae (%)	13.9 ± 0.3	11.7 ± 0.2	84.2[b]
Dry weight gain (mg)	69.7 ± 5.5	29.1 + 2.3	41.8[b]
Dry weight of feces (mg)	220.4 ± 17.4	84.0 ± 5.9	38.1[b]
Dry matter of feces (%)	18.0 ± 0.2	24.8 ± 1.5	131.2[b]
AD(%)	29.3 ± 0.7	38.6 ± 3.4	131.7[b]
ECD (%)	77.5 ± 2.2	55.8 ± 2.8	72.0[b]
ECI (%)	22.5 ± 0.5	20.4 ± 0.9	90.7

[a]Neonate larvae were placed directly on experimental or control diet, and allowed to feed for 11 days.
[b]Significant at $P < 0.01$ level.

Pinitol. Pinitol was tested at the ED_{50} level (for weight gain) of 0.7% (Dreyer et al., 1979). Once again, when larvae were fed control diet for 24 hr before being transferred to treated or control diet, the ED_{50} level did not reduce growth significantly (Table 3). However, when neonate larvae were placed directly on control or pinitol-containing diet, 0.7% pinitol reduced growth to 41.8% of the controls (Table 4). The ECI was not reduced by a statistically significant amount. A reduction in the amount eaten by the larvae was the major factor in reducing larval growth.

Condensed Tannin. H. zea growth can be reduced by cotton condensed tannin (Figure 1). Growth was not reduced to any appreciable extent by a reduction in assimilation or efficiency of conversion of assimilated diet. There was a significant negative correlation between condensed tannin concentration and the amount ingested by the larvae; thus, the major factor in growth reduction was a reduction in ingestion.

DISCUSSION

The bioavailability of nutrients has to be considered in insect dietetics. If an allelochemic reduces ingestion, assimilation, or efficiency of conversion of assimilated material, it has prevented essential nutrients from being available

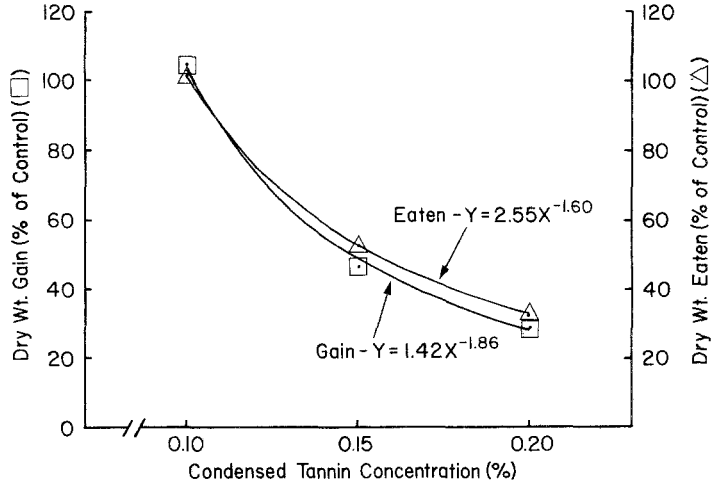

Fig. 1. Effects of cotton condensed tannin on dry weight gain and dry weight eaten in *Heliothis zea* larvae. Neonate larvae were placed on control diet for 24 hr, then transferred to fresh experimental or control diet and allowed to feed for 10 days. Correlation between condensed concentration and dry weight gain was significant at $P < 0.0005$ level ($r = 0.47$, 92 df). Correlation between condensed tannin concentration and dry weight eaten was significant at $P < 0.0005$ level ($r = 0.53$, 92 df).

to the insect. Interactions between nutrients and nonnutrients have been demonstrated with gossypol effects on *Heliothis zea* and *H. virescens* (Fabricius) larvae (Shaver and Parrott, 1970), sinigrin on *Papilio polyxenes asterius* Stoll (Erickson and Feeny, 1974), and with a number of phenolics and related compounds on *Agrotis ipsilon* (Hufnagel) larvae (Reese and Beck, 1976a–c), as well as many vertebrate systems (see references cited by Reese, 1979).

In the experiments reported here we find that maysin, pinitol, and condensed tannin all reduce growth largely through a reduction in ingestion. This is not to say that the mechanism is nonpreference. Nonpreference implies a choice-test situation. Simply eating less does not necessarily correspond with nonpreference in a choice-test situation. Given no choice, insects may quickly become conditioned to a compound and then consume essentially as much as the controls. Likewise, ingesting some compounds may bring about a "loss of appetite" without nonpreference behavior per se. With the type of experiments we have conducted, it would also not be possible to use the word antifeedant. A true antifeedant causes death by reducing food intake until the animal dies from induced starvation (Wright, 1963). Our compounds did not completely prevent feeding at the concentrations we tested. At concentrations sufficient to prevent feeding entirely, there may be toxic effects from the first bites taken, or at relatively high concentrations, death might occur sooner than if the insects were simply starved to death. Our compounds would come closer to fitting the broader requirements of a feeding deterrent (Higgins and Pedigo, 1979), although recent results suggest it is not this simple either (B.G. Chan, unpublished data).

Our experiments also suggest that there is a striking decrease in sensitivity to allelochemics during the first 24 hr of larval life. This may well correspond to the induction of any of several different detoxification systems in the larvae. This is not a new observation (Freedman et al., 1979), but further emphasizes the importance of a carefully designed bioassay. Although apparently less sudden, there is a similar relationship between larval age and biological activity in *Heliothis virescens* (Waiss et al., 1981).

The data for the controls in Tables 1–4 suggest that the larvae are sensitive to handling. In three groups, neonate larvae were placed on control diet and allowed to feed for 24 hr before being transferred to fresh control diet. The 11-day weight for these insects ranged from 23.4 to 31.5 mg. The undisturbed larvae in Table 4 averaged 69.7 mg, or 2.6 times heavier than the heaviest group of disturbed larvae.

The results of our experiments with cotton condensed tannin are of particular interest because of the assumptions made for many years about the probable mechanism of tannin-induced growth reduction, since tannins have been well-known for their ability to form complexes with other compounds,

particularly proteins. Thus, when Feeny (1968) found that oak leaf tannin reduced the growth of winter moth larvae [*Operophtera brumata* (L.)] and subsequently showed that oak leaf tannin forms a hydrolysis-resistant complex with casein in vitro (Feeny, 1969), it was widely assumed that the growth-inhibiting effects of tannins in insects are due to the formation in the gut tract of tannin–dietary protein complexes that are not readily digested. It is also widely assumed that many digestive enzymes may be complexed, further reducing the rate of assimilation across the gut wall. Various investigators have found no evidence to support the hypothesis that tannins reduce growth by reducing assimilation (Fox and Macauley, 1977; Bernays, 1978; Bernays et al., 1981). Chan et al. (1978a) have isolated a condensed tannin from cotton with a molecular weight of about 4850. This tannin inhibits the growth of *H. virescens*, but experiments with condensed tannin–casein or condensed tannin-polyamide complexes showed no reduction in biological activity (Chan et al., 1978a), suggesting that the ability of tannin to inhibit growth involves something other than a reduction in assimilation due to complexing with gut tract proteins. Also, Lawson and Klug (personal communication) found that when *Anisota senatoria* (J. E. Smith) feeds on *Quercus bicolor* Willd. (probably high in tannin), it has a nitrogen utilization efficiency of 39.4%. However, when *Pieris rapae* (L.) feeds on *Barbarea vulgaris* R. (probably low in tannin), its nitrogen utilization efficiency is 36.2% (Slansky and Feeny, 1977). It seems unlikely, therefore, that oak tannins are blocking assimilation in *A. senatoria*.

In our experiments with *H. zea*, we found once again that a tannin could be a relatively potent growth inhibitor, but we found little evidence for a reduction in assimilation. Instead, the primary mechanism through which growth was reduced was an inhibition of ingestion.

In the case of the winter moth, tannins may very well complex with dietary proteins, thus reducing assimilation. The assumption that this is how tannins inhibit growth in species other than the winter moth needs to be carefully reexamined, however. In the experiments cited above and in our work with *H. zea*, mechanisms other than a reduction in assimilation appear to be operating. In fact, after reviewing the literature quite thoroughly, Bernays (1981) concludes that ". . . there is as yet no unequivocal proof of an antidigestive effect of tannic acid or of condensed tannin in insects."

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EFFECTS OF SECONDARY METABOLITES FROM MARINE ALGAE ON FEEDING BY THE SEA URCHIN, *Lytechinus variegatus*

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Abstract—A bioassay was developed to test the hypothesis that secondary metabolites from marine algae affect feeding by sea urchins. During experiments measuring chemoreception and gustation, feeding by the regular sea urchin, *Lytechinus variegatus* (Lamarck), was inhibited by extracts from the green marine alga, *Caulerpa prolifera* (Forsskal) Lamouroux and *Cymopolia barbata* (Linneaus) Lamouroux. Cymopol, a monoterpene-bromohydroquinone component of *C. barbata*, was partially responsible for the inhibited feeding observed in tests of the *Cymopolia* crude extract. Caulerpenyne, an oxygenated sesquiterpene from *C. prolifera*, was responsible for all of the urchin feeding inhibition observed in tests of the *C. prolifera* crude extract. Feeding was not affected by: (1) extracts from several other *Caulerpa* species including *C. mexicana* (Sonder) J. Agardh, *C. ashmaedii* Harvey, *C. racemosa* v. *macrophysa* (Kützing) Taylor, and *C. racemosa* v. *laetevirens* (Montagne) Weber-van Bosse; (2) caulerpin, an indole-containing pigment isolated from all of the *Caulerpa* species except *C. mexicana*; and (3) an extract from the red marine alga, *Gracilaria foliifera* v. *angustissima* (Harvey) Taylor, which has no known secondary metabolites. Feeding inhibition was independent of the test diameter which correlated with the reproductive state of the urchins. Feeding inhibition was also independent of the starvation periods between experiments, and the temperature and salinity in ranges tolerated by *L. variegatus* obtained from the Florida Gulf Coast. The data strongly suggest that at least one alga, *Caulerpa prolifera*, is chemically defended against a dominant omnivore in its community.

Key Words—Chemical defense, feeding deterrents, cymopol, caulerpenyne, caulerpin, *Cymopolia*, *Caulerpa*, *Gracilaria*.

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INTRODUCTION

Food selectivity in most temperate and tropical sea urchins appears to be a compromise between food preferences and availability (Larson et al., 1980; Leighton, 1966, 1968; Ogden, 1976, 1980; Ogden and Lobel, 1978; Vadas, 1977; Vadas et al., 1982). For some sea urchins with generalized food preferences, the availability of food and ease of ingestion are believed to be the most important factors determining food selectivity (Ayling, 1978; Lawrence, 1975a; Lowe, 1974). Tropical sea urchins exhibit more diverse feeding habits than temperate urchins, possibly because of the availability of a more diverse food supply, which can be very patchy in distribution (Lowe and Lawrence, 1976; Ogden and Lobel, 1978). Feeding preferences in tropical urchins are usually characterized by avoidance of certain food items which then become highly suspect of maintaining strong chemical or structural defenses against herbivory (Ogden, 1976; Ogden and Lobel, 1978).

Lytechinus variegatus (Lamarck) is a major invertebrate consumer of plants and detritus in Gulf of Mexico and Caribbean seagrass communities dominated by *Thalassia testudinum* Köenig (turtle grass) (Gilmore and Gore, 1974; Humm, 1964; Kier and Grant, 1965; Moore et al., 1963; Ogden, 1980; Sharp and Gray, 1962). *L. variegatus* feed primarily on fresh *Thalassia testudinum*, *Thalassia* detritus, and sediment. *Lytechinus* also feed on the seagrass *Syringodium filiforme* Kützing and a variety of attached and drift algae that inhabit seagrass communities. The algae include species of *Caulerpa*, *Halimeda incrassata* (Ellis) Lamouroux, and *Ulva lactuca* Linnaeus (Bach, 1979; Keller, 1976; Lawrence, 1975a and references cited therein; Lowe, 1974; Lowe and Lawrence, 1976; Vadas et al., 1982). In laboratory feeding experiments, Lowe (1974) found that *Caulerpa ashmeadii* Harvey, *T. testudinum* (decomposing, living), and *S. filiforme* were preferred over *H. incrassata*, *U. lactuca*, and a *Sargassum* sp. The results correlated moderately well with food availability in the field and ability of *L. variegatus* to feed on food items (Lowe, 1974). From a field feeding study near Nicaragua, Vadas et al. (1982) found that *T. testudinum* was preferred over most species of algae. There was a general convergence in the diets of *L. variegatus* at different study sites toward *Thalassia* (58% of all feeding observations), especially detrital *Thalassia* (40% of diet). Detrital *T. testudinum* was taken on the basis of availability. Most other abundant plant species, especially certain green algae including *Caulerpa prolifera* (Forsskal) Lamouroux, *Penicillus capitatus* Lamarck, *Halimeda opuntia* (L.) Lamouroux, *Rhipocephalus phoenix* (Ellis and Solander) Kützing, and *Avrainvillea* sp., were avoided.

L. variegatus can consume all *T. testudinum* in *Thalassia*-dominated communities (Moore et al., 1963; Moore and McPherson, 1965) and several incidences of overgrazing have been noted (Bach, 1979; Camp et al., 1973;

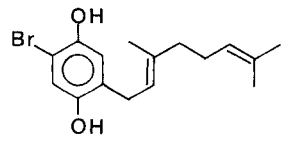
Vadas et al., 1982). *L. variegatus* is not unique in this regard. Examples of increases in urchin density through immigration or recruitment of juveniles by settlement which resulted in urchins exceeding the carrying capacity of plant production or "barren grounds" are well documented (Lawrence, 1975a). Field observation suggests that distance chemoreception often influences migratory drives of sea urchins (North et al., 1963). Because of the availability of drift material, many urchins can persist for years in areas where plants have been completely devoured (Lawrence, 1975a). The diverse feeding habits, including the facultative detrital feeding capabilities of *L. variegatus*, allow it to persist in disturbed areas. However, it is not clear what initially maintains population sizes below levels that usually do not disrupt seagrass communities (Vadas et al., 1982).

To investigate whether secondary metabolites from algae in *Thalassia*-dominated seagrass communities provide chemical defense and are important in determining food preferences of *L. variegatus*, we developed and utilized a bioassay to test the hypothesis that certain macrophytes which are potential food for *L. variegatus* possess chemicals that affect feeding by *L. variegatus*.

METHODS, MATERIALS, AND RESULTS

Preparation of Extracts and Compounds for Assay. The marine green alga *Cympolia barbata* (Linneaus) Lamouroux was collected from shallow waters in the Florida Keys. An ether-soluble extract (1.0% of plant's fresh weight) was obtained by isopropanol–CH_2Cl_2 (1:1) extraction, followed by Et_2O–H_2O partition, of the organic residue (McConnell et al., 1982). Cymopol (2-bromo-5-(3,7-dimethyl-octa-2(*E*),6(*E*)-dienyl)hydroquinone (0.07% of fresh weight, I; Figure 1) isolated from the ether extract by silica gel chromatography (McConnell et al., 1982) exhibited mass spectral, infrared, ultraviolet, and [^{1}H] NMR data identical with published values (Högberg et al., 1976). Of six brominated monoterpene hydroquinones isolated from *C. barbata*, cymopol was present in highest concentration. The remaining five compounds comprised 0.05% of the plant's fresh weight.

The marine green alga *Caulerpa prolifera* was collected from shallow waters in the Florida Keys and at Sarasota, Florida. An ether-soluble extract (1.0% of plant's fresh weight) was obtained by $CHCl_3$–MeOH (1:1) extraction of the fresh frozen algae, followed by removal of solvent in vacuo and Et_2O–H_2O partition of the organic residue. The oxygenated sesquiterpene, caulerpenyne (0.1% of fresh weight, II, Figure 1), was isolated from the ether extract by repeated Si gel thin- (0.2 mm, Si gel 60 F_{254}) and thick-layer (2.0 mm, Analtech-Si gel GF) chromatography. Caulerpenyne exhibited mass spectral, infrared, ultraviolet, and [^{1}H]NMR data identical with published values (Amico et al., 1978).



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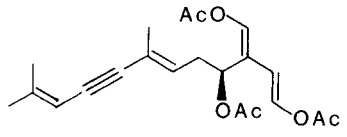

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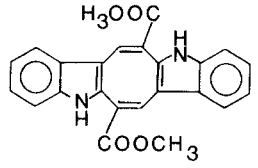

III

FIG. 1. Cymopol (I) from *C. barbata*, caulerpenyne (II) from *C. prolifera*, and caulerpin (III) from *Caulerpa* species isolated and bioassayed.

C. racemosa v. *laetervirens* (Montagae) Weber-van Bosse and *C. mexicana* (Sonder) J. Agardh were also collected at Sarasota, Florida. *C. racemosa* v. *macrophysa* (Kütz.) Taylor was collected at the southern end of Sunshine Skyway, Tampa, Florida, and *C. ashmaedii* Harvey was collected at Howard Park, Tarpon Springs, Florida. Only *C. ashmaedii* and *C. prolifera* were found in beds of *T. testudinum*. The fresh frozen algae were extracted separately with MeOH–$CHCl_3$ (1:1). Et_2O-soluble extracts were obtained from Et_2O–H_2O partition: *C. racemosa* v. *laetevirens*, 0.32% of fresh weight; *C. mexicana*, 0.34%; *C. racemosa* v. *macrophysa*, 0.34%; *C. ashmaedii*, 0.47%.

Caulerpin (III, Figure 1) was isolated from *C. racemosa* v. *macrophysa*, *C. racemosa* v. *laetevirens*, and *C. ashmaedii*. Open, gravity-flow columns of Si gel were employed initially with extracts from *C. racemosa* v. *macrophysa* and *C. racemosa* v. *laetervirens*. Fractions containing caulerpin from *C.*

racemosa and the extract from *C. ashmaedii* were next subjected to Si gel thin-layer chromatography using Et_2O–benzene (1:1). Caulerpin was finally purified by HPLC using μ-Porasil (2 × 30 cm) and Partisil 10 (50 cm) with 100% $CHCl_3$ or EtOAc–hexane (2:3) solvent systems: *C. racemosa* v. *laetervirens*, 0.005% of fresh weight; *C. racemosa* v. *macrophysa*, 0.011%; *C. ashmaedii*, 0.006%. Caulerpin exhibited mass spectral, infrared, ultraviolet, and [^{1}H]NMR data identical with published values (Maiti et al., 1978).

An ether-soluble extract (0.5% of plant's fresh weight) of *Gracilaria foliifera* v. *angustissima* (Harvey) Taylor was obtained by MeOH–$CHCl_3$ (1:1) extraction of fresh algae followed by Et_2O–H_2O partition. *G. foliifera* v. *angustissima* was obtained fresh from outdoor culture tanks at Skidaway Institute of Oceanography (LaPointe and Tenore, 1981; LaPointe, 1981).

Bioassay. Macroalgae with and without a coating of extract or compound were presented to *L. variegatus*. The amount of test algae consumed relative to controls was measured. For measurements of chemoreception and gustation as defined by Vadas (1977), an extract or compound in diethyl ether was evenly pipetted onto 1 or 2 g of the red alga *Gracilaria foliifera* v. *angustissima* (1 ml ether/1 g algae). The *Gracilaria* was readily consumed by *L. variegatus*. Feeding rates of 8–16 g algae/urchin/day on *G. foliifera* v. *angustissima* indicated a preference for the test alga (Table 1). Feeding rates on *Thalassia testudinum* have been reported as high as 6 g/urchin/day (Moore and McPherson, 1965; Ogden, 1980). Control algae were prepared by adding equivalent amounts of ether to *G. foliifera* v. *angustissima*. After the ether evaporated, the basal portions of test and control algae were embedded in paraffin on opposite sides of a disposable (plastic) Petri plate. The algae were moistened frequently with seawater to minimize desiccation. The plates were then placed at the bottom and in the middle of very well aerated (see Moore and McPherson, 1965) 10-gallon aquariums [27 cm (w) × 51 cm (l) × 31 cm (h)] with undergravel filters. The Petri plates were covered with gravel so that just the algae were above the gravel and available to the urchins. To ensure uniformity of surroundings, the sides of each aquarium were covered with aluminum foil. Because *L. variegatus* is negatively geotactic in aquaria and positively phototactic to filtered or artificial light (Sharp and Gray, 1962), many urchins were at the top of the aquariums. For experiments, all urchins were placed on the gravel approximately 10 cm from the algae. Experiments were run for 1–3 hr. At the conclusion of each experiment, the extract or compound was removed from the remaining test algae with ether. The test and control algae were blotted dry, reweighed, and the percentage of test algae consumed relative to control was determined. Weight loss of algae during experiments averaged approximately 5%, but was the same for test and control algae.

Standard error and propagation of error calculations (Shoemaker and

Table 1. Feeding by *L. variegatus* on *G. foliifera* v. *angustissima* With and Without Diethyl Ether Added

Bioassay parameters[a]	Feeding rates: With ether (g/hr ± SE)(*N*)	Feeding rates: Without ether (g/hr ± SE)(*N*)
I[b,c]	0.31 ± 0.13 (6)	0.30 ± 0.12 (6)
II[b,c]	0.63 ± 0.12 (12)	0.58 ± 0.21 (18)
II[c,d]	0.64 ± 0.14 (6)	0.68 ± 0.11 (6)
III[c,e]	0.54 ± 0.06 (12)	0.34 ± 0.10 (24)
		0.42 ± 0.09 (24)
		0.54 ± 0.09 (12)
III[e,f]	0.31 ± 0.08 (18)	0.31 ± 0.12 (18)

[a] I: $T = 26 \pm 1°C$, $S = 35 \pm 1‰$; 2 g test/control algae; 2 test + 2 controls/9-cm plate/aquarium; 6 urchins/aquarium; experiment ran 2–3 hr. II: $T = 20 \pm 1°C$; $S = 31.5 \pm 1‰$; 1 g test/control algae; 2 test + 2 controls/9-cm plate-aquarium; 6 urchins/aquarium, 1–3 aquaria; experiment ran 1 hr. III: $T = 20 \pm 1°C$; $S = 31.5 \pm 1‰$; 1 g test/control algae; $N = 12$–24; 1 piece of algae/5-cm plate/urchin; 6 urchins/aquarium, 2–4 aquaria; experiment ran 1–1.5 hr.

[b] The feeding rates are not g/hr/urchin because six urchins per aquarium competed for four pieces of algae. Also, all algae were either coated with ether or were completely ether free.

[c] Urchin test diameter range: 56–76 mm.

[d] Experiment parameters: 2 test algae with ether + 2 control algae without ether/aquarium.

[e] The feeding rates are g/hr/urchin.

[f] Urchin test diameter range: 38–53 mm.

Garland, 1972) yielded error limits. In experiments with three aquaria and two test and two control algae per aquarium (see below), the two-tailed Mann-Whitney nonparametric statistical test (Zar, 1974) was used to determine significance levels.

Feeding rate experiments were carried out by embedding the algae individually into 5-cm Petri plates and by placing the urchins approximately 2 cm from the algae.

We attempted to use a bioassay in which extracts or compounds were added to a preferred algal homogenate in 2% agar–seawater (Geiselman, 1980; Geiselman and McConnell, 1981) and presented to the urchins to measure chemoreception and gustation. These were not successful because the urchins did not consistently feed on the media itself and traces of unevaporated ethanol used to disperse extracts into the media partially inhibited feeding.

One hundred sea urchins obtained from the Gulf Specimen Company (Panacea, Florida) were used during the course of the experiments. The urchins with test diameters of 38–53 mm and 56–76 mm were infertile and fertile, respectively. Feeding was not corrected for test size, i.e., a "standard test size" was not used (Moore and McPherson, 1965). To prevent condition-

ing of *L. variegatus* on control algae, several grams of "iceberg" lettuce, which were readily consumed, were fed to each urchin between experiments.

To ensure that we were not measuring artifacts and that ether was not acting as an attractant or repellent, we determined feeding rates of *L. variegatus* on *G. foliifera* v. *angustissima* with and without ether added. Table 1 shows that ether had no effect on feeding.

The results of assays where algae were not embedded in agar (Geiselman and McConnell, 1981), but coated with extracts or compounds, as described here, would be invalid if substantial quantities of test material dissolved into the surrounding seawater. Nonpolar extracts from *C. barbata* and *G. foliifera* v. *angustissima* did not dissolve from test algae to a significant extent, but the polar extract from a sponge, *Cliona celata*, did (Table 2). Clearly, the assay we employed cannot be used to test effects of polar organic or water-soluble extracts.

Righting times have been considered a reliable index of the functional state or viability of *L. variegatus* for bioassays. Lawrence (1975b) determined that using its tube feet and spines, *L. variegatus* can right itself most rapidly when placed on its aboral surface to 90° (the position perpendicular to starting position) at 28° C and 35‰S. Lowe and Lawrence (1976) later used righting times to test urchin viability in a study to determine the efficiency with which *L. variegatus* absorbs selected marine macrophytes. If it took longer than 2 min for an urchin to right itself, it was not used. Moore and McPherson (1965) found that feeding rates of *L. variegatus* on *T. testudinum* varied widely between individuals and from day to day.

We determined that righting times were a valuable, but not totally reliable criterion to indicate the functional state of *L. variegatus*. The inverse

TABLE 2. REEXTRACTION OF EXTRACTS AFTER ASSAY[a]

Extract (range of % applied)	% Recovery (*N*)	Experiment duration (hr)
C. barbata (1.5–6.0)	88 ± 12 (12)	2–3
G. foliifera v. *angustissima* (1.0)	100 ± 2 (2)	2
Cliona celata (sponge) (polar EtOAc extract enriched in phenolics) (4.0–6.0)	16.3 ± 10 (2)	2.5

[a] $T = 26 \pm 1°C$, $S = 35 \pm 1$‰; 2 g test/control algae; $N = 1$; 2 test + 2 controls/9-cm plate/aquarium; 6 urchins/experiment/aquarium; experiment ran 2–3 hr; 1 experiment every 24 hr.

TABLE 3. CORRELATION OF RIGHTING TIMES AND FEEDING RATES OF *L. variegatus*.[a]

N	Ranges: Righting times (Y) (min)	Ranges: Feeding rates (X) (gm/hr)	$Y = A + BX$; $B \pm$ SE	Coefficient of determination, r^2 (% correlation)
25	1.6–12.0	0.24–0.61	−17.2 ± 8.4	15.2
24	2.0–12.0	0.27–0.55	3.8 ± 6.3	1.7
18	2.5–10.0	0.43–0.69	−10.6 ± 5.1	21.5
18	2.2–10.0	0.37–0.70	− 8.9 ± 4.4	20.3
18	2.3–10.0	0.25–0.64	−10.5 ± 5.7	17.6
17	3.0–14.5	0.28–0.79	− 4.3 ± 5.5	4.0
18	1.5–10.0	0.10–0.60	4.8 ± 6.6	3.2

[a] $T = 20 \pm 1°C$; $S = 31.5 \pm 1‰$; 1 g test/control algae; 1 piece of algae/5-cm plate/urchin; 6 urchins/aquarium; 3–4 aquaria; experiment ran 1–1.5 hr.

correlation between righting time and feeding rate, which is a more important parameter for bioassay purposes, was, in most experiments, moderately significant (Table 3). Smaller urchins righted quicker than larger urchins and larger urchins generally fed more rapidly than smaller urchins. However, correlations between righting times, test size, and feeding rates were usually only marginally significant (Tables 4 and 5). Righting times were used as a criterion for urchin viability, but with certain limitations. If there were more than two of six urchins in the same aquarium with righting times of >10 min before an experiment, the urchins were not used for that experiment.

. *Assays with* Cymopolia barbata *and* Gracilaria foliifera *v.* angustissima. Following development of the assay, experiments were carried out with the Et_2O-soluble extracts from *C. barbata* and *G. foliifera* v. *angustissima* and with cymopol (Table 6). An extract from *G. foliifera* v. *angustissima* was tested to determine whether it would affect feeding by *L. variegatus* and

TABLE 4. CORRELATION OF RIGHTING TIMES AND TEST DIAMETERS OF *L. variegatus*[a]

N	Ranges: Righting times (Y) (min)	Ranges: Test sizes (X) (cm)	$Y = A + BX$; $B \pm$ SE	Coefficient of determination, r^2 (% correlation)
18	2.5–10.0	5.6–7.2	0.8 ± .06	11.0
18	2.2–10.0	5.6–7.6	0.4 ± .08	1.4
16	3.0–14.5	4.3–5.3	−.06 ± .03	12.5

[a] $T = 20 \pm 1°C$; $S = 31.5 \pm 1‰$; 1 g test/control algae; 1 piece of algae/5-cm plate/urchin; 6 urchins/aquarium; 3–4 aquaria; experiment ran 1–1.5 hr.

TABLE 5. CORRELATIONS OF TEST SIZE AND FEEDING RATES OF *L. variegatus*[a]

	Ranges			
N	Test size (*Y*) (cm)	Feeding rates (*X*) (gm/hr)	$Y = A + BX$; $B \pm$ SE	Coefficient of determination, r^2 (% correlation)
18	5.6–7.2	0.43–0.69	1.1 ± 1.4	3.7
18	5.6–7.6	0.37–0.70	-1.9 ± 1.6	7.8
17	4.3–5.3	0.28–0.79	1.2 ± 0.5	8.2
18	3.8–5.3	0.10–0.60	0.3 ± 1.0	0.5

[a] $T = 20 \pm 1°C$; $S = 31.5 \pm 1‰$; 1 g test/control algae; 1 piece of algae/5-cm plate/urchin; 6 urchins/aquarium, 3–4 aquaria; experiment ran 1–1.5 hr.

possibly complicate interpretation of assay results. Chemical examination of the nonpolar Et_2O-soluble extract revealed that the extract lacked secondary metabolites (<0.001% of fresh weight). To determine if feeding preferences of *L. variegatus* would be affected by changes in several physical and biological parameters, experiments with *C. barbata* and *G. foliifera* v. *angustissima* were carried out with larger, fertile urchins and smaller, infertile urchins and at different temperatures, salinities, and starvation periods between experiments.

The first experiments were carried out with the Et_2O extract of *C. barbata* and cymopol (I) at $T = 26 \pm 1°C$ and $S = 35 \pm 1‰$ using large, fertile urchins. One aquarium with two test and two control algae was employed. Because feeding on either of the two test or control algae was not considered an independent event, consumption data for test and control algae were combined for each aquarium. Similarly, one aquarium with two test and two control algae was employed in experiments with the Et_2O extract of *G. foliifera* v. *angustissima* at $T = 22 \pm 1°C$ and $S = 33 \pm 1‰$ using large fertile urchins. Subsequently, three aquariums were employed for each experiment. For a sample size of three, the highest confidence level in the Mann-Whitney test is 0.1.

G. foliifera v. *angustissima* was a suitable alga for assay because at 0.5% of the alga's fresh weight, the concentration of the nonpolar extract which for *G. foliifera* v. *angustissima* may be defined as the "ecologically significant" concentration, the Et_2O extract neither inhibited nor stimulated feeding significantly by *L. variegatus*. From Table 6, changes in temperature and salinity at values tolerated by *L. variegatus* (Lowe and Lawrence, 1976) and changes in the starvation period between experiments did not affect feeding (Table 6). A slight reduction of feeding on test algae was observed only as the concentration of the Et_2O extract was increased to 3.0–5.0% in experiments at $T = 22°C$ and $S = 33‰$.

TABLE 6. CONSUMPTION BY *L. variegatus* OF *G. foliifera* V. *angustissima* COATED WITH EXTRACTS OF *C. barbata* AND *G. foliifera* V. *angustissima* AND WITH CYMOPOL (I)

% Extract or Compound Coated on *Gracilaria* (w/w)	% test algae consumed relative to control ±SE (*N*, significance level)[a]	*T*(°C)	*S*(‰)	Interval between experiments (hr)
Cymopolia barbata–Et_2O				
0.5	66 (1,-)	26	35	24
1.4	60 (1,-)	26	35	24
1.5	70 (1,-)	26	35	24
3.4	98 (1,-)	26	35	24
4.4	49 (1,-)	26	35	24
5.1	59 (1,-)	26	35	24
5.7	51 (1,-)	26	35	24
0.5	81 ± 27 (3,0.2)	20	31.5	24
0.6	64 ± 36 (3,0.2)	20	31.5	24
2.4	58 ± 14 (3,0.1)	20	31.5	24
0.5	69 ± 20 (3,0.1)	20	31.5	72
1.0	65 ± 19 (3,0.1)	20	31.5	72
1.4	58 ± 18 (3,0.1)	20	31.5	72
1.4	58 ± 18 (3,0.1)	20	31.5	72
2.4	44 ± 39 (3,0.1)	20	31.5	72
0.1[b]	82 ± 40 (3,NS)	20	31.5	72
0.8[b]	69 ± 43 (3,0.1)	20	31.5	72
1.0[b]	62 ± 26 (3,0.1)	20	31.5	72
Cymopol				
0.3	82 (1,-)	26	35	24
0.6	75 ± 24 (3,0.1)	20	31.5	72
1.2	63 ± 31 (3,0.1)	20	31.5	24
Gracilaria foliifera v. *angustissima*–Et_2O				
0.4	125 (1,-)	22	33	24
1.0	97 (1,-)	22	33	24
2.5	120 (1,-)	22	33	24
2.7	92 (1,-)	22	33	24
3.1	66 (1,-)	22	33	24
5.4	76 (1,-)	22	33	24
0.5	105 ± 46 (3,NS)	20	31.5	24
1.0	115 ± 28 (3,NS)	20	31.5	24
1.2	87 ± 23 (3,NS)	20	31.5	24
1.9	88 ± 36 (3,NS)	20	31.5	24
0.6	95 ± 46 (3,NS)	20	31.5	72
2.4	87 ± 52 (3,NS)	20	31.5	72

[a] $N = 1$: experiments with 2 test and 2 control algae in one aquarium – consumption data for test and control combined (see text); $N = 3$: experiments using 3 aquariums with 2 test and 2 control algae per aquarium. NS = inhibition of feeding not significant using two-tailed Mann-Whitney nonparametric test ($U > 0.2$).

[b] Urchin test diameter range: 38–53 mm; in all other experiments, urchin test diameter range: 56–76 mm.

At 1.0% of the alga's fresh weight, the ecologically significant concentration, the Et_2O extract from *C. barbata* inhibited feeding of *L. variegatus* by 35–40% in most experiments. Data from Table 6 indicate that food preferences of *L. variegatus* were unaffected by changes in temperature, salinity, starvation periods between experiments, and test diameter or reproductive state of urchin. Additional reduction of feeding was observed for concentrations of Et_2O extract greater than 1.0%. At concentrations of 10–20 times greater than found in *C. barbata*, cymopol (I) inhibited feeding of *L. variegatus*, but to a lesser extent than the Et_2O extract.

Evidence for feeding inhibition of *L. variegatus* by a secondary metabolite from an alga avoided by *L. variegatus* in the field was obtained from the following experiments.

Assays with Caulerpa *Species, Caulerpin and Caulerpenyne.* Although *C. barbata* was collected just inside the perimeter of a sparse *Thalssia* bed in the Florida Keys, neither *C. barbata* nor *G. foliifera* v. *angustissima* are as commonly encountered by *L. variegatus* as algae of the order Siphonales which includes species of *Caulerpa* and *Halimeda* (Bach, 1979; Dawes, 1974; Dawes et al., 1967; Hamm and Humm, 1976; Humm, 1964; Taylor, 1960; Vadas et al., 1981). However, one species of *Caulerpa*, *C. ashmaedii*, is preferred by *L. variegatus* (Lowe, 1974), while another species, *C. prolifera*, is avoided (Vadas et al., 1981). To investigate the causes for the preferential feeding, extracts and compounds from these algae and other *Caulerpa* species were assayed (Table 7).

Only caulerpenyne (II) and the Et_2O-soluble extract from *C. prolifera* caused significant inhibition of feeding. Even at 0.4% of the alga's fresh weight, the Et_2O extract substantially inhibited feeding whereas at 1.0%, the ecologically significant concentration, feeding was reduced further; only 50% of the test algae was consumed. Caulerpenyne was responsible for all of the activity of the extract; at 0.1% concentration, caulerpenyne reduced feeding by approximately 50%. In contrast, caulerpin (III) and extracts from *C. racemosa* v. *laetevirens*, *C. racemosa* v. *macrophysa*, *C. mexicana*, and *C. ashmaedii* did not inhibit or stimulate feeding significantly. Extract concentrations in assays ranged from 0.4 to 0.9%; concentrations of caulerpin in assays ranged from 0.1 to 0.3%. The latter concentrations were equal to or greater than the highest concentrations of caulerpin isolated from species of *Caulerpa*.

DISCUSSION

Numerous studies have shown that feeding preferences of sea urchins correlate positively with food availability in the field, efficiency of food absorption and assimilation, growth rate, reproductive development, chemo-

TABLE 7. CONSUMPTION BY *L. variegatus* OF *G. foliifera* v. *angustissima* COATED WITH EXTRACTS FROM SPECIES OF *Caulerpa*, WITH CAULERPENYNE (II) and CAULERPIN (III)[a]

% extract or compound coated on *Gracilaria* (w/w)	% test algae consumed relative to control ± SE (significance level)[b]
Caulerpa prolifera–Et_2O	
0.4	57 ± 44 (0.1)
0.4	72 ± 21 (0.1)
0.6	76 ± 54 (NS)
0.9	82 ± 85 (NS)
1.1	49 ± 22 (0.1)
Caulerpenyne	
0.07	68 ± 61 (NS)
0.08	49 ± 54 (0.2)
0.1	44 ± 40 (0.1)
0.1	59 ± 51 (0.2)
C. racemosa v. *macrophysa*	
0.5	111 ± 48 (NS)
C. racemosa v. *laetervirens*	
0.5	137 ± 20 (NS)
0.9	101 ± 17 (NS)
C. ashmaedii	
0.8	96 ± 58 (NS)
C. mexicana	
0.3	73 ± 33 (NS)
Caulerpin	
0.1	170 ± 147 (NS)
0.3	80 ± 47 (NS)

[a] $T = 20 \pm 1°C$; $S = 31.5 \pm 1‰$; $N = 3$; 2 test + 2 control algae per aquarium, 3 aquariums; urchin test diameter = 38–53 mm; each experiment run for 1 hr, 72 hr between experiments.
[b] NS = inhibition of feeding not significant using two-tailed Mann-Whitney nonparametric test ($U > 0.2$).

receptive preferences, and tast preferences (palatability). Feeding preferences, however, do not correlate with the caloric value of food (Ayling, 1978; Larsen et al., 1980; Lawrence, 1975a; Leighton, 1966, 1968; Lowe, 1974; North et al., 1963; Ogden, 1976; Ogden and Lobel, 1978; Vadas, 1977). The extent of each correlation with feeding preferences depends on the feeding habits and habitat of the urchin. In most studies, the utilization of secondary metabolites in chemical defense of algae against herbivory and the effects of these compounds on feeding preferences of sea urchins were suggested, but not proven. Examples of natural products as agents for chemical defense of algae include inhibitors of fish feeding and fish toxins from red marine algae (Paul and Fenical, 1980; Paul et al., 1980; Crews and Kho, 1975), green marine

algae (Sun and Fenical, 1979), brown marine algae (Gerwick et al., 1979; Gerwick and Fenical, 1981), a sponge and its nudibranch predator (Schulte et al., 1980), and a mollusk (Dieter et al., 1979). Polyphenols from two temperate brown marine algae inhibit feeding by the major herbivore, a mollusk, found in the algal community (Geiselman and McConnell, 1981). Our investigation provides evidence that feeding by the tropical and subtropical sea urchin, *L. variegatus*, a dominant plant and detrital consumer in seagrass beds of *T. testudinum*, is affected by secondary metabolites from algae found in *Thalassia* beds.

At concentrations found in the algae, caulerpenyne from *C. prolifera* and the Et_2O-soluble extracts of *C. prolifera* and *C. barbata* significantly inhibited feeding by *L. variegatus*. Cymopol accounted for some, but not all, of the feeding inhibition of the Et_2O extract of *C. barbata*. Other compounds from *C. barbata* may be more active or synergism may be important. These results demonstrate that secondary metabolites from marine algae inhibit feeding by sea urchins and verify that secondary metabolites affect urchin feeding preferences.

Because *L. variegatus* tend to aggregate and feed in groups (Moore et al., 1963; Camp et al., 1973; Lawrence, 1975a), the experiments closely simulated feeding in the field. Larson et al. (1980), Leighton (1966, 1968), Vadas (1977), and Lowe (1974) agree that providing several urchins with a variety of algae in excess in laboratory food preference studies yields the most ecologically significant results. However, Lawrence (1975a) and Carefoot (1967) feel that observing the positions of urchins or other herbivorous or omnivorous invertebrates relative to food (= chemoreception) is a more efficient method of studying food preferences than offering choices of food and measuring feeding rates (= chemoreception + gustation) (Leighton, 1966, 1968; Vadas, 1977). The argument purported by the former investigators is that, although choice is involved in both, the phenomena are distinct; urchins might be attracted to one alga, yet feed more rapidly on a second alga once feeding begins. More rapid feeding might be related to the ability of the urchin to manipulate and eat the food. Leighton (1966, 1968) and Vadas (1977) disagree, and it has been pointed out by Barkman (1959) that attractions other than food preferences (e.g., cover or spawning substrate) may be involved in chemoreception experiments. Our bioassay obviates both arguments since the same alga was used for test and control. The physical parameter of differential feeding due to different algal morphology and texture is normalized except at high extract concentrations where texture changes of test algae were noted. We attribute the inhibition of feeding by *L. variegatus* on, e.g., *G. foliifera* v. *angustissima* at 3.0–5.0%, to the "greasiness" of the extract coating.

Feeding rates of sea urchins vary with type of algae, season, temperature, urchin test diameter, physiological condition, urchin density in the field, and

starvation period between meals (Greenway, 1976; Lawrence, 1975a; Leighton, 1966, 1968; Moore, 1963; Moore and McPherson, 1965). Changes in temperature and salinity at values tolerated by *L. variegatus*, $S = 23–40‰$ and $T < 36°C$ (Lawrence, 1975b), however, did not affect feeding preferences for algae coated with extracts and compounds. Similarly, feeding preferences of larger, fertile urchins were not significantly different than feeding preferences of smaller, infertile urchins.

In many animals, adaptive phenotypic differences in digestive enzyme production are well established (Barnard, 1973). Therefore, it is possible that prestarvation periods much greater than that required for food passage through the gut of *L. variegatus* might result in concentration changes in digestive enzymes and food preferences in experiments. Larson et al. (1980) and Vadas (1977) starved *Strongylocentrotus* spp. for two weeks before experiments, and Leighton (1966) starved *Lytechinus anamesus* for two days. However, the time of food passage through the gut of *L. variegatus* is on the order of 24 hr (Vadas et al., 1982). Compared to *Strongylocentrotus* species in colder temperate waters, metabolic activities, including digestion, in *L. variegatus* from Caribbean waters are probably higher. We found that changes in starvation periods did not affect feeding preferences.

Because *L. variegatus* are capable of consuming all benthic plants in seagrass communities dominated by *T. testudinum* (Bach, 1979; Camp et al., 1973; Moore et al., 1963; Moore and McPherson, 1965), it is not clear what normally maintains population sizes of *L. variegatus* below levels that do not disrupt the communities (Vadas et al., 1982). Greenway (1976) proposed that crowding must inhibit the feeding rate of *L. variegatus* because it was observed that consumption rates of individuals were inversely proportional to density of urchins in *Thalassia* beds near Jamaica. Additionally, defensive strategies of algae against herbivory, including structural and chemical defenses (Ogden, 1976; Ogden and Lobel, 1978), may limit overgrazing. Structurally defended algae are hard, stiff, or calcareous and difficult for *L. variegatus* to manipulate and eat. Lowe (1974) suggested that *S. filiforme* and *T. testudinum* are easily ingested by *L. variegatus*, but that *U. lactuca*, a *Sargassum* sp., and *Caulerpa* spp. are not. Lowe (1974) further suggested that *Sargassum* is unpalatable because of tannin content and *L. variegatus* feed on some *Caulerpa* species because of chemical attractants. Our data show that *C. prolifera* is avoided by *L. variegatus* because of chemical defense. Other algae in *Thalassia*-dominated communities may also limit overgrazing by *L. variegatus* through similar chemical defenses.

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LOOPLURE EFFICACY AND ELECTROPHYSIOLOGICAL RESPONSES IN THREE PLUSIINAE[1] SPECIES

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Abstract—Source concentration differences of (*Z*)-7-dodecen-1-ol acetate, or looplure, were evaluated for field trapping efficiency and electrophysiological responses with male *Pseudoplusia includens* (Walker), *Trichoplusia ni* (Hubner) and *Rachiplusia ou* (Guenné) (Lepidoptera: Noctuidae). Sticky traps baited with 1000 μg of the lure captured a significantly greater ($P < 0.05$) number of male *P. includens* and *T. ni* than any other concentration; *R. ou* males were caught at a greater rate in traps baited with 100 μg of looplure, significantly more ($P < 0.05$) than with 1000 μg. Electroantennogram (EAG) studies demonstrated that antennae of male *P. includens* have a lower response threshold to looplure than either *T. ni* or *R. ou* antennae, the latter demonstrating the highest significant threshold of response. No differences in the stimulus–response functions of the three species were detected.

Key Words—Efficacy, electroantennogram, looplure, response threshold, sex attractant, trapping, *Trichoplusia ni*, *Pseudoplusia includens*, *Rachiplusia ou*, pheromone, Lepidoptera, Noctuidae.

INTRODUCTION

(*Z*)-7-Dodencen-1-ol-acetate, or looplure, has been identified as the sex pheromone for five noctuid moths (Berger and Canerday, 1968; Shorey et al., 1965; Tumlinson et al., 1972) and attracts 11 other species in the field (Tamaki,

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1977). The five noctuids for which looplure is the apparent sex pheromone include *Autographa biloba* (Stephens), *A. californica* (Speyer), *Pseudoplusia includens* (Walker), *Rachiplusia ou* (Guenee), and *Trichoplusia ni* (Hubner), members of the subfamily Plusiinae.

The lack of apparent specificity in the sex pheromone of this group has intrigued researchers for over a decade. Shorey et al. (1965) and Berger and Canerday (1968) demonstrated that not all species of Plusiinae tested exhibit the characteristic sex attractant responses to looplure, but all responded to extracts of all other species. If these different species utilize this single chemical as their sex pheromone, other factors must be involved in the reproductive isolation of each species. These may be differences in geographic distribution, habitat preferences, seasonal or daily cycles and rhythms, morphological differences, male and female behavioral interactions, and pheromone release rates. The possibilities of the presence of secondary chemicals, and consequently multicomponent sex pheromonal systems, are not without merit. Within the Plusiinae group there have been recent reports of laboratory and/or field identification of secondary, or minor, active components (Bjostad et al., 1979; Butler et al., 1977; Steck et al., 1979; McLaughlin et al., 1975).

The three species studied have overlapping geographic distribution, habitat preferences, and seasonal and daily cycles. (Alford and Hammond, 1981, unpublished data). The effects of several source concentrations of looplure were evaluated for field attractancy efficiency for *P. includens*, *T. ni*, and *R. ou*, and the male antennal responsiveness of the three species to the chemical was examined using the electroantennogram technique.

METHODS AND MATERIALS

Field Trapping Tests. From June through September, 1976–1978, four experimental sites were used in an effort to span the entire state of Louisiana. The sites were soybean fields ranging in size from 80 to 200 hectares. Sites included: (1) Niblett, Jefferson Davis Parish, located at latitude ca. 30° N; (2) Krotz Springs, St. Landry Parish (ca. 30° 30′N); (3) Bunkie, Avoyelles Parish (ca. 31° N); and (4) Bonita, Morehouse Parish (ca. 33° N). A site near Angola, Louisiana was used during 1976 instead of the Bunkie site, but due to its isolation, the more accessible Bunkie location was chosen for 1977 and 1978. The Bonita site was sampled during 1977 and 1978 only.

Populations of the three species were monitored with synthetic sex pheromone (Farchan Chemical Co.). Varying amounts of looplure were dissolved in hexane and soaked into rubber septa (5 × 7-mm rubber stoppers, sleeve type, Arthur H. Thomas Co.) and placed in Pherocon® 1C (Zoecon Corp.) sticky traps. The traps were hung from aluminum stakes placed east to

west 15 m apart (as described by Lingren et al., 1978), no more than 30 cm above the plant canopy and adjusted throughout each growing season. Two replicates were used at each site.

Experimental source concentrations of 100, 500, and 1000 μg were selected during the first few weeks of the 1976 season. During 1978 a fourth concentration of 10 μg was also evaluated in an effort to find a lower concentration in which no male moths were captured. An unbaited control trap was included at each site for each replicate.

Adult collections were made, concentrations randomized, and the looplure-impregnated septa replaced weekly. The traps were replaced every two weeks. Male genitalia were used to separate the three species (Eichlin, 1975).

The statistical analyses were based on a model containing the following sources of variation: weeks, years, sites, and concentrations. There were three years, an unequal number of sample dates (weeks) each year (12–22), three (or four) sites, three (or four) concentrations and two concentration replicates. An analysis of variance, with the model: years, week/year, site, concentration, and site $\times$ concentration, was used for the analysis of data for each species (Barr et al., 1976). For the analyses, one basic assumption was made: the sampling efficiency of the sex pheromone system described was absolute. Therefore, if no male moths of a particular species were captured during a sampling period, those zero responses were not included in the analysis. Capture rates of the blank (control) traps were not included in the analyses. but capture rates of the pheromone-baited traps were corrected for control capture.

Electroantennogram (EAG). Electroantennograms (Schneider, 1957) of male *T. ni*, *P. includens*, and *R. ou* to test concentrations were conducted using a modification of the apparatus and procedure described by Roelofs et al. (1971). Freshly excised head capsules were depressed into a wet wax block in a Syracuse watch glass containing insect Ringer's solution (Roelofs and Comeau, 1971). Antennae were exposed to short puffs of air (1 cm^3) directed into an airstream of ca. 250 ml/sec. Silver–silver chloride reference and recording electrodes were placed into the wax block and at the tip of the antenna, respectively. Antennal responses were amplified by a Grass® P 18 microelectrode DC amplifier and displayed on a cathode-ray oscilloscope. EAGs were recorded on a chart recorder. *T. ni* and *P. includens* were reared on an artificial diet (Burton, 1969) and maintained at a 14:10 light–dark cycle. *R. ou* was reared on a similar diet with the addition of 100 g lyophilized young soybean leaves (per 862.5 g soaked pinto beans) to increase pupation rate and kept at a 12:12 light–dark cycle. Specimens utilized ranged in age from 2 to 7 days posteclosion. No emphasis was placed on exact age or conditioning of the moths (i.e, time of exposure in relation to light–dark cycle).

Each antenna was exposed to the same concentrations unless activity and response decreased to a level less than ca. 75% of the initial standard response, at which point antennae were discarded. All pheromonal stimulations were followed by a 1-min interstimulus period of clean air. Each test began with the antenna being exposed to a "standard" 1 μg stimulus followed by a control. Each antenna was then exposed to an ascending series of looplure (Farchan, >98% chemical purity) source concentrations of 0.00001, 0.00005, 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 10, 100, and 1000 μg, 2–3 times per concentration. The concentration series were produced by pipetting the appropriate amounts, dissolved in hexane, onto 2 × 5-cm fluted filter paper slips and inserted into disposable glass pipets for release. Control filter papers were loaded with 5 μl hexane. Control and standard exposures were interspersed three times within the series and at the end.

The evalutation of EAG responses utilized the maximal amplitude elicited by a given stimulus minus the mean response of the preceding and succeeding controls. The sensitivity of the male antenna to concentrations was recorded as percentage of the mean of the preceding and succeeding responses to the 1-μg standard. Thresholds of response (Payne et al., 1970) were determined on an individual specimen basis, the assumption being made that an individual's threshold is unique due to both intrinsic and extrinsic factors which were not controlled or moderated. Mean thresholds of response and dosage–response curves were determined for the three series. From the dosage–response curves, lines were fitted by linear regression for comparative purposes. Regression equations were computed utilizing only the responses to concentrations at threshold levels and greater.

RESULTS AND DISCUSSION

The mean captures of the three species at the four source concentrations are presented in Table 1. Significantly greater ($P < 0.05$) numbers of male *P. includens* and *T. ni* were captured in traps baited with 1000 μg of looplure than any other concentration. However, the greatest numbers of *R. ou* were caught in traps containing 100 μg of the pheromone, significantly more ($P < 0.05$) than with 1000 μg.

The averages of the individual EAG thresholds of response for each species are given in Table 2. *P. includens* males were determined to have a lower threshold to the synthetic sex pheromone (0.12 ng) and a slightly higher mean response to the standard (2.12 mV ± 0.21). *T. ni* males had an intermediate threshold (0.30 ng) and standard response (2.09 mV ± 0.12); male *R. ou* had the highest threshold (0.45 ng) and the lowest response to the standard (1.59 mV ± 0.12). Dosage–response curves and fitted linear regressions are presented in Figure 1. The close relationships in the antennal

TABLE 1. MEAN TRAP CAPTURE OF 3 PLUSIINE SPECIES AT 4 CONCENTRATIONS OF SYNTHETIC SEX PHEROMONE IN LOUISIANA SOYBEAN ECOSYSTEMS

Species	Mean trap capture[a]			
	1000 μg	500 μg	100 μg	10 μ g
P. includens	6.16 a	4.06 b	1.63 c	0.27 c
T. ni	1.64 a	0.92 b	0.49 bc	0 c
R. ou	1.15 a	1.59 b	1.78 b	0.94 a

[a] Means followed by same letter in same row are not significantly different at the 5% significance level (Duncan's multiple-range test).

responsiveness of the three species to the chemical are evidenced by the similarities in the curve and regression characteristics. The slope determined for *P. includens* (13.60) was less than slopes for both *T. ni* and *R. ou* (16.36 and 17.32, respectively), while the intercept was greater for *P. includens* males. Coefficients of determination (R^2) were >0.96 for each species.

Comparisons of the results obtained by the two methods of source concentration evaluation (field trapping vs EAG) reveal that *P. includens* males are captured at highest rates in pheromone traps baited with the highest tested concentration of the synthetic material, and they exhibit a relatively low response threshold to looplure. On the other hand, *R. ou* males are captured more effectively in traps containing 10-fold less looplure. Similar field results were reported involving the two plusiines *T. ni* and *A. californica* which overlap widely in their geographic distributions, seasonal cycles, and daily mating rhythms. *T. ni* males were found to be more attractive to release rates of looplure 10–100 times greater than the most attractive release rates for *A. californica* males (Kaae et al., 1973).

Significant EAG response thresholds and stimulus–response functions for a moth may not be critical to its behavior. Mayer (1973) found the EAG to

TABLE 2. CALCULATED THRESHOLDS OF RESPONSE TO SYNTHETIC SEX PHEROMONE AND MEAN RESPONSE TO STANDARD DOSAGE (1 μg) OF 3 PLUSIINE SPECIES ($N = 15$/SPECIES)

	Average threshold of response (ng ± SE)	Mean response to standard (mV ± SE)
P. includens	0.12 ± 0.02	2.12 ± 0.21
T. ni	0.30 ± 0.05	2.09 ± 0.12
R. ou	0.45 ± 0.07	1.59 ± 0.12

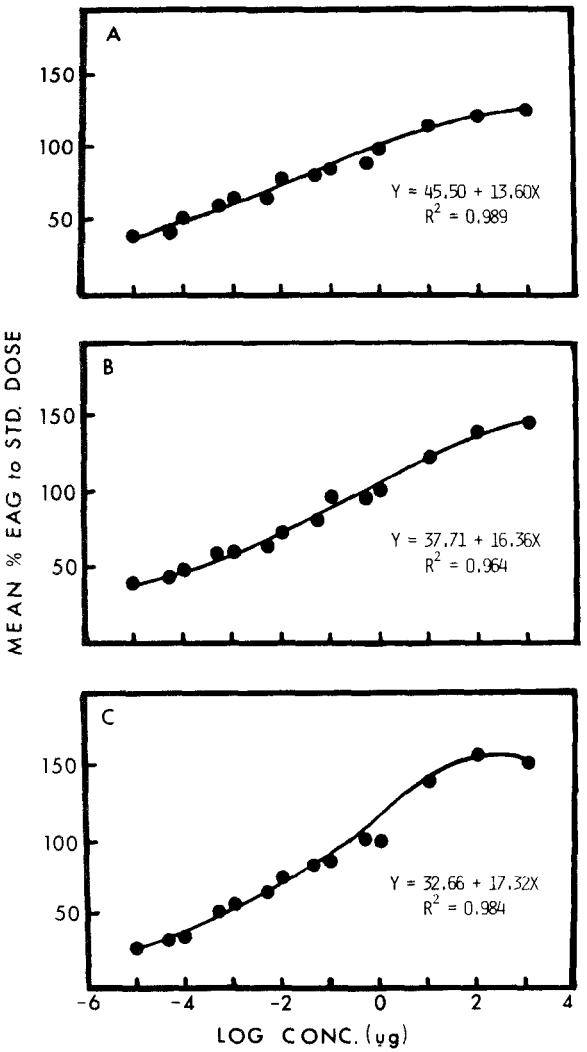

FIG. 1. (A) EAG amplitude expressed as mean percent to standard dose vs. log concentration of looplure and regression equation for male *P. includens*. (B) *T. ni* ($N = 15$/species). (C) *R. ou.* ($N = 15$/species).

be much less sensitive than the behavioral assay; his calculated threshold for the EAG of *T. ni* males (0.33 ng) was over 10^4 greater than that calculated for attractancy. The EAG technique may be most useful in the detection of pheromonal components involved in the natural system (Roelofs, 1978b) and not for behavioral analogies. Conversely, the use of trap capture rates for behavioral analogies may be equally invalid. Cardé (1979) points out that

pheromone trap capture with a synthetic chemical often has little biological validity. Important to consider are the limitations of the trap itself on the numbers of insects caught. Population density, site, behavior, trap density and design, as well as pheromone concentration, influence capture rates (Minks, 1977); the addition of minor pheromone components required for close-range attraction, as with *T. ni* (Bjostad et al., 1979), would certainly enhance trap captures. It is hoped the differences and similarities revealed here in the perception of looplure by the three species will prove more valuable when their natural sexual communication systems and the mechanisms of perception at the antennal sites are more fully understood.

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OLFACTORY RESPONSES OF ADULT *Tribolium castaneum* (HERBST), TO VOLATILES OF WHEAT AND MILLET KERNELS, MILLED FRACTIONS, AND EXTRACTS[1]

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Abstract—Olfactory responses of 72- to 96-hr-old, 24-hr starved adult male, female, or mixed-sex groups of the red flour beetle, *Tribolium castaneum* (Herbst), to volatiles from wheat and millet kernels, certain milled fractions, and solvent extracts were recorded by using a light-sensitive apparatus. Wheat-germ volatiles from intact germ or solvent extracts were generally more attractive than volatiles of wheat endosperm or wheat bran. Volatiles of whole wheat kernels were the least attractive among test materials of wheat origin, whereas there were no significant differences among whole wheat flour, germ, and endosperm. Wheat-germ extracts, however, were more attractive than were extracts of the other fractions. Whole millet flour or fermented millet flour volatiles were more attractive than those from whole millet kernels or millet starch. Beetles reached maximum responsiveness to grain volatiles by 72–96 hr after adult ecdysis. Groups of virgin female beetles generally were more responsive than male or mixed-sex groups to volatiles of substances tested.

Key Words—Red flour beetle, *Tribolium castaneum*, Coleoptera, Tenebrionidae, food volatiles, olfactory responses, olfactometer.

INTRODUCTION

Good (1936) and others reported that the *Tribolium* spp. can infest a wide range of stored commodities such as prepared cereal products, grain, and

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seeds; animal matter such as dry insect specimens; and yeast, nuts, dried fruits, chocolate, certain spices, and plant products. Willis and Roth (1950), Loschiavo (1959, 1965a, b), Schoonhoven (1968), Honda et al. (1969), Tamaki et al. (1971b), Nara et al. (1981) and others have demonstrated that several insect species are able to recognize the odors of their preferred food substances. Two-day-starved adult *T. castaneum* were strongly attracted to volatiles of flour kept below a wire screen (Willis and Roth, 1950), and females under uniform darkness were more responsive to volatiles of foods than were males (Soliman, 1975). Extracts prepared from brewer's yeast, unenriched patent flour, and bran of wheat elicited strong attraction, aggregation, and feeding responses by males and females of *T. castaneum*; the most active of all cereal extracts were those of wheat germ (Loschiavo, 1965a, b), attributed in part to the wheat-germ oils (Tamaki et al., 1971a, b; Nara et al., 1981). The attraction, as reported in most published studies, however, could have been optical and/or tactile as well as olfactory, because the experiments probably were conducted under white light and/or the insects were able to contact the odor source. In our experiments, we used a light-sensitive, event-detector unit (Pinniger and Collins, 1976) for monitoring only the olfactory responses of the adult *T. castaneum*; both optical and tactile stimuli were excluded.

METHODS AND MATERIALS

The red flour beetles, *Tribolium castaneum* (Herbst), used were from stock cultures that had been maintained for several years on whole wheat flour and dry yeast (95:5, w/w) in a room at 27 ± 1°C and 67 ± 3% relative humidity. Sexes were separated at the pupal stage (Good, 1936) to obtain adults of known age and sex. Newly emerged adults (0–24 hr old) in male, female, or mixed-sex groups were provided with food until 48–72 hr old, then starved for about 24 hr until 72–96 hr old. All insects used, except in preliminary tests, were 72–96 hr old.

The olfactory responses of *T. castaneum* were monitored by a light-sensitive, event-detector unit modified from Pinniger and Collins (1976), used in conjunction with a Heath model EUW-20A servo recorder. The detector unit (Figure 1) consisted of a rectangular Plexiglas base (35 × 20 cm), with two recessed light-dependent resistors 15 cm apart and covered with filter paper. A Plexiglas ring (15.2 × 2.6 cm) encircled the test arena and one test sensor, but excluded the other sensor. When the two sensors were equally illuminated by a suspended photographic red light (Loschiavo, 1959, reported that *T. castaneum* was unaffected by red light), the recorder pen was at the zero point on the recorder chart. As an insect passed onto the sensor in the arena, the light-intensity imbalance between the two sensors caused the recorder pen to travel up scale and remain thus until the insect had moved off

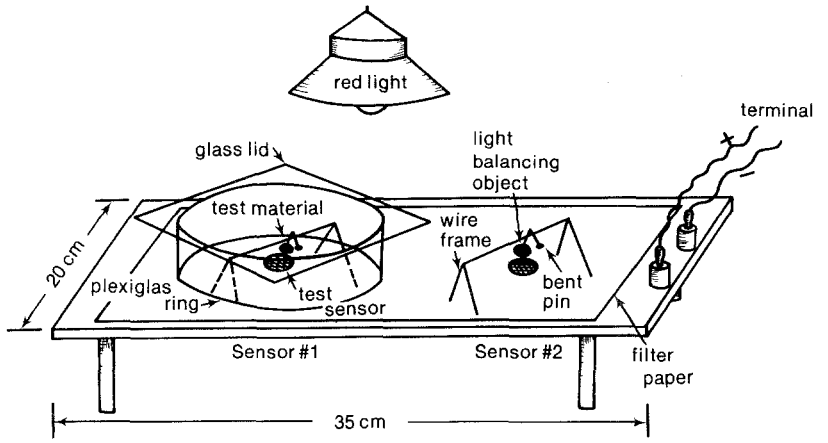

FIG. 1. Light-sensitive equipment used to study attractiveness of food volatiles to red flour beetles, *Tribolium castaneum* (Herbst).

the sensor. The chart speed was 1 inch per minute, which permitted us to calculate the time each insect spent on the sensor as well as the number of visits made per unit test. In groups of ten 72- to 96-hr old, 1-day starved male, female, or mixed-sex (5 + 5), each was placed in the test arena under the red light for 1 hr to acclimate. A control material was suspended over the sensor, and the visits to the sensor were recorded as peaks on the recorder chart during 10 min of observation. The control was then replaced by the test substance, and the visits were again recorded. The recordings were made for three 10-min periods for the control and for the test substance.

Test and control materials in either bagged or impregnated form were suspended directly above the sensor on a hooked, pointed end of an insect pin with a bend of about 60° at the center to hang on a wire frame (13.6 × 2 cm), high enough (1 cm) above the sensor so that beetles could not touch it with their antennae (Figure 1). For suspending whole wheat kernels, the pin point was inserted into the crease of the kernel. Whole wheat kernels, whole wheat flour, wheat germ, wheat bran, wheat endosperm, whole millet kernels, whole millet flour, bran-free fermented millet flour, millet starch, and their solvent extracts were tested. For each grain the whole kernels and their fractions were from the same lot.

The extracts were prepared by placing 100 g of kernels, or kernel fractions, in a funnel lined with glass wool and supported above a flask; 250 ml of fresh diethyl ether were poured, in three portions, over the kernels so that they were completely submerged by each portion. With flours or other grain fractions, a Buchner funnel with Whatman No. 1 filter paper was used. The

extracts were partially concentrated by a rotary vacuum evaporator, then concentrated to 1 ml in a volumetric tube by using a flow of nitrogen gas. A capillary tube was used to impregnate small cotton balls with 1, 5, 10, or 15 μl of extract equivalent to respectively 0.1, 0.5, 1.0, or 1.5 g of grain, flour, or fraction. The solvent was allowed to evaporate before testing.

Styrofoam pellets or clean cotton balls (treated with solvent only) were used as controls with whole wheat kernels or extract-impregnated cotton balls, respectively. There may have been a small degree of cross-contamination of the wheat fractions (germ, bran, and endosperm) during the normal milling process in the Department of Grain Science and Industry, Kansas State University. Data were analyzed for significant differences by Duncan's multiple-range test at the 5% level.

RESULTS

Preliminary tests of insects ranging from less than 24 hr to 144–168 hr old revealed that as they aged the adult beetles increasingly responded to volatiles

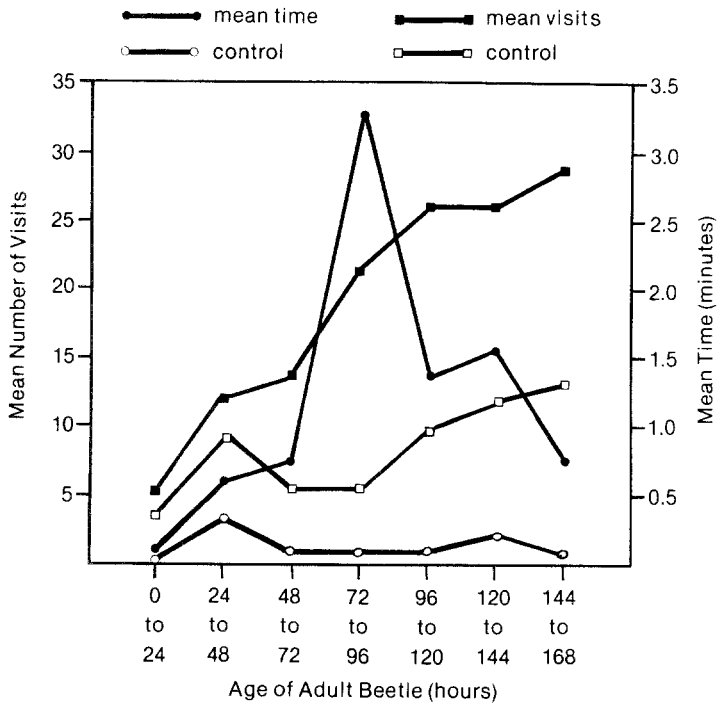

FIG. 2. Effect of age of *Tribolium castaneum* (Herbst) on response to whole wheat kernel volatiles.

of wheat kernels, measured in mean number of visits to the kernels during three 10 min periods. However, their response measured in mean time spent on the sensor during three 10-min periods increased as age increased up to 72–96 hr, then decreased (Figure 2). Thus, beetles between 72 and 96 hr old were considered most responsive and were used in subsequent studies.

Attractiveness of Volatiles of Whole Wheat Kernels and Wheat Fractions. Adult *T. castaneum* were significantly more attracted to wheat kernels and milled fractions than to the control materials (Table 1). The mean numbers of visits by the individuals of the three groups (pooled together) to whole wheat flour, wheat germ, and wheat endosperm, and the mean times spent on the sensor below those substrates were not significantly different. The attractiveness of volatiles of whole wheat kernels, measured in the mean number of visits, was significantly less than the attractiveness of the wheat fractions; the mean time the beetles spent on the sensor below the test materials, however, was not significantly different from that for the wheat fractions. Significant differences among the three groups were observed. Female beetles were significantly more attracted than were males or mixed sex groups to the whole wheat or wheat fractions. Yet males and females did not differ significantly in time spent on the sensor under these test substances.

Attractiveness of Volatiles from Extracts of Whole Wheat Kernels and Wheat Fractions. The responses of the three groups of *T. castaneum* to volatiles from extracts of hard red winter wheat kernels were not significantly

TABLE 1. RESPONSES OF ADULT *Tribolium castaneum* TO VOLATILES FROM WHOLE WHEAT KERNELS, WHOLE WHEAT FLOUR, OR WHEAT FRACTIONS[a]

	Mean number of visits[b]	Mean time spent (min)[b]
A. Sex		
Female	8.033 a	0.687 a
Male	6.233 b	0.457 ab
Mixed sex	6.000 b	0.404 b
B. Test material		
Whole wheat kernel	7.333 c	1.091 ab
Whole wheat flour	12.000 ab	1.374 a
Wheat germ	14.333 a	0.957 ab
Wheat bran	11.444 b	0.689 b
Wheat endosperm	14.444 a	0.949 ab
Control	1.600 d	0.020 c

[a]Means of responses of: (A) beetles (by sex) to 5 test materials considered together; (B) beetles (all sex groups) to each of the test materials. For each test, the 72- to 96-hr-old beetles were observed for three 10-min periods.

[b]Means followed by the same letter are not significantly different at the 5% level (Duncan's multiple-range test).

TABLE 2. RESPONSES OF ADULT *Tribolium castaneum* TO VOLATILES FROM EXTRACTS OF WHOLE HARD RED WINTER WHEAT[a]

	Mean number of visits[b]	Mean time spent (min)[b]
A. Sex		
Female	3.250 a	0.139 a
Male	3.452 a	0.114 a
Mixed sex	3.958 a	0.089 a
B. Extract concentration (g equivalent)		
0.1	2.778 b	0.103 b
0.5	5.278 a	0.183 a
1.0	3.111 b	0.086 b
1.5	3.167 b	0.085 b
C. Test or control material		
Extract of whole wheat kernel	5.611 a	0.195 a
Control	1.556 a	0.032 b

[a]Means of responses of: (A) beetles (by sex) to extracts (all 4 concentrations); (B) beetles (all sex groups) to each extract concentration; (C) beetles (all sex groups) to extracts (all 4 concentrations). For each test, ten 72- to 96-hr-old beetles were observed for three 10-min periods.
[b]Means followed by the same letter are not significantly different at the 5% level (Duncan's multiple-range test).

TABLE 3. RESPONSES OF ADULT FEMALE *Tribolium castaneum* TO VOLATILES FROM EXTRACTS OF SAME-LOT, UNKNOWN-AGE WHEAT KERNELS, WHEAT FLOUR, AND WHEAT FRACTIONS[a]

	Mean number of visits[b]	Mean time spent (min)[b]
A. Extract concentration (g equivalent)		
0.1	6.133 b	0.369 b
0.5	9.300 a	0.802 a
1.0	8.800 a	0.793 a
B. Test or control material		
Whole wheat kernel	4.889 d	0.317 cd
Whole wheat flour	8.889 c	0.550 c
Wheat germ	24.444 a	2.267 a
Wheat bran	14.778 b	1.727 b
Wheat endosperm	16.000 b	1.334 b
Control	2.356 e	0.074 d

[a]Means of responses of: (A) beetles (females) to each extract concentration (all 5 test materials); (B) beetles (females) to each test material (all 3 concentrations). For each test, ten 72- to 96-hr-old female beetles were observed for three 10-min periods.
[b]Means followed by the same letter are not significantly different at the 5% level (Duncan's multiple-range test).

different (Table 2). Significant differences were found between the control and the test substances and also among the different extract concentrations, measured by the mean number of visits and mean time spent on the sensor below the test material. Beetles in each of the three groups made significantly more visits to, and spent more time at, the 0.5-g grain equivalent concentration of extract of the hard red winter wheat than any other concentration. No significant differences were found among 0.1-, 1.0-, and 1.5-g grain equivalents, in either mean number of visits or mean time spent on the sensor (Table 2). Volatiles of wheat-germ extracts were most attractive when only females were exposed to three extract concentrations of each of the same-lot, unknown-age whole wheat kernels, whole wheat flour, wheat germ, wheat bran, or wheat endosperm, as indicated by the mean number of visits and mean time the females spent on the sensor (Table 3). The test substances were significantly more attractive than controls. The differences in mean number of beetle visits and mean time spent on the sensor below wheat endosperm or wheat bran were not significant. Volatiles from extracts of whole wheat kernels or whole wheat flour were less attractive. For all test materials, extract concentrations of 0.5- and 1.0-g equivalents were significantly more attractive than was that of 0.1-g equivalent (Table 3).

Attractiveness of Millet, Its Fractions and Extracts. Significant differences were found between controls and whole millet kernels or processed millet products in the response of the three groups (Table 4). Fermented millet flour and whole millet flour were statistically most attractive to the three

TABLE 4. RESPONSES OF ADULT *Tribolium castaneum* TO VOLATILES FROM WHOLE MILLET KERNELS, WHOLE MILLET FLOUR, OR MILLET PRODUCTS[a]

	Mean number of visits[b]	Mean time spent (min)[b]
A. Sex		
Female	10.750 a	0.445 a
Male	8.083 b	0.713 a
Mixed-sex	9.458 ab	0.728 a
B. Test material		
Whole millet kernel	9.333 c	0.832 b
Whole millet flour	18.556 ab	1.435 a
Fermented millet flour	20.444 a	1.806 a
Millet starch	15.556 b	0.704 b
Control	2.889 d	0.063 c

[a]Means of responses of: (A) beetles (by sex) to 4 test materials considered together; (B) beetles (all sex groups) to each of the test materials. For each test, ten 72- to 96-hr-old beetles were observed for three 10-min periods.

[b]Means followed by the same letter are not significantly different at the 5% level (Duncan's multiple-range test).

TABLE 5. RESONSES OF ADULT *Tribolium castaneum* TO VOLATILES FROM EXTRACTS OF WHOLE MILLET KERNELS[a]

	Mean number of visits[b]	Mean time spent (min)[b]
A. Sex		
Female	3.167 a	0.112 a
Male	3.125 a	0.098 a
Mixed-sex	3.667 a	0.122 a
B. Extract concentration (g equivalent)		
0.1	3.444 ab	0.104 ab
0.5	3.500 ab	0.134 a
1.0	4.056 a	0.161 a
1.5	2.278 b	0.042 b
C. Test or control material		
Whole millet kernel	5.111 a	0.204 a
Control	1.528 b	0.0161 b

[a]Means of responses of: (A) beetles (by sex) to extracts (all 4 concentrations); (B) beetles (all sex groups) to each extract concentration; (C) beetles (all sex groups) to extracts (all 4 concentrations). For each test, ten 72- to 96-hr-old beetles were observed for three 10-min periods.

[b]Means followed by the same letter are not significantly different at the 5% level (Duncan's multiple-range test).

groups pooled together, followed by millet starch and whole millet kernels as measured by either the mean number of visits or mean time spent on the sensor. Males were significantly less responsive than females or mixed-sexes in the mean number of visits, but there were no significant differences among them in the mean time spent on the sensor (Table 4). The differences in the responses among the three groups to volatiles of four extract concentrations of the whole millet kernels (pooled together) were not significant when measured by either mean visits or mean time spent on the sensor. By either measurement, responses of beetles of the three groups, considered together, to 0.1, 0.5, or 1.0 g were not significantly different; these three extract concentrations were more attractive than the 1.5-g equivalent, the highest concentration tested (Table 5).

DISCUSSION

Our experiments differed from most earlier studies of olfactory responses of stored-product insects in that we eliminated optical and tactile stimuli. Thus, responses, even near the source of the food attractants, were olfactory.

Our studies support those indicating that adult *T. castaneum* respond positively to the volatiles of wheat and its fractions. The wheat germ volatiles,

from raw germ, were as attractive as those of whole wheat flour or wheat endosperm, but more attractive than those of wheat bran. The germ volatiles from germ extracts were more attractive than those from whole flour or other fractions. Similar results were reported by Good (1936), Loschiavo (1965a, b), and Nara et al. (1981). Tamaki et al. (1971) attributed that to wheat germ triglycerides. Oosthuizen (1945), however, stated that of the wheat fractions, the germ appeared to be the least attractive to adult beetles.

The maximum response to grain volatiles was reached at 72–96 hr after adult ecdysis. The virgin female beetles were more responsive than males or those of mixed-sex in most experiments, which confirmed the report of Soliman (1975) for *T. confusum*. In contrast, Loschiavo (1965a) stated that the male and female *T. confusum* did not differ in their responses to volatiles from the same source.

The sex groupings tested were also attracted to volatiles of millet kernels and their fractions of solvent extracts. The most attractive components of the millet volatiles were not identified. Studies of Hougen et al. (1971) indicated that the different species and varieties of cereal grains produce largely the same components, but in different relative amounts. The flour from fermented millet no doubt contained different volatiles produced by microorganisms during fermentation and might have been, in part, responsible for the enhanced attractiveness.

Results demonstrate the usefulness of the light-sensitive apparatus of Pinniger and Collins (1976) in testing olfactory attractiveness of food volatiles and its potential for further studies on insect attraction to foods and on related insect behavior.

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IDENTIFICATION OF APPLE VOLATILES ATTRACTIVE TO THE APPLE MAGGOT, *Rhagoletis pomonella*[1,2]

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Abstract—Apple volatiles from whole Red Delicious and Red Astrachan apples were found to be attractive to sexually mature apple maggot flies, *Rhagoletis pomonella* (Walsh), in wind tunnel bioassays. Extracted volatiles elicited directed upwind movement towards the source and significantly increased the number of male and female flies arriving at the source. A behaviorally active fraction was obtained from crude extract by gas–liquid chromatography and assayed in two types of wind tunnels and by electroantennography. The major components in this fraction, identified by chemical derivatization reactions and GLC-mass spectrometry, were hexyl acetate, (*E*)-2-hexen-1-yl acetate, butyl 2-methylbutanoate, propyl hexanoate, hexyl propanoate, butyl hexanoate, and hexyl butanoate in a 35:2 8:12:5:28:10 ratio. Synthetics of the identified compounds and the natural extract elicited similar behavioral and EAG responses. None of the synthetics or natural components elicited full activity when presented alone.

Key Words—Host-plant attractants, Red Delicious, Red Astrachan, apple maggot, *Rhagoletis pomonella*, Diptera, Tephritidae, wind tunnel, electroantennography, hexyl acetate, (*E*)-2-hexen-1-yl acetate, butyl 2-methylbutanoate, propyl hexanoate, hexyl propanoate, butyl hexanoate, hexyl butanoate.

INTRODUCTION

The role of host plant odors in mediating apple maggot behavior has been a subject of much discussion and some study. Although field studies have

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indicated that apple tree volatiles are probably an important cue in the host-finding/selection process (Maxwell and Parsons, 1968; Neilson, 1971; Reissig, 1974), the determination of their exact role through these studies was complicated by the presence of variables such as visual and tactile cues. Prokopy et al. (1973) eliminated these factors to investigate the effect of apple and apple tree volatiles on the behavior of the adult apple maggot. From experiments conducted in a nonfruiting apple orchard, he found that freshly picked whole apples, in a state suitable for oviposition and obscured from view and touch, significantly increased trap catch over a control in three types of traps placed either in nonhost trees or in apple trees. Our study was designed to identify the compounds implicated in these results and to determine their behavioral effects.

METHODS AND MATERIALS

Insect Rearing and Collection. Laboratory insects from a culture (ca. 32 generations) maintained in Geneva were used for all tests. The larvae were reared on Red Delicious apples and adults were maintained on an artificial diet (Neilson and McAllen, 1965). Bioassays were conducted using sexually mature adults, between 7 and 14 days old. Adults were kept at 24° C under a 16:8 light-dark regime. Test flies were not exposed to apples as adults and were assayed only once. Flies were sexed just before testing.

Volatile Collection System. Apple volatiles were collected by passing compressed, charcoal-filtered air through a desiccator filled with apples and onto a column of Porapak Q (10 cm long, 2 cm diameter) (Byrne et al., 1975) for 48 hr. Twenty-five to thirty freshly picked whole apples were used for each collection. Crude extract was obtained by eluting the column with 100 ml of redistilled Skelly B and concentrating the extract under nitrogen to a volume of 1 ml. All extracts were stored in Teflon-lined screw-capped glass vials and kept at −10° C between tests.

Five separate collections were made from Red Delicious and two from Red Astrachan apples during the weeks of September 2–14 and July 1–7, 1979, respectively. At this time, the fruit was at a stage of development susceptable to oviposition. The apples were picked approximately 30 min before use. Each apple was washed with water, and all stems, leaves, and other debris were removed. The apples were not infested or diseased. Between collections, the desiccator was washed and rinsed thoroughly with acetone. The Porapak Q column was cleaned with 500 ml of Skelly B.

Chemical and Physical Determinations. The extracts were fractionated and visualized by GLC. The following glass columns were used: OV-101 (3% methyl silicone on 100–120 mesh Gas Chrom Q, 4 m × 2 mm), SP-1000 (10 %

Carbowax and substituted terephthalic acid on 100–120 mesh Chromosorb W-AW, 2 m × 2 mm), and Porapak Q (100-120 mesh, 2 m × 2 mm).

Electron impact mass spectra (EI-MS) and chemical ionization mass spectra (CI-MS) were obtained with a HP 5985 quadrupole mass spectrometer interfaced with a 46-m OV-101 capillary column. Isobutane was the carrier gas for the CI-MS.

The compounds in the active fraction were characterized by micro-reactions: LAH reduction in diethyl ether, acylation of alcohols with acetyl or propionyl chloride in CS_2, transmethylation of esters in 0.5 M methanolic KOH solution (acidified after 10 min with 1.0 M HCl and extracted with Skelly B) (Litchfield, 1972), and ozonolysis (as described in Geiselmann et al., 1979; Beroza and Bierl, 1967). Synthetic samples were purchased from K & K/ICN, Life Sciences Group (hexyl butanoate and butyl 2-methylbutanoate); Pfaltz and Bauer, Inc., Division of Aceto Chemical Co. (butyl hexanoate); Aldrich Chemical Co. Inc. (hexyl acetate); and California Aromatics and Flavors Co., Div. of Research Organic/Inorganic Chem. Corp. [(*E*)-2-hexen-1-yl acetate]. Unavailable esters were synthesized by adding 100 μg each of alcohol and acid to 1 ml of benzene (acidified by 1 drop of H_2SO_4) and molecular sieve (mesh 4 A°) to ensure dryness. This was heated at 100° C for 1 hr. A 50-μl aliquot was diluted with 1 ml of Skelly B and washed with distilled water (cleaned by extraction with methylene chloride). The product was visualized by GLC and collected from two columns (OV-101 and SP-1000).

Bioassays. Apple volatiles were assayed for behavioral activity in two wind tunnels simiilar to the one described by Miller and Roelofs (1976). Each tunnel was 1.2 m in length and in cross-section measured 61 cm in width at floor level and 52 cm at its highest point. Six Vita-Lite fluorescent bulbs, 1.24 m in length, covered by a translucent plastic diffusing plate, were suspended over each tunnel. The light from these bulbs simulated the full spectrum of sunlight, and the light intensity at the top, midpoint, and floor of the tunnels measured 15.2, 14.9, and 14.4 lux, respectively. The lateral distribution of light from above was very even. The wind velocity was 40 cm/sec, and temperatures were held between 24 and 26° C. Water was provided at the downwind end of both tunnels.

The assay was designed as a two-choice test. The treatments were placed 36 cm apart on a wire screen at the upwind end of the tunnel. A treatment was prepared by placing 100 μg (in a 20-μl aliquot) of the extract to be tested on a cotton wick. Control wicks were prepared with an equal amount of Skelly B. The solvent was evaporated before testing. For each test, 100 flies of the same sex were released into the downwind end of the tunnel. Female flies were used for all tests except those comparing the synthetic mixture to the crude, in which the response of both male and female flies was recorded in separate tests. All bioassays were run between 0800 (2 hr after initiation of photophase)

and 1800 EST. The order in which the treatments were tested and the side of the tunnel on which they were presented were determined randomly. Each pair of treatments was tested five times.

In the first set of assays, Red Delicious and Red Astrachan crude extracts were tested against a control. The treatment and control wicks were each suspended behind a perforated ceresin wax sphere (8 cm diameter) (Prokopy 1966, 1967), which provided a convenient means of egg collection. The total number of visits to each sphere and the number of eggs laid were recorded over a 4-hr period. The first set of GLC fractions and a crude extract were similarly compared to a control but egg counts were not made.

After the major components in the active fraction were characterized, a synthetic mixture of the components (in the same concentration and ratio found in the crude extract), the crude extract, and a control were tested in the wind tunnel. For this experiment, each wick was wired to the top of a white sticky board trap (7 × 20 cm). By eliminating the use of the spheres, which are visually attractive to the files (Prokopy, 1968), the effect of the volatiles alone could be studied. Three pairs of treatments were tested: the crude extract and the synthetic mixture, the crude extract and Skelly B, and the synthetic mixture and Skelly B. Both males and females were tested separately. Each paired set of treatments was tested five times for each sex. Each test ran 10 hr.

Both wind tunnels were used to determine the effects of visual attractants on the response of the flies to volatiles. In this experiment, red artificial florist apples (plastic) covered with Stickem were used in one wind tunnel, while white sticky board traps (as previously described) were used in the other. Each tunnel contained a test treatment of the synthetic mixture and a check. The wicks were wired to the tops of the apples and the tests were run simultaneously. The number of flies caught on each trap was counted every 2 hr for 8 hr. After each count the flies were removed. Treatment placement in the tunnel was determined by random selection, and the type of trap used in each tunnel was alternated for each of the five replicates. Only female flies were tested.

Bioassays were also run in a glass tube olfactometer. This consisted of a glass tube (80 cm long, 10 cm diameter) connected to a glass air-intake system through which filtered, humidified air was pumped (20 cm/sec). This apparatus was designed so that the flies' behavior could be observed more extensively and ensured that all the flies were in close contact to the volatiles being tested. The treatments were presented individually and each test was replicated five times. The order of presentation was determined by random selection. A 20-μg sample (in a 5-μl aliquot) was placed on a disk (2.4 cm) of filter paper, which was stapled onto a metal mesh screen (8 wires/cm) and placed at the the upwind end of the tube. Thirty female flies were placed in the downwind end and allowed to accommodate to the airstream for 5 min before

an extract was introduced. The tube was marked into eight 10-cm sections. The number of flies that touched the filter paper and the number of flies in each section was recorded every minute for 15 min. After a fly touched the source, it was removed to prevent duplicate counts. Other behaviors, such as oviposition attempts, circling, and turning were also monitored. The olfactometer was washed, rinsed thoroughly with acetone, and allowed to dry between tests.

Electroantennograms were conducted as described previously (Roelofs, 1977), except that the entire head was excised and the antenna tip was not snipped. Each set of treatments was tested in random order on antennae from five different insects. Both male and female flies were tested.

RESULTS

Isolation and Bioassay of Active Volatiles. Both the Red Delicious and Red Astrachan crude extracts, tested separately, attracted a significantly ($P < 0.01$, t test) large number of female flies (20.2 ± 4.9 and 25.0 ± 6.4, respectively, $N = 5$) to spheres in the tunnel than did the control (9 ± 3.81 and 8.4 ± 5.59, respectively). More eggs were laid on the treated spheres (6.8 ± 3.42 and 9.8 ± 4.92, respectively, $N = 5$) than on the control spheres (2.4 ± 2.07 and 2.6 ± 1.82, respectively), but if the number of eggs laid per visit was averaged from the total number of eggs deposited and total number of visits, there was no difference between the treatments and their controls in the number of eggs laid per visit or the number of eggs laid per total number of visits to the spheres.

The crude extracts from both varieties were fractionated by GLC (OV-101, temperature programed from 40 to 190° C, 15° / min, 2-min delay, 2-min collections). These fractions were tested in the wind tunnel (Figure 1), in the glass olfactometer (Red Delicious only tested, Figure 2), and by EAG. The 8 to 10-min fraction was the most active for both varieties (Figure 1). In both the wind tunnel and glass olfactometer, significantly more flies landed on the source containing the 8 to 10 min Red Delicious fraction than on all the other fractions and the control, and it was as active as the crude extract. The Red Astrachan 8 to 10 min fraction gave the same results in the wind tunnel. The EAG responses (females tested only) corroborated the bioassay results (Figure 1).

Behavioral observations in the glass tube olfactometer (Figure 2) revealed that females exposed to the 8 to 10 min Red Delicious fraction or the crude extract attempted to oviposit more frequently (perpencidular thrusts of the abdomen with the ovipositor extended against a flat surface). Circling (360° turns with a 5-cm diameter or less) and turning (more than a 90° turn in

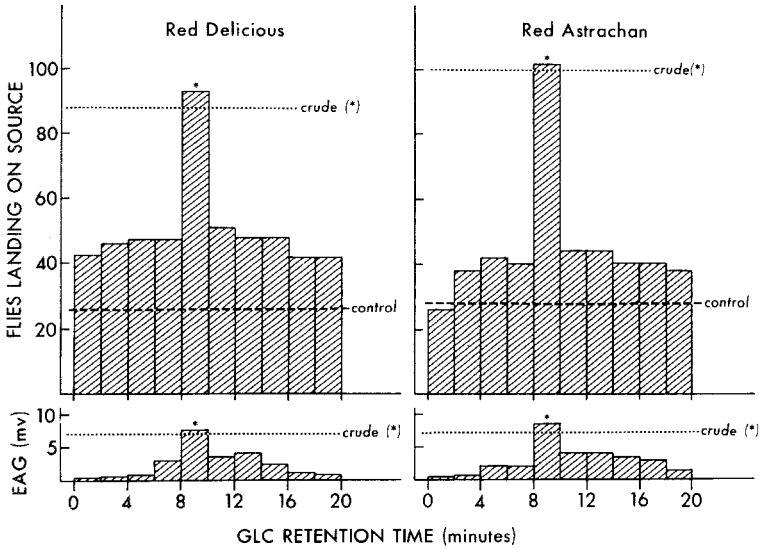

FIG. 1. Wind tunnel bioassay and electroantennogram responses to Red Delicious and Red Astrachan crude extract, GLC-fractioned extract, and a control. Bioassay results are the total number of flies responding in five replicates and the EAG values are the mean results from five different antennae. Treatments marked by asterisks are significantly different from nondenoted treatments according to Dunnett's test, $P < 0.01$ (Dunnett, 1955).

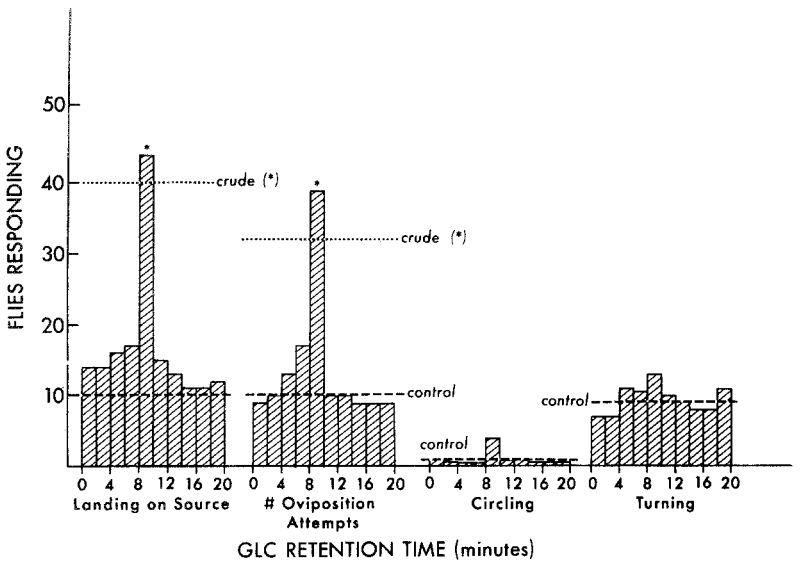

FIG. 2. Behaviorial responses in the glass tube olfactometer to GLC-fractionated Red Delicious extract. Bioassay results represent the total number of responses in five replicates. Treatments marked by asterisks are significantly different according to Dunnett's test, $P < 0.001$.

either direction) were not significantly different among the treatments and the control (Figure 2).

The active Red Delicious fraction was fractionated further by GLC (Ov-101, at 110° C, 10 two-minute collections). The fractions were bioassayed in the glass tube olfactometer. The 4 to 6 min fraction attracted a significantly larger number of female flies to the filter paper than the control. It was not significantly more active than the crude extract. Electroantennogram responses supported the bioassay results (Figure 3).

Identification of Active Components. The 4 to 6 min fraction was injected onto the OV-101 column at 90° C. Five major peaks were observed (Figure 4). These were collected separately and injected onto the SP-1000 column (90°) and the 46-m OV-101 capillary column (temperature programed from 30° to 240°, 2° / min). Peak A separated on both columns into two peaks (A_1 and A_2) and peak E separated into two peaks (E_1 and E_2) on OV-101. All peaks were individually collected and mass spectra (EI-MS) for the natural compounds and synthetic standards were obtained. The spectra indicated the following structures for each peak: A_1:hexyl acetate (35%), A_2:(*E*)-2-hexen-1-

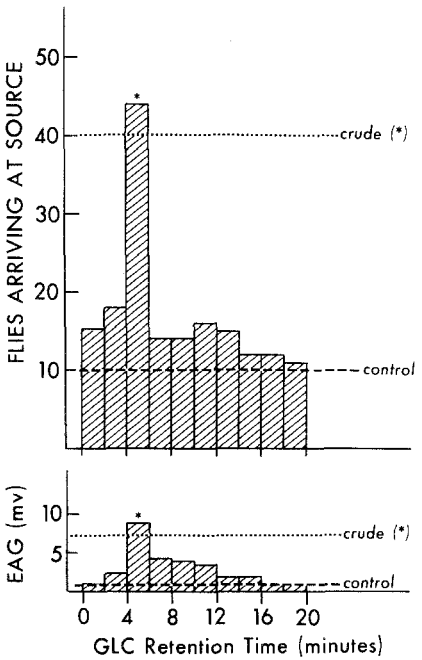

FIG. 3. The active 8-to 10-min GLC fraction (Figures 1 and 2) further fractionated on OV-101 and assayed in the glass tube olfactometer and by electroantennography. Significantly different responses are indicated by asterisks (Dunnett's test, $P < 0.10$).

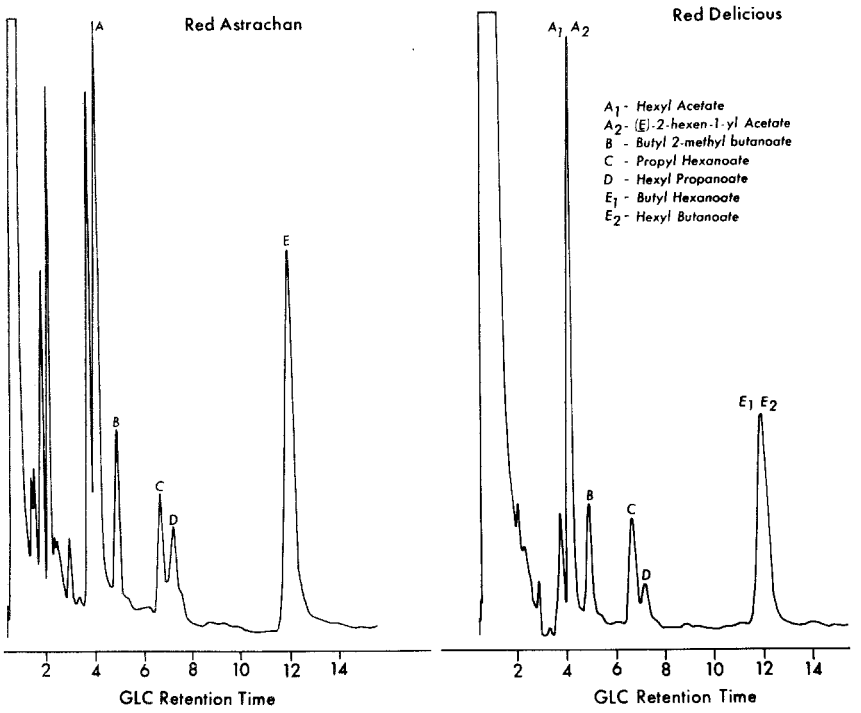

FIG. 4. Gas chromatogram of the behaviorally active 4-to 6-min. Red Delicious GLC-fractionated extract (Figure 3) and the corresponding crude Red Astrachan extract on OV-101 at 90°. The labeled peaks in the Red Delicious extract were characterized as listed. The compounds preceding peak A were not biologically active.

yl acetate (<2%), B: butyl 2-methylbutanoate (8%), C: propyl hexanoate (12%), D: hexyl propanoate (5%), E_1: butyl hexanoate (28%), and E_2: hexyl butanoate (10%). The spectra for each peak were consistent with the corresponding standard.

Each ester, except A_1 and A_2, was methylated to characterize its acid portion. The resulting methyl ester was injected on OV-101 and SP-1000, and the retention times were compared with methyl ester standards of all possible isomers. EI-MS and GLC retention times (OV-101 and SP-1000) of the methylated natural components were identical to those of the corresponding methyl ester standards of the compounds cited above.

The alcohol portion of the ester was also characterized. The esters, including A_1 and A_2, were treated with LAH and the resulting alcohols were acylated. Peaks A_1 and A_2 were acylated with propionyl chloride while the other esters were treated with acetyl chloride. GLC values for the resulting ester products (on OV-101 and SP-1000) were compared to those of synthetic

standards of all possible isomers. EI-MS and GLC retention times of the apple ester derivatives were identical to those of the corresponding standard acetates of the esters listed above.

Ozonolysis of Peak A_2 produced a peak on the Porapak Q GLC column (190°) at 4.2 min. The retention times of the ozonolysis product and that of *n*-butyraldehyde from the ozonolysis of standard (*E*)-2-hexen-1-yl acetate were identical as determined by coinjection.

EI-MS and GLC retention times (on OV-101 and SP-1000) for the natural and synthetic esters were similar in all cases.

The natural ratio of compounds in the active fraction was determined by GLC and EI-MS OV-101 retention times. This ratio was found to change as the season progressed. The profile of the active components found in the Red Astrachan active fraction was similar to that found in Red Delicious (Figure 4).

Bioassays of the Synthetics. Bioassays to determine whether a synthetic mixture of the identified compounds would elicit the same behavioral responses as the crude extract were run in both the wind tunnel and the glass tube olfactometer. The synthetic components A_1, A_2, B, C, D, E_1, and E_2 were prepared volumetrically, equivalent to the natural ratio, and analyzed by GLC.

In the wind tunnel, both the crude extract and the synthetic mixture caught significantly more female flies on the white sticky boards than did the control (Table 1). The crude extract and the synthetic mixture were equally attractive when tested against each other. The response of male and female flies were similar.

TABLE 1. RESPONSES OF MALE AND FEMALE *R. pomonella* TO APPLE VOLATILES IN WIND TUNNEL BIOASSAYS

Treatment	Mean fly catch[a]	
	Females	Males
1. Red Delicious crude extract	56.0 ± 8.1[b]	42.4 ± 5.9[b]
Skelly B control	19.4 ± 4.9	19.8 ± 1.9
2. Synthetic blend[c]	51.6 ± 6.1[b]	59.6 ± 8.8[b]
Skelly B control	19.4 ± 6.4	17.8 ± 2.9
3. Red Delicious crude extract	39.2 ± 5.0	38.4 ± 4.8
Synthetic blend	42.6 ± 6.8	42.6 ± 6.7

[a]Five replicates for each sex in each test.
[b]Significantly different from the control by *t* test analysis at $P < 0.01$.
[c]Consisted of compounds A_1, A_2, B, C, D, E_1 and E_2, and used at a rate similar to that of the crude extract (100 μg in 20 μl of Skelly B).

TABLE 2. WIND TUNNEL RESPONSES OF FEMALE *R. pomonella* TO APPLE VOLATILES ON TWO TYPES OF TRAPS

Treatment	Mean fly catch[a]	
	White panel traps	Artificial apple traps
Red Delicious crude extract	58.4 ± 8.9a	75.8 ± 25.8a
Skelly B control	17.0 ± 2.1b	16.8 ± 2.9b

[a]Five replicates, 100 females/test. Traps were checked and cleaned every 2 hr. No significant differences ($P < 0.01$, t test) in the rate of catch were found between the two types of traps when containing the same treatment. Treatments followed by the same letter are not significantly different by t test analysis, $P < 0.01$.

The use of the red artificial apples did not increase the rate of response to the volatiles as compared to the white sticky traps. The test treatment for both trap types caught significantly more flies than its corresponding check, but the artificial apples containing the test treatment did not catch significantly more flies than the white traps with the same treatment. The same held true for the check traps. No significant differences could be determined for the rate of fly catch on either type of trap (Table 2).

Bioassays in the glass tube olfactometer also showed that the synthetic mixture was as active as the crude extract and that both treatments attracted significantly more flies (7.6 ± 1.8 and 7.0 ± 1.6, respectively) to the source than did the control (1.0 ± 0.7) (Waller and Duncan $P < 0.01$). Additionally, the crude extract and the synthetic mixture elicited a significant change in distribution up the tube toward the source, when compared to the control, throughout the course of the test (Figure 5).

Electroantennogram tests comparing the crude extract with the synthetic mixture did not show a significant difference in antennal response (mV) for either females (7.2 ± 0.1 and 7.4 ± 0.1, respectively) or males (7.0 ± 0.1 and 7.2 ± 0.1, respectively).

When the individual components of the active fraction were tested in the glass tube olfactometer, none of the compounds tested alone were as active as the crude mixture. Hexyl acetate and (*E*)-2-hexen-1-yl acetate were not significantly different from the control. Electroantennogram assays gave similar results with both sexes (Figure 6.).

DISCUSSION

The response of apple maggot flies to various visual stimuli, such as size, shape, and color, has been well documented (Prokopy, 1968, 1977). It appears

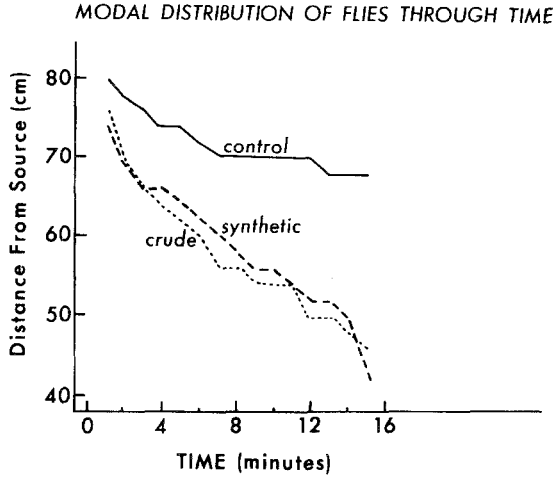

FIG. 5. The movement of flies toward treatments in the glass tube olfactometer. The olfactometer was marked into 8 (10 cm) sections and the number of flies in each section was recorded every minute. Data from five replicates of each treatment were summed for each minute and the arithmetic mean was calculated according to location to designate the location of the majority of the fly population as the test proceeded.

that the flies are attracted over relatively short distances to the visual cues from apples, the site of mating, and the host tree (Prokopy, 1968). Differential damage to various apple cultivars has frequently been observed in the field (Dean and Chapman, 1973), and the long-range migration of flies to susceptable varieties has also been recorded in apple orchards (Maxwell and Parsons, 1968; Neilson, 1971). Although the exact stimuli involved in the orientation of the flies to certain varieties of apple is not known, Prokopy et al. (1973) have demonstrated that apple volatiles may play an important role in the long-range attraction of the flies to the host.

This work has shown that Red Delicious and Red Astrachan apples contain chemicals that elicit both a behavioral and electrophysiological response in laboratory-reared male and female apple maggot flies. In the wind tunnel, the presence of the volatiles significantly increased the number of flies that landed on the source. The same response also occurred in the tube olfactometer in which directed upwind movement toward the source was observed. Although the exact nature of the behavioral responses elicited by the apple volatiles has not been defined, in the tube olfactometer flies were not simply activated in a nondirected pattern to arrive at the source but moved steadily toward the volatiles by walking or short hopping flights. The amount of circling and turning was not significantly different from the checks. Therefore, although klinokinesis (Kennedy, 1965, 1977) may be involved,

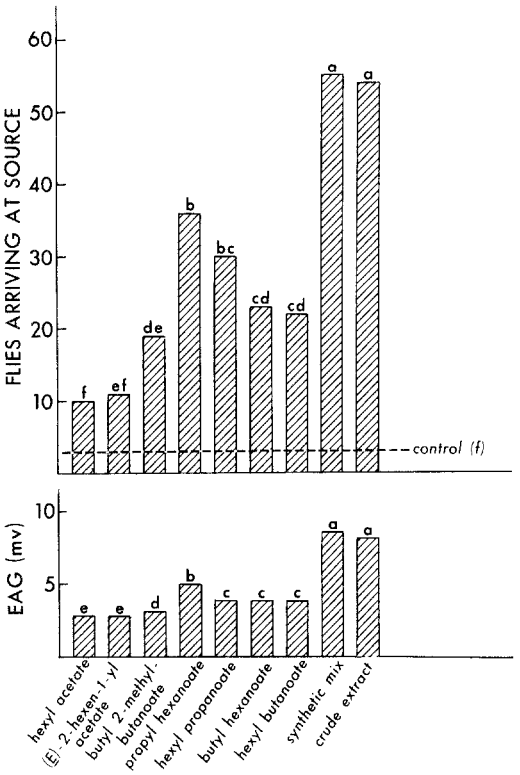

FIG. 6. Responses to synthetic components of the active fraction tested individually, the synthetic mixture, and the crude extract in the glass tube olfactometer and by electroantennography. Bioassay results are the total number of flies responding in five replicates and the EAG values are the mean results from five different antennae. Treatments followed by the same letter are not significantly different at $P < 0.01$ (Waller and Duncan BSD rule).

directed movement toward the source was the main behavior observed that could explain our results. Positive anemotaxis in response to host volatiles has been reported for onion flies, *Hylemya antiqua* (Dindonis and Miller, 1980), cabbage root fly, *Erioschia brassicae* and *Delia brassicae* (Hawkes and Coaker, 1976; Hawkes et al., 1978), desert locust, *Schistocerca gregaria* (Haskell et al., 1962; Kennedy and Moorhouse, 1969), and the Colorado potato beetle, *Lepinotarsa decemlineata* (Wilde et al., 1969). In the present study, in both the wind tunnel and the tube olfactometer, casting across the odor plume was never observed.

In both olfactometers, the active volatiles elicited an increased number of

oviposition attempts. However, in the wind tunnel, when spheres were provided to monitor egg deposits, the number of eggs laid per visit was not significantly different between the crude extract and the check. Inadequate stimuli or the increased amount of interaction among the flies on the test spheres may have been responsible for this result. The number of flies on the test sphere at any one time was generally higher than that found on the check and females may have been interrupted before oviposition was complete. In addition, the use of flies from a colony with a high oviposition drive may have reduced the effect of the volatiles.

The response of the flies to the synthetic blend was not significantly increased by testing the blend with the artificial apples, which indicates that visual stimuli were not necessary for the flies to be attracted to the volatiles. Prokopy (1973) also demonstrated that apple maggot flies responded to apple odors in the absence of visual cues. However, the close proximity of the flies in the wind tunnel to the traps may have reduced interactions between the visual stimuli and the apple volatiles. Therefore, the effect of visual attractants on increasing the response to the volatiles will be evaluated in the field.

The ratio of the active components (Figure 4), determined by GLC analysis from extracts collected throughout the season, changed as the apples matured. This necessitated the use of extracts taken within a very short time interval for our assays. Aliquots were saved from each collection and the ratios were analyzed by GLC. Within the extracts used for the assays and characterization, there was very little change. However, early in the season, when the apples are rarely infested, there is a much higher proportion of hexyl acetate, butyl hexanoate, and hexyl butanoate than seen in the ratio shown in Figure 4. As the apples mature and become susceptable to oviposition, the relative amount of butyl 2-methylbutanoate, propyl hexanoate, and hexyl propanoate increases. In preliminary experiments investigating the effects of these changing ratios, it was found that extracts from ripe apples reduced the number of flies that landed at the source (in both the wind tunnel and tube olfactometer) and extracts from rotten apples sometimes caused flies in the tube olfactometer to move downwind away from the source. This correlates well with field studies (Reissig, 1974) and observations that show that apple maggot flies prefer apples that have not yet reached maturity and are repelled by rotting varieties (Dean and Chapman, 1973).

Further work is underway to determine changes in quantity and ratio of the active volatiles associated with the maturation of different apple varieties and to determine whether these compounds may be used to increase apple maggot monitoring trap effectiveness. In addition, related *Rhagoletis* species and their host volatiles are being studied to understand more about the evolution of races onto new hosts and the host-plant selection process.

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ALLELOPATHIC RESEARCH OF SUBTROPICAL VEGETATION IN TAIWAN II. Comparative Exclusion of Understory by *Phyllostachys edulis* and *Cryptomeria japonica*[1]

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Abstract—On many hillsides of Taiwan there is a unique pattern of weed exclusion by *Phyllostachys edulis* (bamboo) and *Cryptomeria japonica* (conifer) in which the density, diversity, and dominance of understory species are very different. Although the physical conditions of light, soil moisture, and soil nutrients strongly favor the growth of understory in a bamboo community, the biomass of its undergrowth is significantly low, indicating that physical competition among the understory species in the bamboo and conifer communities does not cause the observed differences. However, the biochemical inhibition revealed by these two plants appeared to be an important factor. The growth of *Pellionia scabra* seedlings, transplanted from the study site into greenhouse pots, was evidently suppressed by the aqueous leachate of bamboo leaves but was stimulated by that of conifer leaves. The radicle growth of lettuce, rye grass, and rice plants was also clearly inhibited by the leachate and aqueous extracts of bamboo leaves but not by those of conifer leaves. Six phytotoxins, *o*-hydroxyphenylacetic, *p*-hydroxybenzoic, *p*-coumaric, vanillic, ferulic, and syringic acids were found in the aqueous leachate and extracts of leaves and alcoholic soil extracts of *P. edulis*, while the first three compounds were absent in the extracts of *C. japonica*. The phytotoxicities of extracts were correlated with the phytotoxins present in both leaves and soils. The understory species might be variously tolerant to the allelopathic compounds produced by the two plants, resulting in a differential selection of species underneath. Therefore, comparative allelopathic effects of *Phyllostachys edulis* and *Cryptomeria japonica* may play significant roles in regulating the populations of the understories.

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Key Words—Allelopathy, comparative exclusion, phytotoxins, phytotoxic phenolics, *Phyllostachys edulis*, *Cryptomeria japonica*, bamboo, conifer.

INTRODUCTION

It is often asserted without justification that a deleterious effect of one plant upon another is ascribable to physical competition for light, soil moisture, or mineral nutrients. Muller (1969) thus emphasized that allelopathy should be recognized as one of the environmental parameters to be used to interpret the mechanisms of plant competition, dominance, succession, and productivity. Under some circumstances allelopathy may also interact with other natural stresses, resulting in a synergistic effect upon the plants sharing the same habitat (Muller, 1974; Koeppe et al., 1976; Rice, 1979; Chou, 1980). During the last 20 years, this research has been extensively conducted in many parts of the world (Muller, 1966, Börner, 1971; Grodzinsky, 1971; Rice, 1974a; Friedman et al., 1977; Newman et al., 1977; Anaya et al., 1978; Gliessman and Muller, 1978; Chou, 1980).

Although Chou and his associates have demonstrated several cases of allelopathy in Taiwan (Chou and Chung, 1974; Chou and Chiou, 1979; Chou and Hou, 1981), little is known of the allelopathic interaction in forests. For example, *Phyllostachys edulis* (bamboo) and *Cryptomeria japonica* (conifer) forests, which are often planted close together in many mountainous districts of Taiwan, exhibit a dramatic difference in understory species distribution. Relatively bare areas are easily found under the canopy of bamboo stands, but a luxuriant growth of understory is rather uniformly distributed under that of conifer stands. Chou and Hou (1981) reported that several bamboo species exhibited allelopathic potential among 14 species which they studied. On the hillsides of the Shitou Forest Experimental Station, there are extensive pure stands of bamboo and conifer on a uniform substrate. Among many bamboo species, *Phyllostachys edulis* is a predominant one, under which an understory often reveals a relatively low density and low productivity or bare ground (Figure 1A). However, in an adjacent area, there is a luxuriant growth of understory in the conifer forest, *Cryptomeria japonica*, (Figure 1B). This unique pattern of understory distribution between two forests induced us to do detailed field measurements by using the importance value techniques. It is the aim of this report to evaluate the comparative allelopathic effects of *Phyllostachys edulis* and *Cryptomeria japonica* on the population dynamics of the understory in these forest ecosystems.

METHODS AND MATERIALS

Study Site. Both *Phyllostachys edulis* and *Cryptomeria japonica* are widely distributed on hillsides in Taiwan at an elevation of about 1000 m. A study site

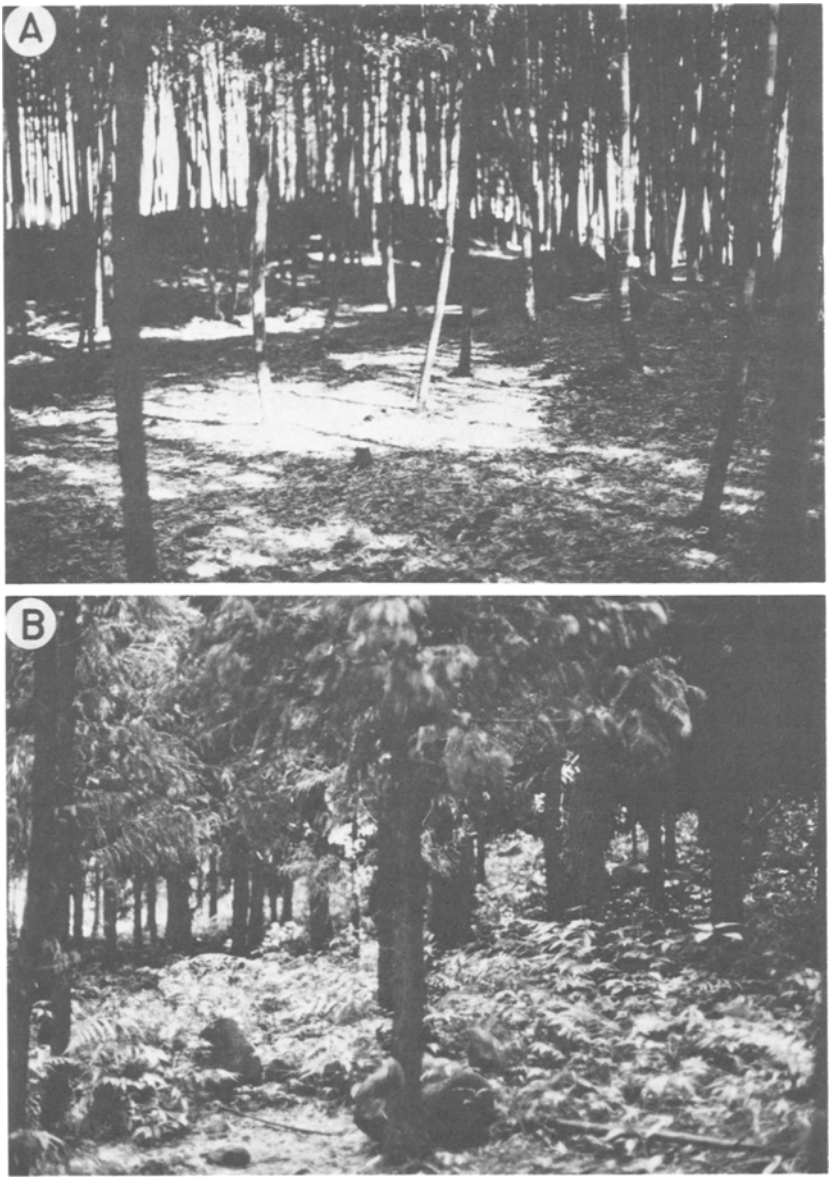

FIG. 1. Vegetation pattern at the Shitou experimental site of Nantou County, Taiwan, showing relatively bare ground underneath *Phyllostachys edulis* (bamboo) (A), and luxuriant plant growth underneath *Cryptomeria japonica* (conifer) (B).

chosen at the Shitou Forest Experimental Station of National Taiwan University is located at 23° 41′N and 120° 47′E and has an elevation of 1150 m. The area is generally occupied by planting forming relatively pure stands of *Phyllostachys edulis* (bamboo) and *Cryptomeria japonica* (conifer). The annual precipitation is about 2900 mm with 90.5% humidity, 16.4°C annual average temperature, and total evaporation of 50 mm. About 4 hectares (2 hectares for bamboo stands, 1.5 for conifer stands, and 0.5 for ecotone) were selected for field measurement and sampling. In this study site animal activity was fairly low.

Materials. Leaves and litter of *P. edulis* and *C. japonica* were collected in the study site in the winter and summer seasons of 1979–1981. These samples were brought back to the laboratory of Academia Sinica and allowed to air dry. At the same time, soil samples were taken down to a depth of 10 cm after leaves and litter were collected. The soil was air dried and screened with a 2-mm sieve to remove all visible plant residues. For the purpose of greenhouse pot experiments, a substantial number of seedlings, about 10 cm high, of each of the four understory species, namely, *Ageratum conyzoides*, *Commelina undulata*, *Pellionia scabra*, and *Pilea funkikensis*, with attached soil were carefully transferred into plastic bags (15 × 20 cm) without disturbing the root system. Five seedlings of each species were transplanted into a clay pot, and watered daily for 3 weeks to allow the seedlings to become established in the greenhouse of Academia Sinica.

Sampling Techniques and Field Measurement. In order to understand the population dynamics of the understory species in the stands of *Phyllostachys edulis* and *Cryptomeria japonica*, five plots (each 2 m in radius) in each stand were randomly selected for sampling. The relative density, relative frequency, and relative dominance of each species in the stand were determined by the methods described by Cottam and Curtis (1949). The data were obtained from 15 plots based on three-year repetitious sampling; thus the importance value (IV) was obtained from the sum of relative density, relative frequency, and relative dominance.

The light intensity underneath the vegetation zones was measured on sunny and cloudy days in September 1981.

Preparation of Leaf Leachate. Because of the extreme difficulty of collecting leaf leachates from bamboo and conifer forests in the study site, an artificial leaf leachate apparatus was designed. The apparatus consisted of three layers of plastic containers (each container 50 × 40 × 15 cm) with about 600 holes (less than 0.1 mm in diameter) on the bottom of the upper two containers. The top container was used to place 7500 ml of deionized water, which passed through the holes like natural rainfall. Two-hundred fifty grams of leaves were placed in the middle container, 30 cm below the upper container, to receive the artificial raindrops. The leaf leachate was collected in the lowest container without holes and was recirculated through the upper

container to repeat the leaching process. The same process was continued for 72 hr in order to concentrate the metabolites of leaf leachate, and then the leachate was harvested. No visible fungus on the leaf surface was found during the leaching process. Subsequently, four leachates were obtained using the same leaves. These leachates were stored in a cold room at 5° C (±1° C) before assaying.

Greenhouse Pot Experiment. Five seedlings of each species were grown in a clay pot under greenhouse conditions. Each set of the experiment consisted of six pots as replicates, and the seedlings were separately irrigated with the leaf leachate of *C. japonica* and *P. edulis* as test solutions and with distilled water as control. At the end of the third and fourth week of irrigation with the test solution, the length of stem and leaf area of each seedling were measured. The percent increase of stem length and leaf area was obtained from the difference between measurements made at the end of the third and the fourth week. The effects of the leaf leachates of *C. japonica* and *P. edulis* on the growth of seedlings were compared.

Aqueous Extracts of Leaves and Soils. To 10 g of chopped leaves of *C. japonica* and *P. edulis* separately 300 ml of distilled water were added, and the mixture was shaken for 2 hr. Each aqueous extract was then obtained by suction filtration and, if necessary, further centrifuged at 5000 rpm to clear the filtrate. One hundred-gram samples of bamboo and conifer soil were shaken with 300 ml of distilled water for 2 hr and the supernatant was obtained by centrifugation at 3000 rpm. These extracts were stored in a cold room at 5° C (±1° C) before analyses.

Physiochemical Analyses of Leachates and Extracts. The leaf leachates and aqueous extracts of leaves and soils were subjected to determination of osmotic concentration by using an osmometer (Fiske G-66) and the pH was determined by a pH meter (Jenco model 671). Other physiochemical properties, such as soil moisture, organic matter content, soil texture, total amount of nitrogen, phosphorus, cation exchange capacity (CEC), and amount of cations (Mn, Mg, Fe, Ca, Zn, Na, and K) were determined by methods described by Rice (1974b). The contents of N and P, and CEC were determined by a titration method, while the cation contents were measured by an atomic absorption spectrophotometer (Perkin-Elmer model 300).

Bioassay of Leachates and Extracts of Leaves and Soils. The phytotoxicity of the leaf leachates and aqueous extracts of leaves and soils of *P. edulis* and *C. japonica* was determined by using the bioassay techniques described by Chou et al. (1974, 1976). Seeds of rice (*Oryza sativa* Taichung 65), lettuce (*Lactuca sativa* var. Great Lakes 366), and rye grass (*Lolium multiflorum*) were used as test materials. The percentage of phytotoxicity of the leachate and extracts was obtained by measuring the percent germination and radicle growth of test seeds against those of the distilled water control.

Statistical Analysis. The data of greenhouse experiment and bioassay

results were analyzed by means of Student's *t* test to find the significant level of treatment compared to control.

Isolation and Identification of Phytotoxins in Leachates and Extracts. A portion of each leachate or aqueous extract of *C. japonica* and *P. edulis* was concentrated in vacuo and futher extracted with ethyl ether, which was evaporated to dryness, and the residue dissolved in spectroscope grade methanol. The isolation of allelopathic substances followed the techniques described by Chou and Young (1975), and then identification was made mainly by paper and thin-layer chromatography and UV-visible spectrophotometry (Vásquez et al., 1968; Mabry et al., 1970). The solvent systems employed in the chromatography were 2% acetic acid (2 ml acetic acid with 98 ml distilled water), 15% acetic acid, *t*-butanol–acetic–water (5:4:1, v/v/v), and acetic–methanol (1:9, v/v). Both one-dimension paper strip (Wang et al., 1967) and two-dimension paper chromatography were used. Phytotoxic substances in soils of *P. edulis* and *C. japonica* were isolated by the methods described by Chou and Hou (1981), and the compounds were identified by the same techniques.

RESULTS

Vegetation Pattern and Floristic Composition of Understory. The floristic composition and its importance values in three vegetation zones, *P. edulis*, ecotone, and *C. japonica* are given in Table 1. Based on three years of sampling, there are about 25 species of understory in *P. edulis* stands, 24 in the ecotone, and 24 in *C. japonica*. The dominant species are different in the three zones. For example, the five most abundant species in the *P. edulis* community are *Ageratum conyzoides* (IV = 18%), *Commelina undulata* (19%), *Pilea funkikensis* (7%), *Pratia nummularia* (27%), and *Tetrastigma formosana* (5%), while in the *C. japonica* community they are *Ficus pumila* (3%), *Pellionia scabra* (97%), *Pilea funkikensis* (7%), *Piper arboricola* (7%), and *Urtica thunbergiana* (3%). Regarding the importance values, *Pilea funkikensis* is the most common species, but its importance value is quite low; *Pellionia scabra* is the most important understory species in the conifer community but is absent in the bamboo forest. In addition, the distributions of nine common species are quite different in the three vegetation zones (Figure 2).

Competition for Physical Factors. The depletion of necessary physical factors by one plant species may possibly result in the disappearance of some understory species sharing the same habitat. Physical factors, such as light, soil moisture, and nutrient availability, were compared between *C. japonica* and *P. edulis* forests. On a sunny day in September 1981, the light intensity ranged from 2000 to 2500 lux under the canopy of *P. edulis*, from 1000 to 1200

TABLE 1. FLORISTIC COMPOSITION OF COMMON UNDERSTORY SPECIES IN THREE VEGETATION ZONES[a]

Understory species	*Phyllostachys edulis* B g/m²	RD (%)	RF (%)	RC (%)	IV (%)	Ecotone B g/m²	RD (%)	RF (%)	RC (%)	IV (%)	*Cryptomeria japonica* B g/m²	RD (%)	RF (%)	RC (%)	IV (%)
Pratia nummularia	4.76	25	1	1	27	2.57	3	0	0	3	0	0	0	0	0
Commelia undulata	—[b]	8	7	4	19	—	1	4	1	6	—	0	2	0	2
Ageratum conyzoides	0.75	7	4	7	18	1.19	3	3	1	7	0	1	0	2	3
Pilea funkikensis	0	2	3	2	7	1.28	4	6	5	15	0	1	3	3	7
Tetrastigma formosana	—	2	1	2	5	—	0	1	0	1	—	0	0	0	0
Diphazium dilatatum	—	1	3	1	5	—	0	2	1	3	—	0	4	0	4
Cayiatia japonica	—	2	1	1	4	—	0	1	0	1	—	0	0	0	0
Alocasia macrorhiza	—	0	2	0	2	—	1	4	1	6	—	0	2	0	2
Polygonum chinensis	—	1	1	1	3	—	0	0	0	0	—	0	0	0	0
Pellionia scabra	0	0	1	0	1	4.60	0	6	0	6	49.18	23	15	59	97
Begonia randaiense	—	0	1	0	1	—	0	1	0	1	—	0	1	0	1
Urtica thunbergiana	—	0	1	0	1	—	0	0	0	0	—	1	0	2	3
Microlepia strigosa	—	0	0	0	0	—	0	1	0	1	—	1	1	1	3
Piper arboricola	—	0	0	0	0	—	0	1	0	1	—	4	1	2	7
Ficus pumila	—	0	0	0	0	—	4	0	0	4	—	3	0	0	3
Total[c]	5.51	46	28	17	91	9.64	16	29	9	54	49.18	35	30	70	135
Total number of species[c]			25				24						24		

[a] The abbreviations are as follows: B = biomass, RD = relative density, RF = relative frequency, RC = relative cover, IV = importance value = RD + RF + RC.
[b] Not detected.
[c] Data were obtained from the sum of all species counted in field.

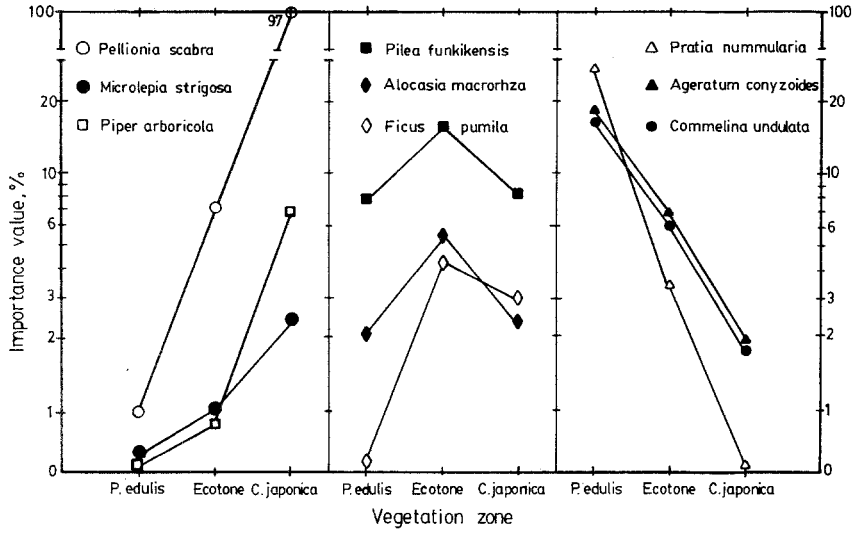

FIG. 2. Quantitative distribution of nine common understory species in three vegetation zones: *Phyllostachys edulis*, *Cryptomeria japonica*, and ecotone (mixed zone of two vegetations).

lux in the ecotone, and about 200 lux under that of *C. japonica*; on a cloudy day, the light intensity was proportionally decreased, ranging from 350 to 300 lux in the bamboo stands and about 50 lux in the conifer stands (Figure 3). The total number and dry weight of seedlings per square meter were much higher in the conifer community than in the bamboo forest (Figure 3). Forty-five understory species were found in the conifer floor in the Shitou forest area regardless of the planting density of the forest (Kuo, 1958). Most of the species reported by Kuo were also found under the canopy of many bamboo species except *P. edulis*. Some facultative sciophytes, such as *Commelina undulata*, *Diphazium dilatatum*, *Pratia nummularia*, and *Polygonum chinesis*, which disappeared in the conifer floor were not due to the low light intensity beneath the canopy of conifer. Other species, such as *Pellionia scabra*, *Begonia randaiensis*, *Ficus pumila*, *Microlepia strigosa*, *Piper arboricola*, and *Urtica thunbergiana*, which revealed significantly lower IV in the bamboo floor than that in the conifer floor, were not likely due to the high intensity of light underneath the bamboo canopy. Because of uncontrollable factors, we were unfortunately unable to conduct field experiments to evaluate the response of individual species to various light conditions. Nevertheless, based on our three-year field observation, we were convinced that the exclusion principle of understories in the two types of vegetation was unlikely due to physical competition for light.

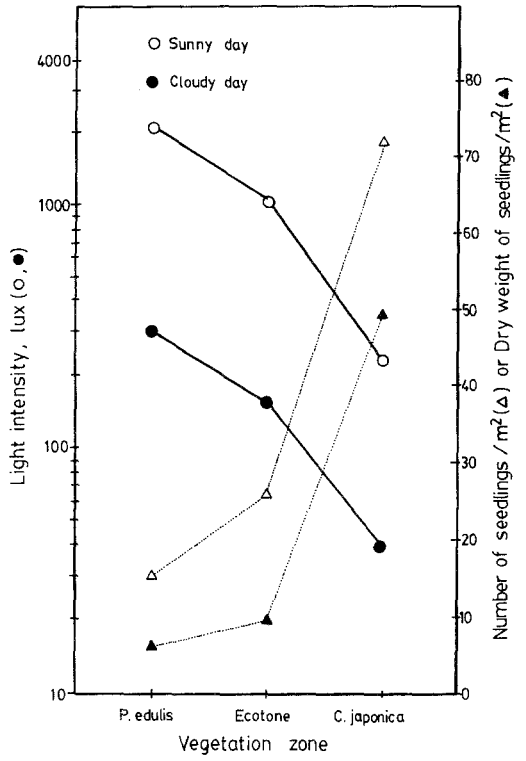

FIG. 3. Quantitative comparison of light intensity and production of understory seedlings in three vegetation zones.

Comparing physical properties of soils in the three vegetation zones, it was found that there was no significant difference in soil pH, moisture, texture, organic matter, total nitrogen, and cation exchange capacity (Chou and Hou, 1981). The contents of phosphorus, Mn, Fe, Ca, K, and Na, showed higher values in the bamboo soil than in the conifer soil, indicating that nutrient availability greatly favors the growth of weeds underneath bamboo vegetation rather than under the conifer (Chou and Hou, 1981). Thus it is concluded that physical competition among understory species of the two major forests is not significant. Other biochemical interaction might be important in determining the population structure of plants under the canopies of these two forests. This hypothesis was tested by the following experiments.

Chemical Properties of Leachate. All aqueous leaf leachates from *P. edulis* and *C. japonica* collected in the winter and summer seasons of 1979-1981 were examined for the chemical properties listed in the earlier section.

TABLE 2. SEASONAL VARIATION OF SOME CHEMICAL PROPERTIES OF LEACHATES OBTAINED FROM LEAVES OF *Phyllostachys edulis* AND *Cryptomeria japonica*[a]

Chemical property	*Phyllostachys* leachate		*Cryptomeria* leachate	
	Summer	Winter	Summer	Winter
pH	7.55	7.82	7.65	7.72
Osmotic concentration (mosmol)	15.2	38.1	4.8	24.0
Cation concentration (ppm)				
Na	57.4	216.0	60.1	323.6
K	8.4	9.0	6.5	8.6
Cu	0.1	3.2	0.1	2.4
Zn	0.3	0.3	0.1	0.4
Fe	0.4	0.5	0.1	0.1
Ca	6.8	3.4	12.8	8.4
Mg	102.2	127.5	117.8	122.2
Mn	0.1	0.1	0	0.1

[a] Data were obtained from the average of five replicates.

Results of the analyses are given in Table 2, which shows that the cation concentrations (except Ca content) in bamboo leachate were generally higher in the winter samples than in the summer samples. The osmotic concentration of leachates was significantly lower in the summer samples of both leachates, reflecting the seasonal difference of metabolic activity. It is interesting to note that the Na content is also four to five times greater in the winter samples than in the summer ones. However, the pH values ranged from 7.55 to 7.82 and the osmotic concentration was below 38 mosmol, which is too low to cause significant inhibition of seed germination and radicle growth of lettuce and rice plants (Chou and Young, 1974; Chou and Chiou, 1979).

Effects of Leachates on Growth of Seedlings Grown in Pots. In order to test the biochemical inhibition of bamboo and conifer leachates on their understory species, seedlings of four tested species (*Ageratum conyzoides*, *Commelina undulata*, *Pellionia scabra*, and *Pilea funkikensis*) were transplanted into pots and were separately watered with leachate using tap water as the control. Results based on the length of stem growth and total leaf area are given in Table 3, which shows that the growth of *P. scabra* was inhibited by bamboo leachate but stimulated by conifer leachate. On the other hand, the growth of *Ageratum conyzoides* and *Commelina undulata* seedlings was much better when they were watered with bamboo leachate than with conifer leachate. These findings agreed with those of field measurement (Figure 2), which showed that the importance values of *A. conyzoides* and *C. undulata*

TABLE 3. COMPARATIVE PHYTOTOXIC EFFECTS OF LEAF LEACHATES OF *Phyllostachys edulis* AND *Cryptomeria japonica* ON GROWTH OF SEEDLINGS COLLECTED FROM STUDY SITES AND GROWN IN POTS.

Test solution	Length of stem growth (% increase)[a]			
	Ageratum conyzoides	*Commelina undulata*	*Pellionia scabra*	*Pilea funkikensis*
Distilled water (control)	44.9 (100)[b]	62.0 (100)	23.4 (100)	21.4 (100)
Phyllostachys leachate	44.2 (98)	66.2 (107)	17.4 (74)b	38.6 (178)b
Cryptomeria leachate	32.7 (73)b[c]	47.3 (76)	24.8 (106)	27.3 (128)
	Total leaf area, % increase[a]			
Distilled water (control)	118.8 (100)	50.5 (100)	69.2 (100)	112.5 (100)
Phyllostachys leachate	159.9 (135)c	138.6 (274)a	63.2 (91)	61.0 (54)b
Cryptomeria leachate	114.9 (97)	54.4 (108)	90.7 (131)c	64.9 (58)b

[a] The data of % increase were obtained by the formula: $[(D_2 - D_1)/D_1] \times 100\%$, where D_1 and D_2 were obtained from the measurements made on the 21st and 28th day after seedlings were watered with leachate and distilled water, respectively.
[b] Data in parenthesis indicate the percent index of the distilled water control.
[c] a, b, and c: Statistical significance at 1%, 5%, and 10% levels, respectively.

TABLE 4. COMPARATIVE PHYTOTOXIC EFFECT OF LEAF LEACHATES OF *Phyllostachys edulis* AND *Cryptomeria japonica* ON GERMINATION AND RADICLE GROWTH OF LETTUCE, RYE GRASS, AND RICE PLANTS.

Tested plants	Season	*Phyllostachys* leachate		*Cryptomeria* leachate	
		Germination % of control	Radicle growth % of control	Germination % of control	Radicle growth % of control
Lettuce	Summer[a]	—[b]	86.5 (−13.5)[c]	—	135.8 (35.8)b[d]
	Winter	78 (−22)	89.4 (−10.6)	102.9 (2.9)	92.2 (−7.8)
Rye grass	Summer	—	81.3 (−18.7)	—	100.6 (0.6)
	Winter	83.7 (−16.3)	63.6 (−36.4)b	90.3 (−9.7)	94.5 (−5.5)
Rice	Summer	—	69.8 (−30.2)b	—	70.8 (−29.2)b
	Winter	66 (−34)	56.5 (−43.5)a	115.0 (15)	84.4 (−15.6)

[a] Leaves were collected in the summer and winter of 1980–1981.
[b] Not detected.
[c] The negative value in the parenthesis indicates the percent inhibition of distilled water control, while the positive value indicates the percent simulation of distilled water control.
[d] a and b: Statistical significance at 1% and 5% levels, respectively.

were higher in the bamboo community than in the conifer, while those of *P. scabra* were significantly lower in the bamboo community than in the conifer. This correlation indicates that the understory species may exhibit different responses to the action of leached metabolites.

Comparative Phytotoxic Effects of Leachates and Aqueous Extracts. The leachates obtained from the bamboo and conifer leaves were also bioassayed by using lettuce, rye grass, and rice seeds as test materials. Results of bioassays are shown in Table 4 and indicate that the phytotoxicity is generally higher in the bamboo leachate than in the leachate from conifer and is significantly higher in the winter than in the summer leachates. The subsequent leachates of bamboo leaves also showed significant inhibition of the radicle growth of lettuce and rice seedlings in an experiment that lasted for 15 days. But the conifer leachates did not show toxicity to the growth of these plants (Figure 4).

The 5% aqueous extracts of bamboo and conifer leaves were also bioassayed. The bioassay results showed that the inhibition of seed germina-

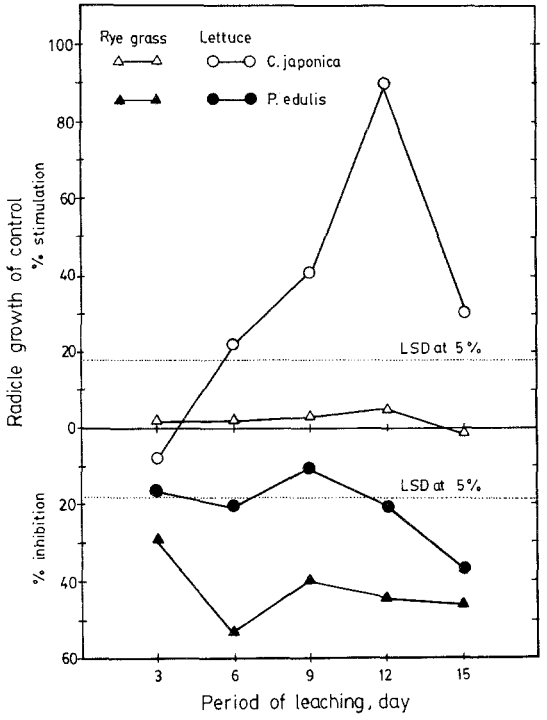

FIG. 4. Effects of leaf leachate of *P. edulis* and *C. japonica* on the radicle growth of rye grass and lettuce plants. The leachates were obtained at 3-day intervals from the same plant samples.

TABLE 5. COMPARATIVE PHYTOTOXIC EFFECTS OF AQUEOUS LEAF EXTRACTS OF *Phyllostachys edulis* AND *Cryptomeria japonica* ON GERMINATION AND RADICLE GROWTH OF LETTUCE, RYE GRASS, AND RICE PLANTS.[a]

Tested plants	Extraction sequence	*Phyllostachys* extract		*Cryptomeria* extract	
		Germination (%inhibition)	Radicle growth (% inhibition)	Germination (%inhibition)	Radicle growth (% inhibition)
Lettuce	I	14	75a[b]	63	70a
	II	0	35a	0	49a
	III	0	8	0	17
Rye grass	I	28	31b	48	43a
	II	33	23c	30	0
	III	25	10 NS	30	16
Rice	I	66	40a	45	43a
	II	57	20c	22	22c
	III	22	5	25	12

[a] The aqueous extract of chopped leaves was obtained by using 5 g leaves added to 95 ml distilled water and the extract was designated as I. The subsequent extract II was obtained by extracting the first residue with 95 ml water and III was obtained from the second residue in the same way.
[b] a, b, c: Statistical significance at 1%, 5%, and 10% levels, respectively.

tion and radicle growth was higher in the extracts from bamboo than in those from conifer, and the phytotoxicity decreased with time of subsequent extractions (Table 5). This finding was in good agreement with the results of the leachates.

Relative Quantity of Phytotoxins in Extracts of Leaves and Soils. Inasmuch as phytotoxicity was present in the leachates and extracts of bamboo and conifer leaves, it seemed worthwhile to isolate and identify responsible phytotoxins by chromatography using known compounds for references. Since the phytotoxicity was present in the aqueous fractions, attention was concentrated on water-soluble phenolics. Six phytotoxins, namely, *o*-hydroxyphenylacetic, *p*-coumaric, *p*-hydroxybenzoic, ferulic, vanillic, and syringic acids, were present in the aqueous extracts and leachates of bamboo leaves, but the first three compounds were either absent or in very low concentration in those from conifer leaves. Quantitatively, we found almost two to three times as much phytotoxin in the leaves of *P. edulis* as in *C. japonica* (Table 6). Phytotoxins present in the two soil samples were also determined by using alcoholic extraction methods. Except for syringic acid, the other five compounds were present in the bamboo soil; on the other hand, only vanillic, ferulic, and syringic acid were present in the conifer soil (Table 6). The amount of phytotoxin was also greater in the bamboo soil than in the conifer soil. The distribution of soil phytotoxins correlated well with the phytotoxins found in the leaves.

It is concluded that the greater inhibitory action found in the aqueous leaf leachates and extracts of *Phyllostachys edulis* than in those of *Cryptomeria japonica* is primarily due to the quantity of phytotoxins present. Allelopathic interaction therefore plays a significant role in regulating species distribution and production under the canopy of the two forests studied.

DISCUSSION

In the Shitou experiments site, as well as on many hillsides of Taiwan, the vegetational composition is very different under bamboo and conifer forests, the importance value of the understory being evidently higher in the conifer stands than in the bamboo stands. The productivity of undergrowth based on the dry weight of seedlings was significantly greater in the former stands than in the latter, although the light intensity was very much lower under the conifer canopy (Figures 1–3). Other physical factors, such as soil moisture and nutrients were shown not to be limiting for the understory species in either community. However, allelopathic influence on the understory proved to be important. The distribution of understory species and quantity of phytotoxins produced by both vegetation types revealed that a differential allelopathic effect existed between them.

TABLE 6. RELATIVE QUANTITY OF PHYTOTOXINS IN AQUEOUS LEAF LEACHATES AND EXTRACTS OF *Phyllostachys edulis* AND *Cryptomeria japonica* AND ALCOHOLIC EXTRACTS OF ASSOCIATED SOILS

	Concentration ($\times 10^{-7}$mol/g sample)[a]						
	Leaf leachate		Leaf extract		Soil extract		
	P. edulis	*C. japonica*	*P. edulis*	*C. japonica*	*P. edulis*	Ecotone	*C. japonica*
o-Hydroxyphenylacetic acid	3.2	0	120	0	80	80	0
p-Coumaric acid	0	0	80	0	320	80	0
p-Hydroxybenzoic acid	4.8	0	40	0	320	80	0
Vanillic acid	3.2	0	40	40	240	160	320
Ferulic acid	1.6	6.4	40	40	80	160	240
Syringic acid	1.6	1.6	40	40	0	80	40
Total	14.4	8.0	360	120	1040	640	600

[a] The relative quantity of phytotoxins was based on paper chromatography compared with known compounds and calculated on dry weight basis of leaf or soil samples.

The allelopathic substances produced from the bamboo plants differ greatly from those produced by the conifer. Since the most ecologically significant compounds are water soluble, we have not attempted to extract other groups of allelopathic compounds with nonpolar solvents instead of distilled water. Therefore, most of the terpenoid phytotoxins present in the *Cryptomeria japonica* tissue were not isolated in the present study, although ecologists have identified some such allelopathic compounds in various coniferous plants, such as *Abies concolor*, *A. sibirica*, *Juniperus* spp., *Larix decidua*, *L. sibirica*, *Picea odorata*, *Picea excelsa*, and *Pinus* spp. (del Moral and Cates, 1971; Jameson, 1970; Lee and Monsi, 1963; Peterson, 1972; Rice, 1974a, 1979). Nevertheless, by using distilled water as the extraction solvent, we were able to obtain a substantial quantity of phytotoxin from the leaves of *P. edulis* (Table 6). Chou and Hou (1981) evaluated the allelopathic potential of 14 bamboo species and isolated various allelopathic compounds from them. Besides phytotoxic phenolics (shown in Table 6), we have found several flavonoids with phytotoxic potential.

Local silviculturists have always found that *Phyllostachys* is a very aggressive plant and often invades its adjacent forests, such as *Cryptomeria japonica*, which is eventually eliminated by the bamboo plants. Bamboo plants have two types of rhizomes, namely, sympodial rhizocauls and horizontal rhizomes with lateral culms. *Phyllostachys edulis* has the latter type. The rapid invasion of *P. edulis* into its adjacent woodland forests is primarily due to: (1) the fast growth of rhizomes, which may possibly release phytotoxic root exudates, and (2) allelopathic substances produced by the bamboo leaves and the decomposing litter. The continuous release of water-soluble phytotoxins from the *Phyllostachys edulis* and accumulation of the compounds in the soil may result in suppression of the growth of understory or in elimination of neighboring plants. The distribution gradient of phytotoxins in the three vegetation zones studied indicates that the distribution of understory species is controlled by phytotoxins. It is also possible that physical factors may interact synergistically with allelopathic substances so as to produce a more complex interaction in the field. Some field experiments are in progress in order to clarify the role of allelopathy in bamboo and conifer reforestation.

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Erratum

SEX PHEROMONE OF FALL ARMYWORM: Laboratory Evaluation of Male Response and Inhibition of Mating by Pheromone Components

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On page 267, in the Abstract section, insert "acetate" before "(TDA)."

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