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GENERALIZATION IN OLFACTORY DETECTION OF CHEMICAL CUES CONTAINING CARBONYL FUNCTIONS BY TIGER SALAMANDERS (*Ambystoma tigrinum*)

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Abstract—Tiger salamanders generalize behaviorally between carbonyl-containing odorants (e.g., aldehydes or cycloalkanones). However, responding does not generalize from such odorants to stimulus compounds with comparable molecular shapes and dimensions but different functional groups. Discrimination between aldehydes and a ketone is temporarily impaired by two-step covalent modification of the olfactory epithelium. Two-step modification of the olfactory epithelia also impairs, but does not obliterate, olfactory detection, and generalization persists even during the period of impairment. These results are interpreted as implying the existence of carbonyl-binding, ''generalist'' olfactory receptors in addition to other classes of ''generalist'' receptors that are not affected by two-step modification. Generalization is inferred to require overlap in the response profile of more than one class of receptor.

Key Words—Tiger salamander, *Ambystoma tigrinum*, carbonyl function, generalist receptor, olfactory discrimination.

INTRODUCTION

Chemoreceptors may be portrayed as falling into two categories, ''specialists'' and "generalists" (Schneider, 1969). Specialists represent receptors that respond to a specific stimulant molecule (and, perhaps, to a few closely related

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analogs). Pheromones, for instance, may interact with such specialized receptors. Generalists, on the other hand, represent receptors that respond to a range of molecules, which share some common feature. Animals might use such generalist receptors, for example, in detecting volatile food cues and to identify and distinguish potential hosts or prey.

Although the distinction between specialist and generalist may, in practice, be less clear-cut than it is in principle, it raises the issue of how one might ascertain experimentally whether two different molecules interact with a given receptor site. In studies of pheromone detection, it is sometimes assumed that if a molecule triggers a receptor, then the response will be the same regardless of the identity of the stimulus. By the same token, if two different molecules elicit the same response, it is often concluded that they must have interacted with the same receptor (Chapman et al., 1978). Such assumptions may not be warranted in the case of generalist receptors. We would like to draw parallels between generalist receptors and the behavioral phenomenon of generalization, which can be combined with chemical manipulation of the receptor in an effort to assess whether two different molecules bind at the same site.

In human subjects, the phenomena of adaptation and cross-adaptation have been explored in an effort to answer this type of question (Köster, 1971). However, the facts that: (1) adaptation appears to involve both central and peripheral mechanisms, and (2) the ability to recognize or distinguish odors does not seem to be necessary for the central component to function, (Eichenbaum et al., 1983) complicates the interpretation of experimental data on human beings. Behavioral experiments with animals cannot ordinarily ask the detailed sorts of psychophysical questions that can be studied with humans. But a major advantage of studying animals is that the subjects' sensory systems or CNS can be modified in the course of experimentation. This paper will discuss the application of chemical modification of the olfactory epithelium to the study of generalization.

Choice of species for experimentation was based on the following considerations. First, the test animals had to be air-breathing vertebrates for which behavioral assays had been developed. Second, the olfactory epithelium in the species of choice had to be accessible to chemical manipulation. Adult tiger salamanders (*Ambystoma tigrinum*) were chosen because they are air-breathing vertebrates that can be classically conditioned to discriminate and generalize among reagent-grade odorants (Mason et al., 1980, 1981; Mason and Stevens, 1981a,b). Also, their simple nasal cavities permit direct chemical treatment of the olfactory epithelium (Mason and Morton, 1982, 1984; Mason et al., 1984, 1985).

METHODS AND MATERIALS

Subjects. Adult tiger salamanders were purchased from Charles D. Sullivan Company, Inc., Nashville, Tennessee. All of these animals had been land-

phase (air-breathing) for two or more years and were collected from two ponds about 2.5 km apart in Wilson and Sumner Counties, Tennessee (Sullivan, personal communication).

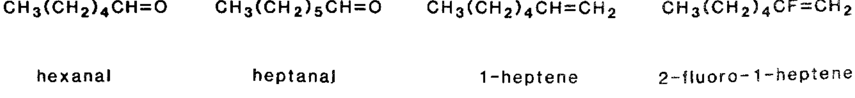
While these animals are probably all from the same breeding population, we believe that their data are representative of the species. There are several grounds for this inference. First, animals collected in other geographic locations (e.g., New Mexico, Arizona) are readily conditioned to respond to simple odorants and show discrimination and generalization among odorants similar to that reported here (e.g., Mason and Stevens, 1981a; Mason et al., 1984). Second, larval tiger salamanders that metamorphosed in our laboratory show similar response patterns to those purchased from Sullivan, Inc. (Arzt et al., 1986).

While in the laboratory, the salamanders were housed in plastic boxes in a refrigerator at 5–8 °C. Refrigeration permitted the animals to be kept without weight loss and essentially disease-free for several months. Mealworms were hand-fed to animals every 14 days.

The boxes in which the salamanders were housed were lined with paper towels moistened with dechlorinated water. Liners were changed every third day. All animals were used within two months of arrival in the laboratory. Thirty minutes before each experimental session, the salamanders were removed from the refrigerator and placed in plastic tubs. Each tub contained about 100 ml of dechlorinated water. Testing occurred in a room with an ambient temperature of 23° ± 1 °C.

Chemicals. Molecular structures of odorants used in these studies are drawn in Figure 1. Cyclohexanone, cyclopentanone, 1-butanol, ethyl acetoacetate, 1-heptene, hexanal, heptanal, 2-heptanone, and pristane (2,6,10,14-tetramethylpentadecane) were purchased commercially. Cyclohexanone and ethyl acetoacetate were redistilled at atmospheric pressure. The other odorants were used without additional purification.

Among the odorants used for generalization studies, a fluoroalkene was included as an example of an odorant molecule with molecular dimensions similar to an aldehyde, which also possesses a large permanent dipole moment, but without an aldehyde's ability to bind covalently as a Schiff base. 2-Fluoro-1-heptene was prepared from 2-bromo-1-heptene, which in turn, was prepared by reaction of 2,3-dibromopropene with *n*-butylmagnesium chloride (Lespieau and Bourguel, 1941). *N*-Bromoacetamide (1.66 g, 0.012 mol) was suspended in a mixture of 15 ml dry tetrahydrofuran and 15 ml dichloromethane and cooled in a Dry Ice–acetone slush. Excess liquid hydrogen fluoride (approximately 5 ml) was added, followed by addition of 2-bromo-1-heptene (1.77 g, 0.010 mol) in a mixture of 25 ml dry tetrahydrofuran and 15 ml dichloromethane. The reaction mixture was kept cold for 3 hr and allowed to warm slowly to room temperature. Stirring was continued for 16 hr at room temperature, and the reaction mixture was then worked up by adding 50 ml of ether and washing with water, saturated sodium bicarbonate solution, and brine. Removal of solvent afforded



$CH_3(CH_2)_4COCH_3$ $CH_3COCH_2COOCH_2CH_3$ O O

2-heptanone ethyl acetoacetate cyclohexanone cyclopentanone

FIG. 1. Structures of odorant molecules used in the present study.

2.62 g of 1,2-dibromo-2-fluoroheptane (9.5 mmol, 95% yield), for which [^{19}F]NMR showed a multiplet 99 ppm upfield from $CFCl_3$; [^{1}H]NMR ($CDCl_3$): δ 4.3 (d, J_{HF} = 10 Hz, 2H), 4.0 (m, 2H), 16–1.6 (m, 6H), 0.85 (t, 6 Hz, 3H).

The dibromo compound was then dehalogenated (Daub et al., 1985). A 5.0-g portion of zinc–copper couple (prepared according to Lambert et al., 1971) was added to a stirred solution of 9.0 g (33 mmol) 1,2-dibromo-2-fluoroheptane in 50 ml ether at 0°C, and the reaction mixture was allowed to warm to room temperature and stirred for 12 hr. The mixture was then filtered, the ether carefully distilled, and the residue vacuum-distilled at room temperature through a succession of three vacuum traps cooled to −23°C, −63°C, and −195°C. The first efficiently trapped 2-bromo-1-heptene and the last ether. The −63°C trap contained 1.1 g (10 mmol, 29% yield) of 2-fluoro-1-heptene: IR (neat film) 3140, 2900, 1675 cm^{-1}; [^{19}F]NMR (acetone-d$_6$): δ −94.0 (m); [^{1}H]NMR δ 4.53 (m, J^{cis}_{H-F} 17.6 Hz, J^{trans}_{H-F} 50.3 Hz, J_{H-H} 2.4 Hz, 3H), 2.8 (J_{H-F} 16.1 Hz, 2H), 1.7–1.1 (m, 6H), 0.89 (t, J = 7 Hz, 3H); [^{13}C]NMR δ 167.0 (J_{CF} = 256.3 Hz), 89.1 (J_{CF} = 19.5 Hz), 31.8 (J_{CF} = 26.8 Hz), 31.1, 25.7, 22.4, 13.9; mass spectrum (70 eV) m/z (rel intensity), 116 (11), 96 (10), 88 (10), 81 (14), 73 (24), 61 (26), 60 (22), 59 (18), 58 (19), 56 (100), 55 (22), 41 (90); $M^{\cdot+}$ 116.1003 (calc'd 116.1001). Presence of a small impurity attributed to trace 1,2-heptadiene was inferred on the basis of a variable band in the infrared spectrum at 1970 cm^{-1}.

Apparatus. The apparatus was similar to that described by Mason and Morton (1982). A flow dilution olfactometer generated volatile stimuli from neat samples of hexanal, cyclohexanone, cyclopentanone, 1-butanol, and ethyl acetoacetate, or from pristane solutions of other odorants. Stimuli were delivered in an airflow of approximately 100 ml per minute via an elliptical glass funnel that passed through the front wall of a stainless-steel conditioning chamber, as shown in Figure 2. Unless otherwise specified, odor stimuli from neat samples were presented at approximately 2.0–2.5% of vapor saturation. Salamanders

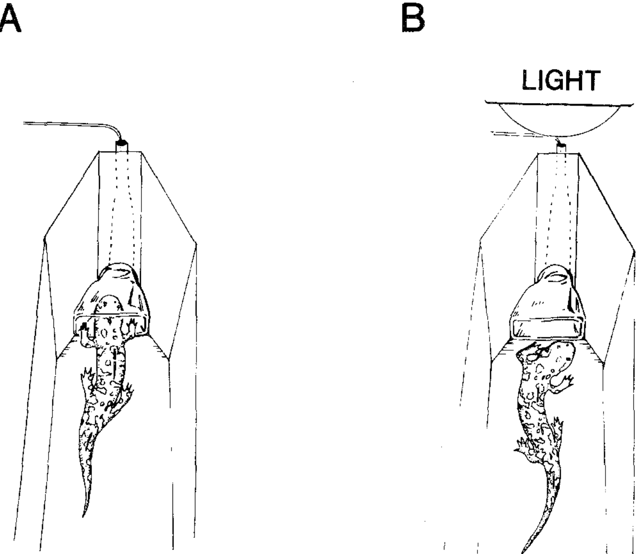


FIG. 2. Conditioning chamber for classical avoidance training of a tiger salamander. (A) Experimental animal positioned in odorant airflow. If the subject remains in this position, with its head in the glass sniffing port during presentation of odorant, a lack of avoidance is scored. (B) Experimental animal removing its head from sniffing port. If the subject removes its head during odorant presentation, an avoidance is scored. A light above the sniffing port is used as negative reinforcer. If the subject removes its head during reinforcement, an escape response is scored.

were placed in the conditioning chamber so that their heads rested in the sniffing port, and odorants were delivered to the port via separate pieces of Teflon spaghetti tubing (Mason and Stevens, 1981a). Concentrations of odorant stimuli in pristane were calculated using Raoult's Law. Specifically, for the 2% (w/v) solutions in pristane, the percent vapor saturation of the odorant at equilibrium with the solution (relative to neat odorant) was estimated to be 6%, equal to the mole fraction of the odorant in the solution, 0.06.

Procedure. Training involved placing the animals in the conditioning chamber with their heads resting in the sniffing port (Figure 2). Approximately 15 sec later, a 15-sec pulse of odorant–air mixture was delivered. Immediately after presentations of S+ odorant, a 300-W projector lamp was turned on to produce an aversive light reinforcer (Kuntz, 1923). The lamp was fitted with a condensing lens and was directed into the front of the conditioning chamber from 12 cm above. Although a small amount of radiant heat reaching the animal might have contributed to the aversive character of the stimulus, the light presentations did not appreciably change the temperature near the sniffing port, even though they continued for 60-sec or until the animal backed away from

the sniffing port. If backing away occurred during presentations of the S+ odorant, an avoidance response was scored. However, if backing away occurred after light onset, an escape response was scored. Unreinforced presentations (S–) of *n*-butanol or filtered air were randomly interspersed with presentations of the S+ odorant. Such presentations provided a day-to-day measure of differences in the animal's level of responsivity and an assessment of whether conditioning to the S+ odorant had occurred. Training continued until animals showed ≥80% avoidance to presentations of S+, and ≤20% avoidance to presentations of S–.

Experiment 1. Twelve salamanders were randomly assigned to two groups ($N = 6$ per group). The first group was trained to respond to hexanal (as S+), presented at 2.5% of vapor saturation. The S– was filtered air. After criterion responding was achieved (eight days), concentration–response tests were conducted over five consecutive days. For these tests, animals were presented with varied S+ odorant concentrations using a temporal forced-choice method of limits (Mason and Stevens, 1981b). Unreinforced presentations of air passed over pristane (as S–) were randomly interspersed with the S+ presentations. On each day of testing, animals were initially presented with hexanal at 2.5% of vapor saturation. Over subsequent trials, the hexanal concentration was reduced until animals failed to exhibit avoidance on two successive trials. At that point, odorant concentrations were increased until avoidance responding was again exhibited.

Following the response–concentration tests using vapors from neat hexanal, additional training trials (two days) were given using a 2.0% (w/v) hexanal solution for which the diluent was pristane, an odorless alkane of low volatility. When criterion responding was achieved, additional response–concentration trials were administered (three days) using the same procedures as those described above. After this second series of tests, three days of generalization trials were given among hexanal, 1-heptene, 2-fluoro-1-heptene, and pristane. The odorants were dissolved in pristane at a concentration of 2.0% (w/v), and volatiles above these solutions were presented (five presentations of each, in a randomized order, per session) to animals without further air dilution. All hexanal presentations during generalization were reinforced. Generalization among these odorants was followed by three days of generalization among hexanal, heptanal, 2-heptanone, and pristane. In all of these generalization tests (1) odorants were dissolved in pristane at a concentration of 2% (w/v), and (2) volatiles from these solutions were presented to animals without further air dilution.

The second group ($N = 5$) was trained to respond to 2% (w/v) hexanal in pristane, interspersed with unreinforced presentation of clean air blown over pristane. Once all subjects had reached criterion (after five days), generalization trials were run with heptanal and 2-heptanone, as described above. After three days, the animals were anesthetized, and lavage was administered to both ol-

factory sacs of each subject. Animals were anesthetized by immersion in a 0.5% aqueous solution of Tricaine. Anesthetized animals were randomly assigned to two subgroups. One subgroup ($N = 2$) was given nasal lavage with 50 mM aqueous sodium cyanoborohydride, while the other ($N = 3$) received lavage with 0.5 mM ethyl acetoacetate, followed by 50 mM sodium cyanoborohydride (Mason et al., 1985). Lavage with either ethyl acetoacetate or sodium cyanoborohydride involved injection of fluids into the olfactory sacs via the external nares. Excess fluid was absorbed at the internal nares with paper wicks. Each naris was rinsed with 100 μl of saline immediately following experimental treatments, and 2 min later, each animal was rinsed in dechlorinated water and placed in a tub containing 100 ml of dechlorinated water to recover from anesthesia. After recovery, the animals were returned to the refrigerator. On each of the six days following lavage, the animals were given generalization trials identical to those described above.

Experiment 2. Twelve salamanders was randomly assigned to three groups ($N = 4$ per group). Each group was trained to respond to one odorant (as S+) at 2% of vapor saturation. Group 1 was trained with cyclohexanone, while groups 2 and 3 were given training with cyclopentanone and ethyl acetoacetate, respectively. Group 3 did not exhibit acquisition of conditioned avoidance, as though insensitive to ethyl acetoacetate. When this odorant was replaced by a cycloalkanone, avoidance acquisition was observed (see below). For all three groups, unreinforced presentations of *n*-butanol (as S−) were randomly interspersed with presentations of S+ odorants.

After training to criterion, groups 1 and 2 were given generalization trials between cyclohexanone and cyclopentanone for six days. On each of these days, animals were given 20 unreinforced test trials (10 per odorant), preceded by five reinforced S+ trials interspersed with five S− presentations. These daily pretest trials served to prevent extinction and to provide a measure of each animal's level of activity.

Following generalization tests, the animals that had been presented with ethyl acetoacetate during the original training trials were randomly assigned to either the cyclohexanone (CH) or cyclopentanone (CP) group. These two groups of animals ($N = 6$ per group) were given five additional days of training, and then each group was divided into two subgroups ($N = 3$ per subgroup); CH-E, CH-C, and CP-E and CP-C, respectively. CH-C and CP-C animals were anesthetized and given a control lavage of 50 mM $NaBH_3CN$ (100 μl into each naris). CH-E and CP-E animals were anesthetized and given nasal lavages of 0.5 mM ethyl acetoacetate followed by 50 mM $NaBH_3CN$ (100 μl of each per naris). After lavage, all animals were rinsed in dechlorinated water and placed in their home cages to recover. On each of the six days following lavage, the animals were given generalization trials identical to those described above.

Analysis. For experiment 1, a series of two-way repeated measures analyses of variance (ANOVAs) were used to assess training periods, response-

concentration curves, and generalization tests. Tukey b post-hoc tests were used to isolate significant differences among means (Winer, 1962). For experiment 2, data from both acquisition and generalization periods were assessed using three-way analyses of variance with repeated measures on two factors (days and odorants). The independent factor in each analysis was groups.

RESULTS

Experiment 1

Group 1. In the first set of experiments, subjects were trained to respond to hexanal. During initial training, there were significant differences among days (F (7,35) = 4.5, $P < 0.001$), and between hexanal and filtered air (F (1,5) = 308.5, $P < 0.0001$). The interaction between days and stimuli was also significant (F (7,35) = 4.2, $P < 0.002$), and the analysis was interpreted in terms of that effect. Tukey tests showed that avoidance responding to presentations of hexanal increased over days (Ps < 0.01), while responding to filtered air remained consistently low ($P > 0.25$).

Analysis of the initial response-concentration test period (neat hexanal) revealed significant differences in responding over days (F (4,20) = 8.5, $P <$ 0.001) and among hexanal concentrations (F (4,20) = 56.7, $P < 0.0001$; Figure 3). The interaction of these terms was also significant (F (16,80) = 14.3, $P < 0.0001$), and the analysis was interpreted in terms of that effect. Post-hoc tests revealed that, over days, lower concentrations of hexanal elicited avoidance responses (Ps < 0.01). Once responding stabilized, however, a concentration between 1 and 1.5% of vapor saturation was found to elicit avoidance responses 50% of the time. This concentration was operationally defined as threshold (Mason and Stevens, 1981b).

During the second training period (hexanal in pristane), the only significant difference was in responding to hexanal versus pristane (F (1,5) = 783.6, $P <$ 0.0001). There were no significant differences between days ($P > 0.25$), and no interaction ($P > 0.25$). Animals exhibited nearly perfect transfer of training, and exhibited 9.3 ± 1.0 responses (out of 10) to hexanal but only 0.70 ± 1.0 (out of 10) responses to pristane on the first day of training.

During the second concentration–response period, the only significant difference was among odorant concentrations (F (5,25) = 55.9, $P < 0.0001$). Post-hoc tests revealed a pattern of results similar to those obtained with air dilutions of pure pristane, but a concentration estimated between 2 and 3% of vapor saturation was found to elicit avoidance responses 50% of the time. These higher threshold values were taken to indicate that the effective vapor concentration above the solutions was less than the equilibrium vapor pressure calculated for an ideal solution.

Results for unreinforced trials are summarized in Table 1. Analysis of generalization among hexanal, 2-fluoro-1-heptene, 1-heptene, and pristane re-

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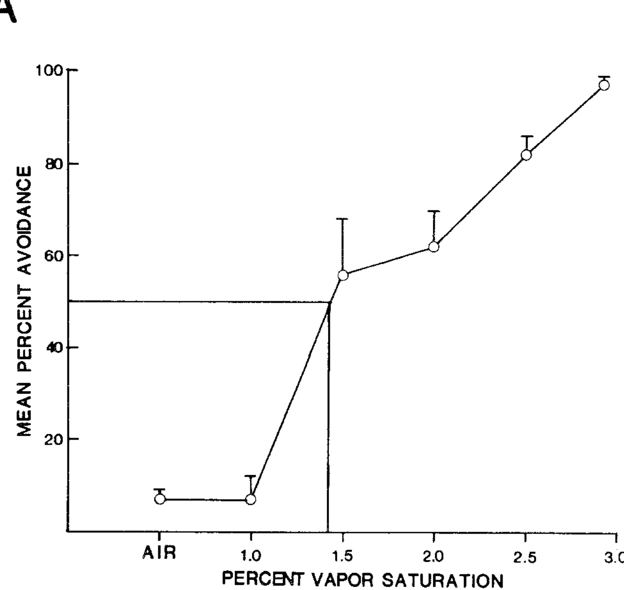

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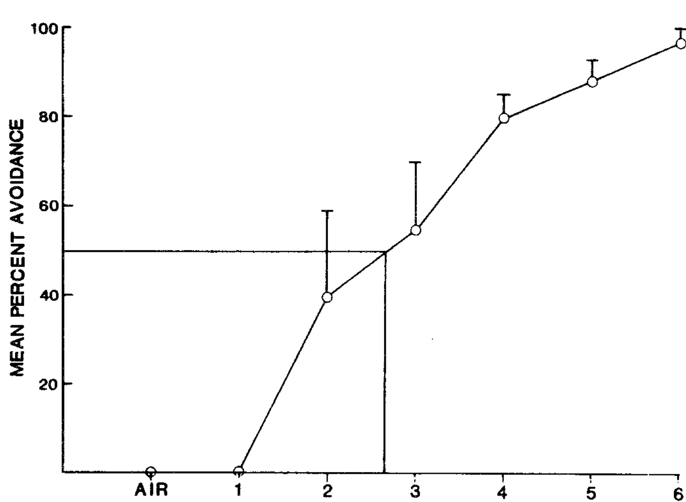

FIG. 3. Concentration–response data for hexanal. (A) Mean percent avoidance for tiger salamanders (N = 6) presented with various air dilutions of vapors from neat hexanal. Threshold for 50% avoidance as represented by the intersection of the horizontal and vertical lines, lies between 1.0 and 1.5% of vapor saturation. Capped vertical bars represent standard errors of the means. (B) Mean percent avoidance for tiger salamanders (N = 6) presented with various air dilutions of a 2.0% (w/v) solution of hexanal in pristane. From Raoult's law, the equilibrium vapor pressure over this solution is estimated to be 6% of vapor saturation. Threshold for 50% avoidance, as represented by the intersection of vertical and horizontal lines, corresponds to a value between 2% and 3% of vapor saturation. Capped vertical bars represent standard errors of the means.

TABLE 1. MEAN RESPONSES (OUT OF 5 PRESENTATIONS PER SESSION) FOR TWO GENERALIZATION EXPERIMENTS ($N = 6$ FOR EACH) BETWEEN HEXANAL AND OTHER ODORANTS[a]

	Session			Session		
Odorant	1	2	Odorant	3	4	5
Hexanal	5.00	5.00	Hexanal	5.00	5.00	5.00
2-Fluoro-1-heptene	0.00	0.33	Heptanal	**3.50**	**3.33**	3.17
1-Heptene	1.00	0.33	2-Heptanone	0.67	0.67	0.83
Air (passed over pristane)	0.00	0.00	Air (passed over pristane)	0.00	0.00	0.00

[a] Significant generalization observed only for heptanal. Entries in boldface meet significance criterion $P < 0.001$ for comparison between responding to 2-heptanone and heptanal. Responding to 2-fluoro-1-heptene, 2-heptanone, or 1-heptene failed to meet the significance criterion $P < 0.05$ when compared to air (except for 1-heptene in session 1, which failed to meet significance criterion $P < 0.02$).

vealed a significant difference among odorants ($F(3,15) = 630.0$, $P < 0.0001$). There were no differences among days ($P > 0.25$), and no interaction between days and odorants ($P > 0.25$). Post-hoc tests revealed that while animals continued to show avoidance of hexanal presentations, they failed to generalize such avoidance to the other odorants ($P < 0.01$).

Analysis of generalization among hexanal, 2-heptanone, and heptanal revealed a significant difference among odorants ($F(3,15) = 59.5$, $P < 0.0001$) but, again, no difference among days ($P > 0.25$), and no interaction between days and odorants ($P > 0.25$). Post-hoc tests revealed that animals exhibited the highest rate of avoidance to presentations of hexanal, an intermediate rate to heptanal, and almost no avoidance of 2-heptanone or pristane (Ps < 0.01). Also, animals failed to exhibit significant generalization of avoidance between hexanal and three other compounds with similar molecular shape and dimensions: 1-heptene, 2-fluoro-1-heptene, and 2-heptanone. As Table 1 summarizes, responding to heptanal is significantly less than responding to hexanal. We infer that these two odorants were perceived as similar, but not identical.

Group 2. Analysis of acquisition revealed that there were differences among days ($F(3,12) = 14.2$, $P < 0.0001$) and between responses to hexanal and pristane ($F(1,4) = 321.8$, $P < 0.0001$). The two-day interaction between days and odorants was also significant ($F(3,12) = 51.6$, $P < 0.0001$), and Tukey tests indicated that avoidance responses to hexanal presentations increased over days (Ps < 0.01) while avoidance responses to pristane remained consistently low.

Analysis of generalization (combined pre- and postlavage) revealed significant differences between subgroups ($F(1,4) = 17.9$, $P < 0.01$), among

odorants (F (6,24) = 5.1, $P < 0.002$), and among days (F (2,8) = 101.7, $P < 0.0001$). Also, there were significant two-way interactions between subgroups and days (F (6,24) = 6.4, $P < 0.001$), subgroups and odorants (F (2,8) = 5.4, $P < 0.05$), and days and odorants (F (12,48) = 2.4, $P < 0.02$). Because the three-way interaction among subgroups, days, and odorants was significant (F (12,48) = 2.95, $P < 0.05$), the analysis was interpreted in terms of that effect. Tukey tests revealed that, prior to lavage, animals exhibited high rates of avoidance responding toward hexanal and heptanal, but low rates toward 2-heptanone (Figure 4; Ps < 0.05). During tests on the first three days after lavage, animals given lavages with ethyl acetoacetate followed by $NaBH_3CN$ exhibited low responding toward all odorants (relative to animals lavaged with $NaBH_3CN$ alone; Ps < 0.05). Animals given lavage with $NaBH_3CN$ alone exhibited no change in responding and continued to avoid presentations of hexanal and heptanal but not presentations of 2-heptanone (P

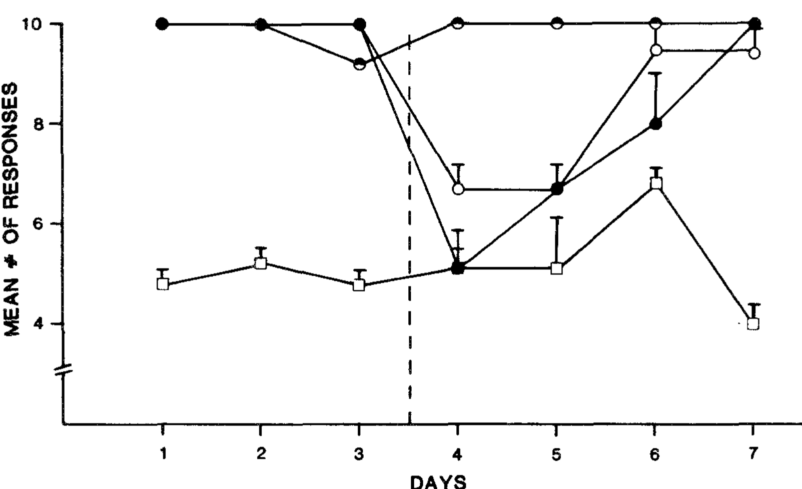

FIG. 4. Effects of chemical modification on generalization among aldehydes for subjects (N = 5) trained on hexanal as S+. In postlavage sessions, half-shaded circles without error bars represent controls (N = 2) that received lavage with 50 mM $NaBH_3CN$ alone. Circles with error bars represent experimental subjects (N = 3) that received two-step lavage (ethyl acetoacetate followed by $NaBH_3CN$). Squares represent mean response to 2-heptanone for all five animals. Dashed line represents point at which lavage was administered.

< 0.05). Responding by animals given combined lavages of ethyl acetoacetate and $NaBH_3CN$ gradually increased over days $P < 0.05$), and by the fourth postlavage test, there were no differences between subgroups ($P > 0.25$).

Experiment 2

Analysis of the initial training period using cyclopentanone and cyclohexanone as stimuli showed that there were no differences between groups ($Ps > 0.25$), although there were significant differences among days ($F(5,30) = 18.3$, $P < 0.0001$), and between responding to S+ and S− ($F(1,6) = 1193.5$, $P < 0.0001$). The interaction between days and odorants was significant ($F(5,30) = 39.4$, $P < 0.0001$), and Tukey tests showed that while avoidance responding to S+ presentations increased over days ($P < 0.01$), responding to S− presentations remained consistently low (Figure 5). Analysis of the initial generalization period (Figure 5) showed that there were no differences between groups ($P > 0.25$), or among days ($Ps > 0.25$). The only significant effect was a two-way interaction between groups and odorants ($F(1,6) = 15.8$, $P < 0.007$). Tukey tests indicated that animals exhibited relatively higher overall levels of responding to their respective training odorant ($Ps < 0.01$). However, both groups showed high levels of responding to both cyclohexanone and cyclopentanone.

During the second training period, there were significant differences between groups ($F(3,8) = 9.3$, $P < 0.006$), between stimuli ($F(1,8) = 1048.9$, $P < 0.0001$), and across days ($F(4,32) = 3.2$, $P < 0.027$). Also, there was a significant interaction between odorant stimuli and days ($F(4,32) = 30.2$, $P < 0.0001$). Tukey tests showed that animals trained with cyclohexanone exhibited higher overall levels of responding than animals trained with cyclopentanone ($P < 0.05$). However, both groups exhibited high response levels to S+ presentations that increased across days while responding to S− presentations remained consistently low ($P < 0.01$).

Analysis of the second series of generalization tests revealed significant differences among subgroups ($F(3,8) = 39.9$, $P < 0.0001$), among odorants ($F(2,16) = 513.5$, $P < 0.0001$), and across days ($F(6,48) = 22.9$, $P < 0.0001$). Also, there were significant two-way interactions between subgroups and odorants ($F(6,16) = 20.6$, $P < 0.0001$), between subgroups and days ($F(18,48) = 5.6$, $P < 0.0001$), and between odorants and days ($F(12,96) = 16.8$, $P < 0.0001$). Because the three-way interaction between subgroups, odorants, and days was significant ($F(36,96) = 4.3$, $P < 0.0001$), the analysis was interpreted in terms of this highest order effect. Post-hoc tests indicated that CH-E and CP-E animals exhibited avoidance decrements ($Ps < 0.01$), while CH-C and CP-C animals did not ($Ps > 0.25$; Figure 6). CH-E and CP-E performance decrements were not permanent, however, and avoidance of cyclohexanone and cyclopentanone increased over days ($Ps < 0.01$). Perfor-

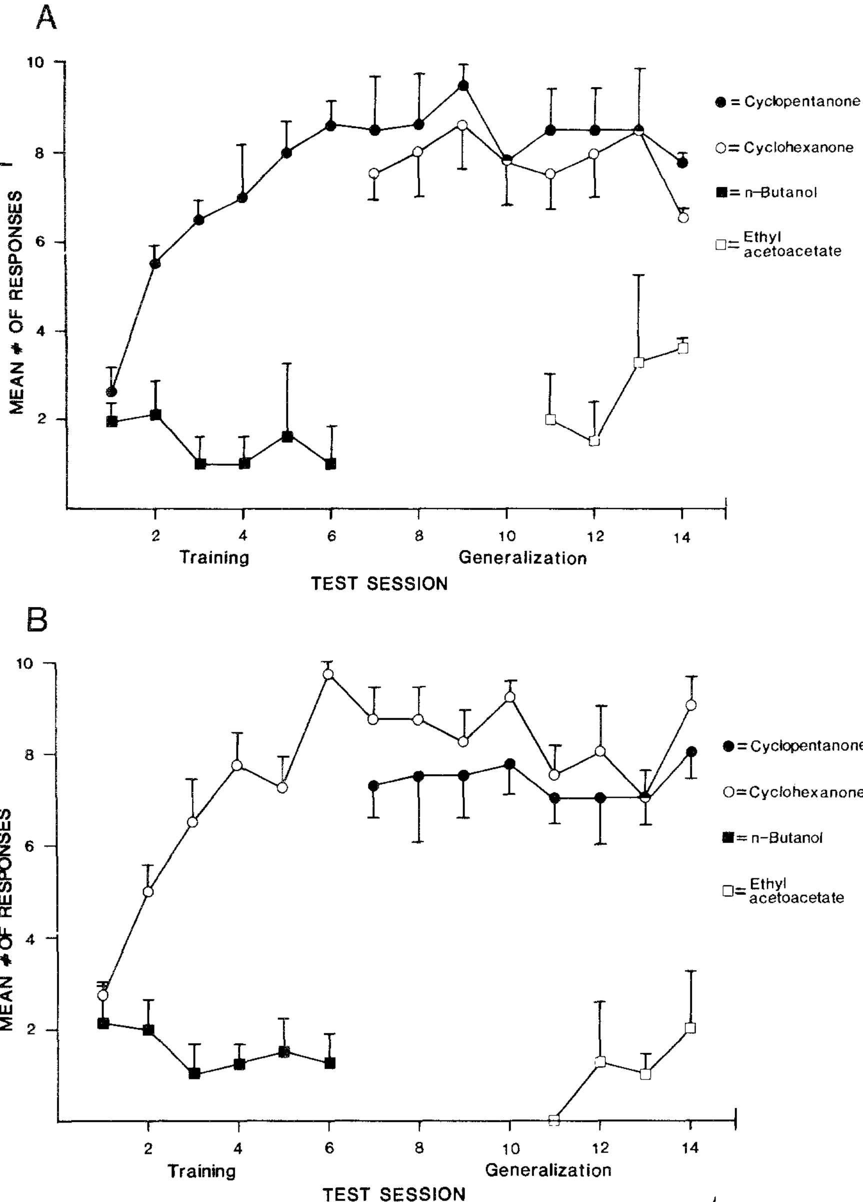

FIG. 5. Generalization between two cycloalkanones over a period of eight days. (A) Results for tiger salamanders ($N = 4$) trained on cyclopentanone (as S+) concurrently with unreinforced presentations of *n*-butanol. Responding to unreinforced presentations of cyclohexanone is significantly lower ($P < 0.01$ by ANOVA of repeated measures) than to unreinforced presentations of cyclopentanone, but generalization between the two odorants is apparent. No generalization to ethyl acetoacetate is observed. (B) Results for tiger salamanders ($N = 4$) trained on cyclohexanone (as S+) concurrently with unreinforced presentations of *n*-butanol. Responding to unreinforced presentations of cyclopentanone is significantly lower ($P < 0.01$ by ANOVA of repeated measures) than to unreinforced presentations of cyclohexanone, but generalization between the two odorants is apparent. No generalization to ethyl acetoacetate is observed.

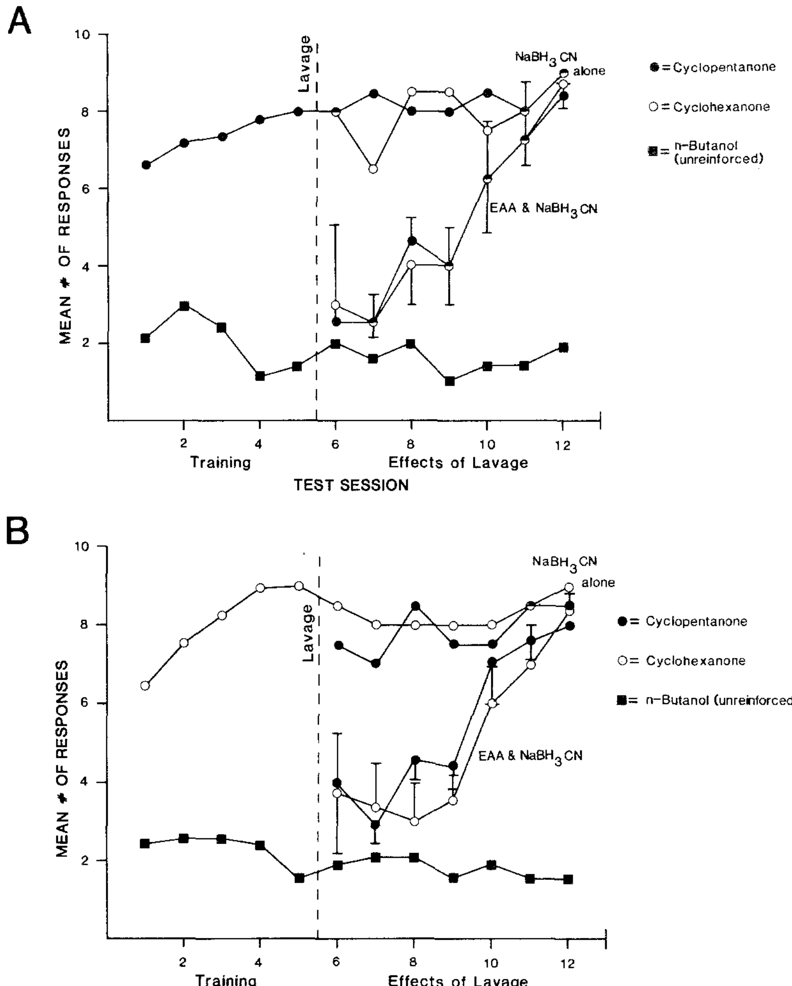

FIG. 6. Effects of chemical modification on olfactory detection and generalization for cycloalkanones. (A) Results for tiger salamanders ($N = 5$) trained on cyclopentanone (as S+) concurrently with unreinforced presentations of *n*-butanol. Lavage with 0.5 mM ethyl acetoacetate followed by 50 mM sodium cyanoborohydride was administered to three animals (represented by points with error bars), and responding to cyclopentanone and cyclohexanone in unreinforced postlavage trials dropped off significantly for four postlavage sessions. Lavage with 50 mM sodium cyanoborohydride alone was administered to two animals (represented by points without error bars). Responding to cyclopentanone and generalization to cyclohexanone were unaffected during postlavage sessions. Responding to *n*-butanol presentations were monitored throughout. (B) Results for tiger salamanders ($N = 5$) trained on cyclohexanone (as S+) with unreinforced presentations of *n*-butanol. Experimental conditions as in A, but with the roles of cyclohexanone and cyclopentanone transposed.

mance improvements were not different between CH-E and CP-E animals ($P > 0.10$).

DISCUSSION

Tiger salamanders generalize from one aldehyde, hexanal, to its next highest homolog, heptanal. However, because presentations of the latter elicit significantly fewer avoidances than presentations of the former, we infer that these odorants are discriminable. Likewise, we infer that hexanal is discriminable from 1-heptene, 2-fluoro-1-heptene, and 2-heptanone, insofar as little or no avoidance generalization to these odorants was observed. Tiger salamanders generalize between cyclopentanone and cyclohexanone, but we infer that these two odorants are discriminable, because groups show significantly greater responding to their respective training odorant during generalization tests.

Two-step modification impairs detection of both aldehydes and of cycloalkanones. Figure 6 shows that, after lavage of the olfactory epithelium, there is no significant difference between responding to S+ and responding to the new odor. Significant impairment of detection occurs only if blocker and fixer are both applied. As shown for the controls in Figure 6, lavage with fixer by itself does not reduce the level of avoidance significantly. We have previously shown that lavage with ethyl acetoacetate alone at a concentration ≤ 1.0 mM has no effect on responding (Mason and Morton, 1982, 1984; Mason et al., 1984, 1985). An important feature of Figure 6 is that discrimination of cycloalkanones from *n*-butanol is not completely abolished by two-step modification. Although dramatically reduced, significant levels of avoidance to both cycloalkanones persist after lavage with the blocker fixer sequence. Generalization persists, as well.

The results of two-step modification are consistent with the hypothesis that the two aldehydes and the two cycloalkanones interact with the same class of receptor. The two steps consist of a reversible covalent attachment of a site-specific ligand (blocker) followed by conversion to an irreversible linkage (via a fixer). In these experiments the blocker was ethyl acetoacetate and the fixer was sodium cyanoborohydride, which have previously been shown to label Schiff base-forming proteins specifically in vitro (Mason et al., 1985). This same procedure, when applied to the olfactory epithelium, produces a selective hyposmia to ketones (Mason et al., 1984, 1985) and aldehydes. The control animals, treated with fixer by itself, exhibit no evidence of impaired olfactory function.

Several investigators have proposed that covalent modification of specific amino acid residues can impair chemoreceptor function (Cagan, 1981; D'Ischia, et al., 1982; Mason et al., 1984, 1985). Antifeedant activity of 9β-polygodial and its 9α-hydroxy analog, warburganal, has been attributed to a specific reaction with primary amino groups, such as lysine residues (D'Ischia et al.,

1982). Two-step modification with acetoacetic ester as blocker followed by cyanoborohydride as fixer has been shown to selectively label the active site of a Schiff base-forming enzyme in vitro and to selectively impair detection of ketone-containing odorants in vivo (Mason et al., 1984, 1985). The suggested mechanism invokes irreversible covalent attachment to especially reactive lysine residues. One advantage of using a blocker–fixer sequence is that control experiments can be performed, in which one of the steps is omitted. As Figures 4 and 6 summarize, omission of blocker prevents impairment.

When an animal is trained to avoid one carbonyl compound, it may generalize to another. Lavage with acetoacetic ester followed by cyanoborohydride impairs responding to both odorants. This suggests that the two different molecules interact with the same class of receptor. Both aldehydes and ketones interact with this class of receptors, but generalization does not occur among all simple carbonyls. For instance, animals trained on hexanal do not generalize to 2-heptanone, even though they do generalize to heptanal.

The effect of covalent modification may be agonistic rather than antagonistic. Chemical steps by which Schiff base-forming sites are blocked by two-step modification have been described (Mason et al., 1985), and it seems unlikely that a very large fraction of olfactory receptor sites can be labeled in this fashion. Since, as previously reported, increasing the dose of labeling reagent increases the duration of selective hyposmia, and not its profundity, we are led to propose the following hypothesis. When an olfactory receptor site is covalently modified, it generates a continuous output. If the covalent modification is irreversible, this output continues unabated and causes a high level of background signal, which effectively masks the response to any new odorant molecules that interact with that channel. As can be seen from Figure 6, covalent blockade did not completely obliterate responding to either cycloalkanone. This is taken to imply that the cycloalkanones also interact with receptors that are unaffected by covalent modification. As might be expected (since cyclohexanone and cyclopentanone are similar in molecular volume, dipole moment, etc.), responding to both odorants is about the same, even during the postlavage interval, where olfactory detection of ketones is greatly impaired. We infer that both cycloalkanones interact with Schiff base-forming receptor sites, but that this is not the sole basis for generalization.

Results presented here add to an increasing body of data that supports the existence of carbonyl-binding, generalist receptors in tiger salamanders. The effects of two-step modification suggest that these receptors attach odorant molecules covalently as Schiff bases. A role for other types of receptors in detection and discrimination of ketones and aldehydes is also deduced, since chemical modification impairs responding, but neither obliterates detection nor eradicates generalization. We infer that generalist olfactory receptors have the following characteristics. A given class of receptors will bind a number of odorants. At

the same time, any given molecule can interact with more than one class of receptors. For generalization to take place, two different compounds must have similar interactions with more than one receptor class. Since nature rarely presents pure compounds as stimuli, the ability to generalize, even in the presence of distractors, must be balanced against the ability to suppress false alarms and discriminate against mimics. This picture may offer an optimum strategy for dealing with natural fluctuations in the composition of olfactory cues.

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PHYTOTOXIC PROPERTIES OF CNICIN, A SESQUITERPENE LACTONE FROM *Centaurea maculosa* (SPOTTED KNAPWEED)

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Abstract—Water and solvent extracts from the aerial tissues of *Centaurea maculosa*, spotted knapweed, inhibited the root growth of lettuce. Column chromatography and lettuce bioassay of a chloroform extract led to the isolation of cnicin, a sesquiterpene lactone. Pure cnicin was bioassayed at 0, 1, 2, 4, 6, 8, and 10 mg/5 ml water with lettuce, created wheatgrass, bluebunch wheatgrass, rough fescue, western larch, lodgepole pine, and spotted knapweed. Germination was inhibited at one or more concentrations for all species except lodgepole pine and spotted knapweed. Growth, particularly of the roots, was retarded between 1 and 4 mg of cnicin. Lettuce, bluebunch wheatgrass, and spotted knapweed were inhibited significantly at all concentrations tested.

Key Words—Allelopathy, spotted knapweed, *Centaurea maculosa*, sesquiterpene lactones, cnicin, phytotoxin, germination inhibitor, growth inhibitor.

INTRODUCTION

Spotted knapweed (*Centaurea maculosa* Lam.) was introduced to the Pacific Northwest from Eurasia around the turn of the century (Strang et al., 1979). First reported in Montana during the 1920s (French and Lacey, 1983; Chicoine, 1984) plants can now be found in every county of the state, and they infest an estimated 0.8 million hectares. The annual rate of spread has been calculated at 27.4% (Lacey, 1983), and man's activities have been an important component in this rapidly expanding distribution (Strang et al., 1979).

Spotted knapweed is a pioneer species that thrives on dry disturbed sites (Watson and Renney, 1974). Being a prolific seed producer (Schirman, 1981, 1984) and efficient competitor for nutrients (Belles et al., 1980) and water,

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knapweed has proven to be an aggressive invader of dry pastures and native rangelands (Harris and Cranston, 1979). Part of its success has been attributed to the lack of natural herbivores and diseases that were left behind in the Old World. Productivity of desirable forage plants decreases significantly as the knapweed density increases (Harris and Cranston, 1979; Maddox, 1979). Annual forage losses in Montana from the present infestation have been estimated at $4.5 million. Recent studies indicate that Montana has 13.7 million hectares of range and grazable woodlands vulnerable to knapweed invasion (Bucher, 1984; Chicoine, 1984). Forage losses could reach $155.7 million annually if all of these sites were invaded.

In 1963 Fletcher and Renney demonstrated that Russian knapweed (*Centaurea repens* L.), diffuse knapweed (*C. diffusa* Lam.), and spotted knapweed tissues, particularly the leaves, contained phytotoxic compounds that inhibited the germination and growth of barley and lettuce. Extracts from Russian knapweed were the most potent inhibitors, and those from spotted knapweed the weakest. Soils naturally infested with Russian knapweed retarded the growth of tomato and barley; however, soils infested with diffuse and spotted knapweed were never tested for toxicity. Fletcher and Renney (1963) were unable to identify the active compounds. As part of a project to evaluate the allelopathic influence of spotted knapweed, in this paper we report the isolation of a phytotoxic sesquiterpene lactone, cnicin, and its effects on the germination and growth of several native plant species.

METHODS AND MATERIALS

Preparation of Knapweed Extracts. Knapweed plants were collected during the last week of April 1983 at a study site in the Rattlesnake Creek drainage, about three miles north of the University of Montana campus. Whole plants, possessing a previous year's stem, were removed from the soil with approximately 15 cm of root attached. After discarding dead stems and leaves, the green rosette leaves and roots were returned to the laboratory on ice. Ten grams of fresh roots and leaves, cut into small pieces before weighing, were each extracted by soaking in 90 ml of distilled water for 15 hr (moisture content was determined with duplicate samples oven dried at 100°C overnight, desiccated for 30 min, and weighed). These solutions were filtered through paper into a 100-ml graduated cylinder. The extracted residue was rinsed with distilled water and filtered, bringing the final volume to 100 ml. An equal volume of distilled water was filtered for the control. Five milliliters of each solution was added to 10 Petri dishes and bioassayed with lettuce. Five dishes from each treatment were randomly selected for measurement and statistical analysis.

Unused leaves and roots were air-dried. Leaves were crushed by hand and three 5.0 g samples weighed (moisture content was measured as described

above). One sample was Soxhlet extracted for 8 hr with diethyl ether, then filtered through paper giving about 40 ml of extract. A white residue on the sides of the distillation–collection flask would not redissolve in either but was soluble in chloroform, which was added to the ether giving a final volume of 100 ml. A second 5 g of leaves were washed in chloroform for 5 min, with slow stirring. After filtering, the final volume was adjusted to 100 ml by rinsing the leaf residue. A water extract was prepared from the third leaf sample following the same procedure used for fresh tissue. Five-milliliter aliquots of these solutions were bioassayed with lettuce (five Petri dishes per treatment). Distilled water, ether, and chloroform were used in separate controls.

Isolation and Identification of Cnicin. In mid-June, green leaves and stems were collected from the Rattlesnake study site. Fresh tissue (649 g), cut into 7- to 10-cm pieces, was washed for 5 min in chloroform (7000 ml for extraction and 1000 ml for rinse), filtered, and the solvent evaporated. A white crystalline material precipitated from the concentrated extract and was removed by filtration (2.39 g). The remaining chloroform was evaporated and the residue dissolved in 50 ml of hot ethanol. After transferring to a separatory funnel, an equal volume of water was added. This solution was washed with hexanes (4×50 ml) and then extracted with chloroform (2×100 ml). The initial chloroform extract, combined hexane washes, final chloroform extract, and hexane and chloroform controls were bioassayed with lettuce (two Petri dishes per treatment). Germination and root growth were most inhibited by the final chloroform extract, which was further fractionated by column chromatography on silica gel eluted with chloroform and increasing proportions of ethyl acetate. Ethanol was used to clear the column. Fractions containing similar chemical components by TLC were combined to give a total of seventeen. After adjusting to equal volumes, each fraction was bioassayed with lettuce (two Petri dishes per fraction). Five milliliters of chloroform were added and dried in the control dishes.

Crystals isolated from fraction 15 were purified by recrystallization. Thin-layer chromatography indicated it was the same compound that had crystallized from the original chloroform extract. It was identified as the sesquiterpene lactone, cnicin, by TLC, NMR, and direct IR comparison with a reference sample. Toxicity of recrystallized cnicin was tested by bioassaying with lettuce, crested wheatgrass, and five native species including knapweed. In each experiment, 40 seeds from one species (20 per Petri dish) were exposed to six different concentrations of cnicin; the entire experiment was then duplicated.

Bioassay Procedures. Petri dishes were lined with three sheets of Whatman No. 1 filter paper, followed by 5 ml of the test solution. Crystalline cnicin has limited water solubility, so solutions were prepared fresh in chloroform–ethanol (23 : 2) to deliver 0, 1, 2, 4, 6, 8, and 10 mg of cnicin per 5-ml aliquot. Dishes containing organic solvents were air-dried in a fumehood, and then remoistened with 5 ml of distilled water. If cnicin were completely soluble in

water, these concentrations would range from 5.29 to 52.9 $\times 10^{-4}$ M. Twenty Black Seeded Simpson lettuce seeds were sown in each dish and the dishes placed into a germinator (continuous fluorescent light, 24–26°C for 72 hr).

Seeds from two tree species, western larch (*Larix occidentalis* Nutt.), and lodgepole pine (*Pinus contorta* Dougl. ex Loud.), were stratified before use. They were soaked for 24 hr in tap water, drained, sealed in a Ziploc plastic bag, and placed in the refrigerator at 0°C for a minimum of two weeks (34 days was the longest cold treatment). Seeds of rough fescue (*Festuca scabrella* Torr.), a native grass, were soaked for 24 hr in tap water and then sown into Petri dishes containing the cnicin-treated filter paper with 5 ml water. Dishes were placed directly into the refrigerator for stratification (a minimum of two weeks). Other species bioassayed without stratification included crested wheatgrass [*Agropyron cristatum* (L.) Gaertn.], bluebunch wheatgrass [*Agropyron spicatum* (Pursh) Scribn. & Smith], and spotted knapweed. All seeds, except lettuce, were surface sterilized with Clorox solution (3% solution of commercial Clorox) before sowing, and Captan fungicide (0.25 g/L) was added to the distilled water to retard fungal growth.

Bluebunch wheatgrass, western larch, and lodgepole pine were grown in continuous light with the same conditions as lettuce. Spotted knapweed was grown with 9 hr dark (20°C) and 15 hr fluroescent light (26°C). Crested wheatgrass and rough fescue were bioassayed in one experiment with continuous light and the other with alternating day/night cycles, with temperatures the same as knapweed. Except for lettuce, the length of time in the germinator varied (5–12 days) to meet the requirements of a particular species. During an experiment additional water was added to all dishes, as needed.

Measurements. After recording germination, seedlings were photographed with black and white film. Their negative image was projected onto a Ladd Graphical Digitizer. Initially root lengths were to be measured for all species, but in experiments where root growth was severely inhibited it was difficult to clearly distinguish root tissue, so seedling lengths (from cotyledon attachment to root tip, or seed coat to root tip) were measured instead. Root lengths were measured for grass species and some lettuce experiments. Since root growth was very sensitive to cnicin, ungerminated seeds were recorded as zero growth and used to determine average length.

Data Analysis. Percent germination was calculated in each Petri dish, then transformed (arcsine), and analyzed by one-way analysis of variance (ANOVA). In nearly all experiments the variances were positively correlated with the mean root length, or mean seedling length, and logarithmic or square root transformations did not remedy the problem. Consequently, seedings were ranked by Kruskal-Wallis, compensating for ties. Rank averages were calculated for each Petri dish and then analyzed by one-way ANOVA. When root or seedling length variances were homogeneous, one-way ANOVA was used. The

0.05 level of probability was applied in all cases, and significantly different means were detected by Duncan's multiple-range test.

RESULTS

A 10% water extract of fresh knapweed roots (3.93 g dry weight) had no effect on the germination and growth of lettuce (Table 1). A similar extract of fresh leaves (1.94 g dry weight) also had no effect on lettuce germination, but limited root growth to 68% of the control. In an attempt to approximate the earlier results of Fletcher and Renney (1963), 5 g of crushed air-dried leaves (4.57 g dry weight) were extracted with water, ether, or chloroform. All extracts inhibited germination, but not to any great extent (Table 2). Seedling growth was severely retarded, particularly the roots, in all treatments with levels of inhibition similar to that reported by Fletcher and Renney (1963).

To isolate the active compound(s), 649 g of fresh knapweed tissue was washed with excess chloroform. While concentrating the extract, a white crystalline precipitate was filtered from the solution. The concentrated extract was

TABLE 1. EFFECT OF FRESH KNAPWEED WATER EXTRACTS ON GERMINATION AND GROWTH OF LETTUCE

	Lettuce		
Knapweed extract	Germination (% of control)	Hypocotyl growth (% of control)	Root growth (% of control)
Leaf	100.0	95.1	68.2[a]
Root	99.0	102.1	100.8

[a] Mean significantly different from the control at $P < 0.05$.

TABLE 2. EFFECT OF DRIED KNAPWEED LEAF EXTRACTS ON GERMINATION AND GROWTH OF LETTUCE

	Lettuce		
Knapweed extract	Germination (% of control)	Hypocotyl growth (% of control)	Root growth (% of control)
Water	95.0[a]	15.2[a]	9.3[a]
Ether	92.0[a]	12.8[a]	3.6[a]
Chloroform	80.0[a]	14.9[a]	4.5[a]

[a] Mean significantly different from the control at $P < 0.05$.

redissolved in ethanol–water, washed with hexanes, and then extracted back into chloroform. Lettuce bioassays indicated the toxin(s) were concentrated in the final chloroform solution. Column chromatography provided 17 fractions for bioassay. Only a few fractions inhibited lettuce germination, whereas nearly all of them retarded seedling growth (Fig. 1). A crystalline compound was isolated from the fraction most inhibitory to growth, and subsequently identified as the sesquiterpene lactone, cnicin. This was the same compound that had precipitated from the initial chloroform extract.

The toxicity of impure and recrystallized cnicin was tested with lettuce and found to be very active. Germination was unaffected by 1 mg of cnicin, but gradually decreased as the concentration went up (Table 3). Seedling growth was more severely affected (Table 4). At 2 mg of cnicin, roots were approximately 1 mm or less in length and brown in color up to the hypocotyls, which were also very short compared to the controls, but otherwise still green and healthy looking. At higher concentrations, the hypocotyls also turned dark brown. It was difficult to accurately distinguish root tissue at higher concentrations, so seedling lengths were measured instead.

Germination of the three grass species was largely unaffected by 1 or 2 mg of cnicin (Table 3). At higher concentrations their responses varied. With 4 mg or greater, crested wheatgrass germination was about 50% of control or lower, whereas bluebunch wheatgrass seldom dropped much below 70%. Rough fescue demonstrated decreasing germination between 4 and 10 mg of cnicin, but with different intensities between duplicate experiments; at 8 and 10 mg, germination was below 50% of control in both experiments. Root growth was more sensitive to cnicin with some inhibition at nearly all levels tested (Table 4).

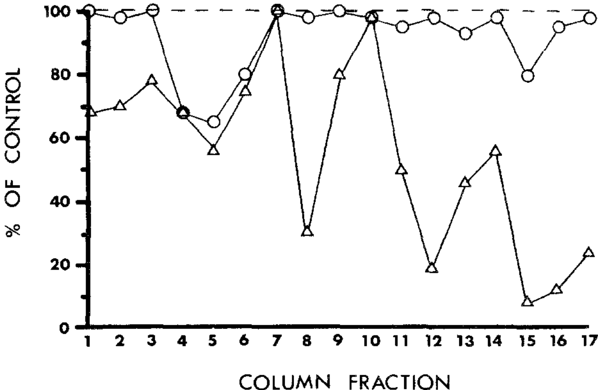

FIG. 1. Effect of column chromatography fractions, from a knapweed chloroform extract, on the germination (○) and growth (△, seedling length) of lettuce. Germination of fractions 4–6, 11, 13, and 15 are significantly different from the control. Seedling lengths in all fractions are significantly different from the control, except 7 and 10.

TABLE 3. EFFECT OF CNICIN ON GERMINATION

Species tested	Experiment No.	Germination (% of Control)[a]						
		Cnicin (mg)						
		0	1	2	4	6	8	10
Lettuce	1[b]	100 a	100 a	100 a	88 b	NT[b]	NT	20 c
	2	100 a	100 a	93 b	93 b	65 c	50 c	30 d
Crested wheatgrass	1	100 a	97 a	97 a	54 b	49 b,c	34 c,d	23 d
	2	100 a	93 a	97 a,b	40 b,c	53 a,b,c	30 c	43 a,b,c
Bluebunch wheatgrass	1	100 a	110 a	114 a	90 a	83 a	79 a	83 a
	2	100 a	86 a,b	94 a,b	91 a,b	77 a,b,c	66 b,c	51 c
Rough fescue	1	100 a	80 a,b	84 a,b	52 a,b,c	32 b,c	8 c	28 b,c
	2	100 a	121 a	107 a	86 a	79 a	43 a	29 a
Western larch	1	100 a,b	118 a	95 a,b	73 a,b	86 a,b	59 a,b	50 b
	2	100 a,b	133 a	124 a	129 a	76 b,c	43 c	38 c
Lodgepole pine	1	100 a,b	106 a,b	100 a,b	135 a	94 a,b	95 a,b	71 b
	2	100 a	100 a	115 a	85 a	85 a	108 a	92 a
Spotted knapweed	1	100 a,b	106 a	106 a	94 a,b	103 a,b	100 a,b	85 b
	2	100 a,b	97 a,b	100 a,b	103 a	95 a,b	92 a,b	74 b

[a] Means followed by different letters are significantly different at $P < 0.05$.
[b] Cnicin used in this experiment was the crude crystalline precipitate obtained during the concentration of the chloroform extract. The 6- and 8-mg concentrations were not tested (NT).

TABLE 4. EFFECT OF CNICIN ON GROWTH

Species tested	Experiment No.	Measurement	Growth (% of Control)[a] Cnicin (mg) 0	1	2	4	6	8	10
Lettuce	1[b]	TS[c]	100 a	18 b	11 c	8 d	NT[b]	NT	1 e
	2	TS	100 a	17 b	9 c	6 d	4 e	3 e,f	2 f
Crested wheatgrass	1	R	100 a,b	209 a	92 b	19 c	14 c	1 c	0 c
	2	R	100 a	81 a	77 a	5 b	11 b	2 b	4 b
Bluebunch wheatgrass	1	R	100 a	84 a	63 a	29 b	20 b	20 b	15 b
	2	R	100 a	62 b	53 b	32 c	16 d	11 d	11 d
Rough fescue	1	R	100 b	50 a	66 a	5 b	1 b	1 b	6 d
	2	R	100 a,b	139 a	79 a	21 a,b	9 a,b	11 a,b	16 b
Western larch	1	TS	100 a	39 a,b	27 a,b,c	13 c,d	13 b,c,d	5 c,d	4 d
	2	TS	100 a	59 a	44 a	29 a,b	16 b,c	7 c	6 c
Lodgepole pine	1	TS	100 a	53 a,b	37 b,c	45 a,b	22 c,d	24 c,d	14 d
	2	TS	100 a	69 a	59 a	45 a	38 a	32 a	29 a
Spotted knapweed	1	TS	100 a	60 b	52 b	31c	30 c	16 d	11 d
	2	TS	100 a	60 b	52 b	32 c	20 d	11 c	9 e

[a] Means followed by different letters are significantly different at $P < 0.05$.
[b] Cnicin used in this experiment was the crude crystalline precipitate obtained during the concentration of the chloroform extract. The 6- and 8-mg concentrations were not tested (NT).
[c] TS = total seedling length, r = root length.

Greatest variation was recorded with 1 mg, where growth was stimulated rather than inhibited in two experiments. Cnicin had signficant impact on all grass roots starting at the 4-mg level; however, none of them exhibited the dark brown discoloration or severe damage as did lettuce.

Of the two tree species tested, western larch appeared more sensitive to cnicin; germination began to decrease about 6 mg and was near 50% of the control at 8 and 10 mg (Table 3). Seedling size (Table 4) was much smaller than the control at all concentrations, with significant differences occurring at 4 mg cnicin or greater. At these higher levels, roots appeared heavily damaged. Lodgepole pine germination did not decrease much even at the highest cnicin concentrations (Table 3). Seedling size was inhibited at all levels, but significant differences were recorded in only one of the experiments (Table 4). Again the roots appeared heavily damaged at the high concentrations. In the mixed conifer forests of northwestern Montana, field observations suggest that western larch has difficulty regenerating from seed in the presence of dense knapweed stands (B. Wilson, personal communication). Lodgepole pine is apparently more competitive and is capable of seedling establishment within the knapweed.

One of the most interesting results of these experiments was the response of spotted knapweed when bioassayed with cnicin. Germination was unaffected (Table 3), whereas seedling growth was strongly retarded (Table 4). At 1 mg cnicin, seedlings were 60% of the control and significantly different, in both experiments. Size decreased with increasing concentrations, reaching only 10% of the control at 10 mg of cnicin. Under the conditions of these experiments knapweed is just as sensitive, if not more sensitive to cnicin, as the other species tested.

DISCUSSION

Politis (1946a,b) reported the isolation of bitter-tasting cnicin from the glandular trichomes of holy thistle (*Cnicus benedictus* L.) Its structure was difficult to solve and required several revisions (Suchy and Herout, 1962; Suchy et al., 1959, 1962, 1965). The currently accepted structure was assigned by Samek et al. in 1969. Cavallito and Bailey (1949) were probably the first to obtain crystalline cnicin from *Centaurea maculosa,* although they were unable to identify it. Data from their chemical analyses leaves little doubt that it was cnicin they had isolated. Cnicin has been extracted from numerous other *Centaurea* species in recent years (Suchy and Herout, 1962; Gonzalez et al., 1978; Rustaiyan et al., 1982; Seaman, 1982; Geppert et al., 1983; Muir and Majak, 1983).

Cavallito and Bailey (1949) were interested in cnicin because of its antimicrobial properties toward both gram-positive and gram-negative organisms. It inhibited the growth of some bacterial species at 25–50 μg/ml. It was bac-

tericidal to the sensitive species at higher concentrations. Similar experiments have been repeated and gave comparable results (Vanhaelen-Fastré, 1972; Vanhaelen-Fastré and Vanhaelen, 1976). In cytotoxicity tests, cnicin was found to be active toward KB and HeLa human carcinoma cells in vitro (Vanhalen-Fastré and Vanhaelen, 1976; Gonzalez et al., 1978). In vivo it was active against L-1210 leukemia (Vanhaelen-Fastré and Vanhaelen, 1976). This is not unusual since sesquiterpene lactones exhibit a variety of such activities (Rodriguez et al., 1976), including phytotoxicity.

The allelopathic potential of sesquiterpene lactones has been documented by numerous bioassay experiments (Dalvi et al., 1971; Garciduenas et al., 1972; McCahon et al., 1973; Kanchan, 1975; Amo and Anaya, 1978; Asakawa and Takemoto, 1979; Picman and Picman, 1984a; Spencer et al., 1984; Fischer and Quijano, 1985; Stevens and Merrill, 1985). The most thoroughly investigated example of an allelopathic sesquiterpene lactone is parthenin in the tropical weed *Parthenium hysterophorus* L. (Kanchan, 1975; Kanchan and Jayachandra, 1979a,b, 1980; Picman and Picman, 1984a). It was the most abundant inhibitor in the aerial tissues of this plant and was present in leaf washes and the rhizosphere soil.

Very little information is available on the precise mode of action of sesquiterpene lactones within plant tissues; however, there is substantial data from other organisms that provides insight for potential mechanisms. The presence of an α-methylene-γ-lactone (Dupuis et al., 1980; Hall et al., 1980a,b; Spring et al., 1982; Elissalde et al., 1983; Picman and Picman, 1984b; Abeysekera et al., 1985;), and/or an α,β-unsubstituted cyclopentenone (Lee et al., 1971, 1977b, 1981) is frequently associated with biological activity, but not always (Picman and Towers, 1983; Harmatha and Nawrot, 1984; Marchant et al., 1984; Picman, 1984). Within cells the lactones can inhibit DNA synthesis (Lee et al., 1977a; Woynarowski and Konopa, 1981; Woynarowski et al., 1981), RNA synthesis (Spring et al., 1982), protein synthesis (Liou et al., 1983), respiration (Van Aswegen et al., 1982), and can interfere with the normal functioning of various enzymes (Hall et al., 1980a,b), the net result being impaired growth and development. α-Methylene-γ-lactones, and α,β-unsubstituted cyclopentenones are reactive sites because they bind easily with free —SH groups in amino acids and proteins, resulting in nonreversible alkylation (Rodriguez et al., 1976; Lee et al., 1977a; Dupuis et al., 1980; Spring and Hager, 1982) that can destroy enzymatic activity. There is no doubt that these same sort of reactions take place in plant cells.

Since the initial isolation of phytotoxic extracts from the tissues of three knapweed species (Fletcher and Renney, 1963), there have been no studies to thoroughly evaluate the ecological significance of allelopathy in any of these species, even though their infestations exhibit allelopathic characteristics, and they are rapidly becoming some of the most noxious weeds in the Pacific Northwest. Several phytotoxic sesquiterpene lactones have been isolated from Rus-

sian knapweed (Stevens, 1982; Stevens and Merrill, 1985), but it is not known if they are responsible for the soil toxicity associated with this species.

A study very similar to ours was just completed with diffuse knapweed (Muir and Majak, 1983). A polar and a nonpolar inhibitor were detected in the aerial tissues. Root extracts also had activity. Column fractionation of a chloroform extract from leaves and stems bioassayed with ryegrass (*Lolium multiflorum* L.), lead to the isolation of cnicin. Impure cnicin, and the column fraction it was isolated from, both inhibited ryegrass seedlings growth at 0.4 mg/ml (the same as our 2 mg/Petri dish). Purified cnicin, at the same concentration, failed to retard germination or growth and was rejected as a major inhibitor by itself. The polar inhibitor was not identified.

In our study cnicin was phytotoxic for all species tested, root growth was most sensitive, and the level of inhibition was concentration- and species-dependent. Cnicin's potential to function as an effective allelopathic agent will be determined by the concentrations in the soil and the associated species in the community. Knapweed root growth is very sensitive to this compound and, unless these plants have some mechanism for eliminating, avoiding, or minimizing cnicin's effect under natural conditions, then knapweed' competitive ability could be diminished. Secondary metabolites (including the two sesquiterpene lactones, parthenin and coronopilin) from *Parthenium hysterophorus* are allelopathic and autotoxic, inhibiting the germination and growth of *Parthenium* seedlings and older plants (Picman and Picman, 1984a).

It was hypothesized that the autotoxicity is a mechanism for population regulation and timing the process of germination. Autotoxicity does not appear to be functioning within knapweed populations. Cnicin does not retard knapweed germination. In the field, germination can occur after seed dispersal in the fall, if water is adequate, otherwise large numbers of seeds germinate the following spring. Knapweed has no apparent difficulty reestablishing itself in the presence of older plants. In Montana, Chicoine (1984) reported 62 mature plants with 178 seedlings per square meter at one study site, and 60 mature plants with 1808 seedlings per square meter at another. Competition could account for eventual decreases in plant numbers.

Other toxic compounds, besides cnicin, were also present in spotted knapweed. As indicated in Figure 1, most column fractions from the chloroform extract inhibited lettuce growth. Their concentrations were not as high as cnicin, but their effects could be additive (Mandava, 1985).

In addition to allelopathy, cnicin might be important in other ecological interactions. As discussed above, cnicin is antimicrobial and could possibly function as a defense against disease organisms, as suggested for the sesquiterpene lactone parthenolide in *Chrysantemum parthenium* Bernh. (Blakeman and Atkinson, 1979). As with many sesquiterpene lactones, cnicin is bitter tasting (Wagner, 1977) and could influence palatability. Cattle and horses limit their consumption of knapweed, particularly when more desirable forage species are

available, and none of the New World insects defoliate spotted knapweed to any significant extent. Glaucolide-A is a bitter tasting sesquiterpene lactone from *Vernonia* that affects the feeding preference of wild rabbits and whitetail deer. Both animals avoid eating *Vernonia* species containing, or artificially coated with glaucolide-A (Burnett et al., 1977; Mabry and Gill, 1979). This compound has also been extensively tested with insect herbivores. In the laboratory it deterred the feeding of various lepidopterous larvae, particularly the southern armyworm, *Spodoptera eridania* (Cramer), and the fall armyworm, *S. frugiperda* (J.E. Smith) (Burnett et al., 1974). Their growth and survival was reduced significantly when reared on diets containing this lactone (Jones et al., 1979). Field experiments failed to substantiate glaucolide-A as a chemical defense against insect herbivores, but it is still considered an effective mammalian feeding deterrent (Burnett et al., 1977, 1978).

CONCLUSION

Green aerial tissues of spotted knapweed contain the sesquiterpene lactone cnicin that is phytotoxic in laboratory bioassays. It retarded the germination of some plants at the higher concentrations tested, but was not a strong germination inhibitor. Cnicin was most active toward seedling growth, particularly roots, causing significant inhibition in all species tested, including spotted knapweed. This compound could be allelopathic under the appropriate environmental and biotic conditions, possibly contributing to the ecological success of spotted knapweed.

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DEFENSIVE STEROIDS FROM A CARRION BEETLE (*Silpha novaboracensis*)[1]

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Abstract—Two novel steroids, 3α,7β-dihydroxy-14β-pregn-4-en-15,20-dione (1) and 3α,7β,20ξ-trihydroxy-14β-pregn-4-en-15-one (4), have been characterized from the rectal gland of *Silpha novaboracensis* (Coleoptera: Silphidae). Judging from the function of comparable pregnanes in another species of *Silpha*, the compounds may play an antipredator role.

Key Words—Coleoptera, Silphidae, *Silpha novaboracensis*, steroids, pregnanes.

We recently described a series of oxygenated pregnanes that serve a defensive role in *Silpha americana*, a carrion beetle (Meinwald et al., 1985). The compounds are produced by a glandular annex of the rectum and are discharged from the anus, together with enteric fluid, when the beetle is disturbed. Bioassays of a representative pair of these pregnanes showed the compounds to be deterrent to jumping spiders. We now report the isolation of two new pregnanes from *Silpha novaboracensis*, also produced by the rectal gland of the beetle. Several hundred glands from beetles collected at bait (dead chickens) near Ithaca, New York, were extracted with 1 : 1 dichloromethane–methanol mixture (ca. 15 μl per beetle). Both thin-layer chromatography and high-performance liquid chromatography (HPLC) showed the extract to contain two major components, totaling ca. 10 μg per beetle, designated as A and B. These were isolated in pure form by preparative HPLC (C-18 column; linear gradient elution with 25% acetonitrile–75% water to pure acetonitrile).

High-resolution mass spectrometry showed A, the major component, to

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have the molecular formula $C_{21}H_{30}O_4$ (m/z 346.2129, calc. 346.2144). The 300-MHz ($CDCl_3$) [^{1}H]NMR spectrum of A suggested a steroidal structure, with characteristic singlet angular methyl group resonances at δ 1.17 and 0.94 (Jackman and Sternhell, 1969). A vinylic proton at δ 5.53 and two protons geminal to hydroxyl groups, at δ 4.41 and 4.04, were also apparent. In addition, the infrared spectrum of A showed two carbonyl absorption bands at 1733 and 1704 cm^{-1}. From these data, A can be recognized as a C_{21} steroid with one trisubstituted double bond, two secondary hydroxyl groups, and two carbonyl groups.

The [^{1}H]NMR spectrum of A showed an additional methyl singlet at δ 2.27, characteristic of a C-21 methyl group in a C-20 keto-steroid. The 1704 cm^{-1} carbonyl absorption supports this assignment, and the remaining carbonyl group (1733 cm^{-1}), clearly in a five-membered ring, must be at C-15 or C-16.

An extensive series of proton decoupling experiments (the results of which are summarized in Table 1) allowed the measurement of chemical shifts and coupling constants such that the substitution patterns and stereochemistry at C-2 to C-9 and C-14 to C-17 could be deduced. These data led to the characterization of A as 3α,7β-dihydroxy-14β-pregn-4-en-15,20-dione (I).

Chemical support for this assignment was obtained by sequential conversion of I to II and III as described below. Oxidation of a 20-μg sample of A with manganese dioxide produced a single product with characteristic conjugated enone absorption in the ultraviolet (λ_{max} 240 nm), and a mass spectrum showing an intense fragment at m/z 124 diagnostic of steroidal 3-ene-4-ones (Zaretskii, 1976). These data, along with characteristic [^{1}H]NMR chemical shifts of the angular methyl groups (δ 1.21 and 1.16), provide excellent support for structure II. Treatment of II with methanesulfonyl chloride in triethylamine

TABLE 1. [^{1}H]NMR SPECTRAL DATA FOR I

Proton	$\delta CDCl_3$	δC_6D_6	J (Hz, C_6D_6)			
3β	4.04	3.98	$J_{3\beta,4} = 4.8$	$J_{3\beta,6\beta} = 1.8$		
4	5.53	5.45	$J_{4,6\beta} = 1.8$	$J_{4,3\beta} = 4.8$		
6α		2.20	$J_{6\alpha,7\alpha} = 5.2$	$J_{6\alpha,6\beta} = 12.1$		
6β		2.18	$J_{6\beta,4} = 1.8$	$J_{6\beta,7\alpha} = 11.0$	$J_{6\beta,6\alpha} = 12.1$	$J_{6\beta,3\beta} = 1.8$
7α	4.41	4.68	$J_{7\alpha,6\alpha} = 5.2$	$J_{7\alpha,6\beta} = 11.0$	$J_{7\alpha,8\beta} = 11.0$	
8β		1.60	$J_{8\beta,7\alpha} = 11.0$	$J_{8\beta,14\beta} = 4.8$	$J_{8\beta,9\alpha} = 11.0$	
14β	2.82	3.10	$J_{14\beta,16\beta} = 1.8$	$J_{14\beta,8\beta} = 4.8$		
16α		2.03	$J_{16\alpha,17\alpha} = 8.5$	$J_{16\alpha,16\beta} = 18.8$		
16β		2.35	$J_{16\beta,17\alpha} = 1.4$	$J_{16\beta,14\beta} = 1.8$	$J_{16\beta,16\alpha} = 18.8$	
17α	2.95	2.27	$J_{17\alpha,16\alpha} = 8.5$	$J_{17\alpha,16\beta} = 1.4$		
18	1.17	0.87				
19	0.94	0.77				
21	2.27	1.75				

FIG. 1

gave a product with intense dienone ultraviolet absorption at 280 nm, as expected for loss of the esterified 7-hydroxyl substituent of II to give 14β-pregna-4,6-diene-3,15,20-trione (III).

Spectral data led us to characterize component B (ca. 10% of A) as 3α,7β,20ξ-trihydroxy-14β-pregn-4-en-15-one (IV). The electron impact mass spectrum (EI-MS) of B shows a parent ion at *m/z* 348, corresponding to the molecular formula $C_{21}H_{32}O_4$, a dihydro derivative of A. The EI-MS also exhibits fragments corresponding to the loss of three molecules of water, suggesting the presence of three hydroxyl groups. The peak at *m/z* 141 (33%), attributed to the D-ring fragment formed by scission of the C-8,C-14, and C-12,C-13 bonds, suggested that B is a 15-keto-20-hydroxy steroid (a corresponding fragment at *m/z* 139 is observed in the mass spectra of I and II). The $[^1H]$NMR spectrum of B (in C_6D_6) is similar to that of I, with a few critical differences. Chief among these is the replacement of the C-21 methyl singlet by a methyl doublet (δ 0.64; note large upfield shift attributable to aromatic solvent), confirming the change from a C-20 ketone to a C-20 alcohol.

In summary, the major defensive steroid from *S. novaboracensis* is characterized as I; the minor constituent is the closely related pregnane IV, whose configuration at C-20 remains unassigned.

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GROWTH OF *Leptinotarsa decemlineata* LARVAE IN RESPONSE TO SIMULTANEOUS VARIATION IN PROTEIN AND GLYCOALKALOID CONCENTRATION

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Abstract—Growth of larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), as measured by weight gain, was determined when larvae were reared on synthetic diets containing measured quantities of protein and one of five *Solanum* glycoalkaloids. Data were analyzed to determine if growth was affected by these two aspects of phytochemical variation independently, or if there was a significant interaction component in larval weight gain to simultaneous variation in protein and glycoalkaloid concentration. For four of the five glycoalkaloids examined, a bivariate regression model without interaction accurately accounted for growth of *L. decemlineata* larvae. For alpha-tomatine, however, a regression model with the addition of a (protein × glycoalkaloid) interaction term more accurately accounted for larval growth. The influence of this interaction was less than either protein or glycoalkaloid concentration alone. The variation in protein concentration accounted for most of the variation in larval growth, and high protein concentrations could more than offset the effect of high concentration of any of the five glycoalkaloids.

Key Words—*Solanum*, glycoalkaloid, *Leptinotarsa decemlineata*, protein, growth, Coleoptera, Chrysomelidae, α-tomatine, α-solamarine, β-solamarine, α-solamine, α-chacomine.

INTRODUCTION

Feeding activity in phytophagous insects is mediated in part by the presence and quantity of particular phytochemicals eliciting or suppressing feeding be-

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havior (Schoonhoven, 1982). Insect feeding and growth rates are also affected by the nutritional quality of the host, and insects may adjust their feeding rates in order to compensate for variation in nutrient (e.g., nitrogen) concentration in ingested food (Slansky and Feeny, 1977; Scriber and Slansky, 1981). Seasonal variation in the concentration of most phytochemicals occurs widely, and the acceptability as well as the nutritional quality of host plants often varies simultaneously over time (Denno and McClure, 1983).

In order to predict how phytophagous insects utilize these varying resources, the following questions must be answered: (1) How do insects respond to quantitative variation in the concentrations of phytochemicals affecting feeding behavior? (2) How do insects respond to variation in the concentration of primary nutrients? (3) Is there any synergism or interference in the insects' responses to these two aspects of phytochemical variation?

Feeding behavior in the Colorado potato beetle, *Leptinotarsa decemlineata* is influenced by a number of phytochemicals. Several sugars and amino acids stimulate feeding (Hsiao and Fraenkel, 1968), and the insect has specialized chemosensory receptors to detect these compounds (Mitchell and Schoonhoven, 1974). Other plant compounds, notably some of the steroidal glycoalkaloids, deter feeding (Hsiao, 1974), although the insect apparently has no chemoreceptors specific for these phytochemicals (Mitchell and Harrison, 1985). Therefore, feeding and growth in *L. decemlineata* is thought to be mediated by the insects's joint responses to a number of plant compounds, only some of which are required for normal growth and development (Hsiao, 1974).

In a previous investigation on the responses of *L. decemlineata* to seasonal phytochemical variation in *Solanum dulcamara* L., Hare (1983) showed that larval survival increased with increasing concentration of extractable foliar protein and declined with increasing concentration of two glycoalkaloids, α- and β-solamarine. In a preliminary study, these two glycoalkaloids also reduced feeding and growth of *L. decemlineata* larvae when incorporated into a synthetic diet. The present study was undertaken to examine in more detail how simultaneous variation in the quantities of protein and glycoalkaloids affected the growth of *L. decemlineata* larvae in the laboratory. The primary objective was to determine if variation in growth of *L. decemlineata* larvae could be modeled as a simple additive function of protein and glycoalkaloid concentration or if it were necessary to include a protein $\times$ glycoalkaloid interaction term into the regression equation in order to accurately model larval growth.

Five glycoalkaloids were examined. Two, α- and β-solamarine, were extracted from *S. dulcamara*, the most common wild host of *L. decemlineata* in Connecticut (Hare, 1983). The responses of *L. decemlineata* larvae to these glycoalkaloids were compared to α-solanine and α-chaconine, the two glycoalkaloids characteristic of the cultivated potato, *Solanum tuberosum* L., *L. decemlineata's* most suitable host, and to α-tomatine, the characteristic glycoal-

kaloid of a less-suitable host plant, the cultivated tomato, *Lycopersicon esculentum* Mill. α-Tomatine has been reported previously to be a strong feeding deterrent to *L. decemlineata* (Hsiao and Fraenkel, 1968) and therefore was included as a reference point to evaluate the activities of the other glycoalkaloids.

METHODS AND MATERIALS

Each of five glycoalkaloids was incorporated at four concentrations in synthetic diets containing one of three concentrations of protein, and the growth of *L. decemlineata* larvae, as defined as the gain in weight after feeding on these diets for 24 hr, was determined. The range of glycoalkaloid concentrations (2, 4, 8, and 16 mg/ml plus a 0 mg/ml control) was chosen to encompass the reported range of concentration in intact plants. For example, Hare (1983) reported the concentration of α- and β-solamarine to vary between 5.9 and 41.6 mg/g (dry wt). This is equivalent to 1.5–10.5 mg/g fresh weight, based upon the observed water content of ca. 75% (Hare, 1983). Similar ranges of 4.6–51.0 mg/g (dry wt) have been reported for tomatine in tomato (Sinden et al., 1978). Glycoalkaloid concentration in commercial and hybrid potato varieties is somewhat lower, ranging from ca. 0.4 to 5.5 mg/g (fresh wt) (Sinden et al., 1980).

Chemical Procedures. α-Tomatine was obtained commercially (Sigma Chemical Co., St. Louis, MO., lot 24F-0090). The other glycoalkaloids were extracted from plant tissue and separated by preparative TLC using methods described previously (Hare, 1983). Identities of the glycoalkaloids from *S. tuberosum* and *S. dulcamara* were determined by hydrolyzing the glycoalkaloid and identifying and quantifying the resulting sugars and aglycones using methods modified from Sinden et al. (1980). Samples (5 g) of each were hydrolyzed under reflux in 10 ml HCl–methanol (1 : 8) for 4 hr. The sample was then evaporated to dryness under vacuum at 18°C. Aglycones were dissolved in chloroform, and the sugars were dissolved in distilled water. The aglycones were identified by their TLC mobility relative to tomatidine (ICN Pharmaceuticals, Cleveland, OH.) and solanidine [prepared by hydrolyzing commercially obtained α-chaconine (Sigma Chemical Co., lot 48C-7520)]. The TLC solvent systems used were hexane–ethyl acetate (1 : 1), CH_2Cl_2–methanol (9 : 1), and CH_2Cl_2–acetone (4 : 1). Identities of the aglycones were confirmed by spraying the plates with 50% aqueous sulfuric acid and noting their characteristic colors before and after heating the plates to 80°C (Hunter et al., 1976).

Identities of the sugars from the hydrolysis (glucose, galactose, and rhamnose from α-solanine and α-solamarine, and glucose and rhamnose from α-chaconine and β-solamarine) were determined by TLC on microcrystalline cellulose plates in *n*-butanol–benzene–pyridine–distilled water (5 : 1 : 3 : 3) (Har-

TABLE 1. COMPOSITION OF EXPERIMENTAL DIET

Constituent	Grams/100 ml of Finished Diet
Powdered cellulose[a]	4.00
Sucrose	2.50
Fructose	1.60
Wesson's salts[a]	0.80
Casein hydrolysate	0.80
Cholesterol	0.05
Vanderzant's vitamin mix[a]	1.00
Corn oil	0.5 ml
Casein Solution	50.0ml
Agar solution (2.5 % w/v)	To 100 ml

[a] ICN Nutritional Biochemicals.
[b] Casein solutions were made up at 50, 100, or 200 mg/ml in 3% KOH (w/v).

borne, 1973). Plates were sprayed with aniline hydrogen phthalate and heated at 105°C for 5 min. The mobilities and colors of the sugars from hydrolysis were compared to those of reference standards. The separation of glucose and galactose was achieved by running the plate twice in solvent after allowing the plate to dry between runs.

Glycoalkaloids were incorporated into a completely defined agar-based diet modified from Hsiao and Fraenkel (1968) (Table 1). Protein concentrations used in the present experiment (25, 50, and 100 mg/ml) were $\frac{1}{2}\times$, $1\times$, and $2\times$ the concentration used in the initial study. Because these glycoalkaloids are only soluble in water at acidic pH (e.g., Mitchell and Harrison, 1985), the glycoalkaloids were ground to a fine powder, mixed with the powdered cellulose, and the dry ingredients were thoroughly mixed into the diet. Diets were poured into sterile Petri dishes set and rapidly cooled at 5°C in a refrigerator so that the dry ingredients would not settle out before the diet solidified.

Fourth-instar *L. decemlineata* larvae weighing between 60 and 80 mg were collected from a colony maintained on potted potatoes (cv. 'Katahdin') in the greenhouse. Larvae were starved for 6 hr, weighed, placed in labeled Petri dishes, and given ~0.3 ml of one of the diets. Petri dishes containing larvae and diets were then placed on enamel trays and enclosed in a clear polyethylene bag containing moist paper towels to maintain ~100% humidity. Trays with larvae were placed in a constant-temperature room (25°C, 16-hr photoperiod). Petri dishes were cleaned, diets were replaced after 12 hr, and final weights of larvae were obtained after 24 hr. Relative weight change was computed for each larva as the difference between the final and initial weights divided by the initial weight. Two groups of 15 larvae were used in each of the 15 combinations of protein and glycoalkaloid treatments.

Multiple regression was used to analyze larval growth as a function of protein concentration, glycoalkaloid concentration, and the protein × glycoalkaloid interaction. Regressions were calculated without transformation and with the (log + 1) transformation applied to protein and glycoalkaloid concentrations. Regression analyses were performed first using only protein and glycoalkaloid concentrations as "main" effects, then regression analyses were repeated after incorporating a protein × glycoalkaloid interaction term into the model. Regression analyses yielded similar results for both the untransformed and transformed independent variables. Because the concentrations of protein and glycoalkaloids were equally spaced after transformation, regression analyses on transformed data are probably more valid.

RESULTS AND DISCUSSION

In general, larval growth increased with increasing protein concentration and decreased with increasing concentration of all glycoalkaloids (Table 2). The regression coefficients between larval growth and protein concentration were statistically significant at $P \leq 0.001$ in all cases (Table 3). Regression coefficients between larval growth and glycoalkaloid concentration were statistically significant ($P \leq 0.05$) for all glycoalkaloids except alpha α-solamarine. These two "main" effects accounted for 67–77% of the variation in the means of larval growth in all experiments. Protein concentration was always the more important factor, accounting for 55–70% of the variation in larval growth alone.

The protein × glycoalkaloid interaction term did not significantly improve the fit of any regression model except for α-tomatine (Table 3). In this one case, the protein × glycoalkaloid interaction accounted for 5.3% of the total variation and raised the total percent of variation explained to 76.4%.

The significant inhibition of larval growth by all five glycoalkaloids, including those from potato, is not entirely unexpected. Although Hsiao and Fraenkel (1968) concluded that solanine would have little, if any effect on *L. decemlineata* at concentrations occurring in potato foliage, *L. decemlineata* growth was reduced in their experiments more than 20% by solanine at concentration of 0.9%. Absolute larval growth declined significantly with the logarithm of solanine concentration ($b = -14.64$, $P \leq 0.05$, calculated from published data); the effect of chaconine was not examined.

Although tomatine was a stronger inhibitor of larval growth than the potato alkaloids in the present study, it was not the "order of magnitude" more effective as was found in previous studies (Hsiao and Fraenkel, 1968; Hsiao, 1974). It is not clear whether these differences might have been due to differences in the diets used or variation among *L. decemlineata* populations in their susceptibility to tomatine.

Finally, the variation in casein content was clearly the more important.

TABLE 2. MEAN (AND STANDARD ERROR)[a] PERCENT WEIGHT CHANGE AFTER 24 HR OF LARVAE FED SYNTHETIC DIETS CONTAINING SPECIFIED CONCENTRATIONS OF GLYCOALKALOIDS AND CASEIN

Protein conc. (mg/ml)	Glycoalkaloid conc. (mg/ml)				
	0	2	4	8	16
α-Tomatine					
25	11.5 (6.4)	2.9 (1.6)	7.2 (4.8)	6.6 (0.1)	4.1 (2.5)
50	18.9 (3.4)	20.9 (5.9)	17.0 (6.3)	19.7 (5.2)	5.4 (1.9)
100	39.9 (3.3)	37.7 (4.5)	24.3 (7.1)	20.1 (4.1)	17.8 (5.4)
α-Solamarine					
25	2.6 (4.6)	4.2 (4.6)	6.7 (2.1)	3.8 (0.7)	2.2 (5.3)
50	25.8 (10.0)	20.7 (7.5)	21.4 (7.2)	16.2 (6.3)	15.4 (9.0)
100	36.3 (14.9)	34.1 (7.3)	38.0 (2.8)	35.0 (13.0)	21.2 (6.2)
β-Solamarine					
25	4.5 (6.4)	8.2 (0.6)	6.4 (10.1)	2.6 (3.8)	1.6 (0.0)
50	34.1 (1.6)	22.2 (1.0)	21.0 (3.3)	10.7 (1.0)	9.3 (2.3)
100	41.0 (10.3)	52.8 (5.3)	38.7 (7.7)	44.7 (10.9)	23.8 (6.4)
α-Solanine					
25	3.7 (3.8)	−4.7 (6.2)	0.4 (0.7)	−4.3 (2.7)	−2.5 (3.0)
50	11.9 (6.8)	10.4 (1.8)	7.2 (1.6)	7.0 (0.1)	6.4 (2.1)
100	29.3 (10.1)	19.2 (6.3)	20.0 (2.6)	23.8 (4.3)	12.7 (4.4)
α-Chaconine					
25	3.7 (3.8)	6.1 (5.1)	1.6 (3.3)	1.8 (2.2)	−1.7 (1.0)
50	11.9 (6.8)	4.5 (1.3)	8.8 (5.6)	5.1 (3.7)	−0.4 (1.0)
100	29.3 (10.1)	28.7 (0.8)	26.8 (4.2)	30.7 (8.0)	18.3 (5.6)

[a] Values are the mean and standard error of means of two groups of 15 larvae per treatment.

TABLE 3. REGRESSION COEFFICIENTS BETWEEN LARVAL GROWTH (PERCENT WEIGHT CHANGE OVER 24 HR) AND PROTEIN OR GLYCOALKALOID CONCENTRATION (mg/ml OF DIET) WITH AND WITHOUT A PROTEIN × GLYCOALKALOID INTERACTION TERM.

Variable	Without Interaction[a]	With Interaction[a]	R^{2b}
α-Tomatine			
Protein	15.8 ***	23.6 ***	0.552
Glycoalkaloid	−4.9 ***	15.0 NS	0.159
Interaction		−5.1 *	0.053
α-Solamarine			
Protein	21.4 ***	25.9 ***	0.643
Glycoalkaloid	−2.6 NS	9.0 NS	0.030
Interaction		−3.0 NS	0.012
β-Solamarine			
Protein	26.2 ***	30.7 ***	0.687
Glycoalkaloid	−5.3 **	6.2 NS	0.086
Interaction		−2.9 NS	0.008
α-Chaconine			
Protein	18.1 ***	18.9 ***	0.645
Glycoalkaloid	−2.8 *	−0.6 NS	0.048
Interaction		−0.6 NS	<0.001
α-Solanine			
Protein	16.6 ***	19.4 ***	0.701
Glycoalkaloid	−2.9 *	4.2 NS	0.064
Interaction		−1.8 NS	0.008

[a] * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; NS = not significant.
[b] R^2 is the proportion of the total variance "explained" by each factor.

Although the nutritional suitability of casein may differ from that of *Solanum* leaf protein, results presented here suggest that the absolute level of growth inhibition by any glycoalkaloid may depend upon the "background" nutritional quality of the substrate used. Because of the great likelihood of inter- and intraspecific variation in protein content of different host species (e.g., Hare, 1983), attempts to relate differences in susceptibility of *Solanum* species or cultivars only to variation in glycoalkaloid concentration without considering the possibility of simultaneous variation in host nutritional quality may lead to erroneous conclusions. With one exception, these results indicate that the use of simple bivariate regression models without interaction (e.g., stepwise multiple regression) are appropriate for the analysis of growth responses of *L. de-*

cemlineata to simultanenous variation in quantities of protein and glycoalkaloids.

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CARDENOLIDE CONTENT AND THIN-LAYER CHROMATOGRAPHY PROFILES OF MONARCH BUTTERFLIES, *Danaus plexippus* L.,[1] AND THEIR LARVAL HOST-PLANT MILKWEED, *Asclepias viridis* WALT.,[2] IN NORTHWESTERN LOUISIANA

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Abstract—This paper is the first in a series on cardenolide fingerprinting of monarch butterflies and their host-plant milkweeds in the eastern United States. Spectrophotometric determinations of the gross cardenolide content of 60 *Asclepias viridis* plants in northwestern Louisiana indicate a positively skewed variation ranging from 95 to 432 μg/0.1 g dry weight with a mean of 245 μg/0.1 g. Butterflies reared individually on these plants contained a normal cardenolide distribution ranging from 73 to 591 μg/0.1 g dry weight with a mean of 337 μg/0.1 g. The uptake of cardenolide by the butterflies best fit a logarithmic function of the plant concentration. Female monarchs (385 μg/0.1 g) contained significantly greater mean cardenolide concentrations than did males (287 μg/0.1 g). No indications of a metabolic cost for either cardenolide ingestion or storage were adduced from size or dry weight data. Thin-layer chromatograms of 24 individual plant–butterfly pairs developed in two solvent systems resolved 21 individual spots in the plants and 15 in the butterflies. *A. viridis* plants appear to contain several relatively nonpolar cardenolides of the calotropagenin series which are metabolized to the more polar 3′-hydroxy derivatives calactin and calotropin as well as to calotropagenin in the butterflies. The epoxy cardenolides labriformin and labriformidin were absent, although desglucosyrioside (a 3′-hydroxy derivative) appeared present in both plants and butterflies. Quantitative evaluation of the R_f values, spot intensities, and probabilities of occurrence in the chloroform–methanol—formamide TLC system produced a cardenolide finger-

[1]Lepidoptera: Danaidae.
[2]Apocynales: Asclepiadaceae.

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print clearly distinct from those previously established for monarchs reared on other *Asclepias* species, supporting the use of fingerprints to make ecological predictions concerning larval host-plant utilization. *A. viridis* is the predominant early spring milkweed throughout most of the south central United States and may be important in providing chemical protection to spring and early summer generation monarchs in the eastern United States.

Key Words—*Asclepias viridis*, milkweed, Asclepiadaceae, *Danaus plexippus*, Lepidoptera, Danaidae, monarch butterfly, cardenolide, cardiac glycoside, digitoxin, chemical ecology, chemical defense, thin-layer chromatography, plant–insect interactions, coevolution.

INTRODUCTION

While other danaid butterflies are restricted to subtropical and tropical climates and food resources, the monarch butterfly, *Danaus plexippus* L., has developed a migratory strategy which enables it to avoid competition for larval host-plants by utilizing the abundant temperate milkweeds in the genus *Asclepias* L. (Asclepiadaceae). Unable to overwinter in freezing temperate climates, each fall millions of monarchs in the eastern United States leave their North American breeding grounds and fly southward to the Gulf Coast and to the volcanic plain of eastern Michoacan, Mexico (Urquhart and Urquhart, 1978; Brower, 1977). Nesting in the trees at elevations of 11,000–12,000 ft, as many as 50 million monarchs hibernate in a few dense colonies in a forested region only a few square miles in area (Brower et al., 1977). Monarchs which breed in the western United States comprise an entirely separate population long known to overwinter along the California coast.

Since the discovery of the Mexican overwintering site in 1976 (Urquhart and Urquhart, 1976), much effort has been made to document the arrival, overwintering behavior, and spring breakup of these spectacular colonies (Brower et al., 1977; Calvert and Brower, 1982; Calvert et al., 1979, 1982; Fink and Brower, 1981). Little is known, however, about the ecology of these monarchs as they recolonize their temperate breeding grounds in eastern North America. For those monarchs remigrating through Texas and Louisiana, *Asclepias viridis* and *A. asperula* probably represent the first common and widespread milkweeds they encounter. From western Florida eastward and northward along the Atlantic coastal plain *A. viridis* is more localized and *A. humistrata* is more heavily utilized for oviposition by monarchs (Brower, 1961, 1962; Cohen and Brower, 1982). Clearly, these three species are the principal milkweeds which migrant monarchs encounter as they reach the temperate United States and serve as larval host-plants for many, if not most, of the first spring generation monarch larvae.

The breeding cycle in the southern states is short lived, however, since the first monarchs arrive in late March and by early May monarchs in all life stages

are essentially absent. The fates of both the original remigrants and the first spring generation are unclear. By mid-summer monarchs are widely dispersed throughout much of the northeastern U.S. and southeastern Canada. *A. syriaca* and *A. speciosa* are probably the major food source for monarch larvae during the summer breeding season (Urquhart, 1960; Fink and Brower, 1981), although several other milkweed species are abundant and serve as monarch host-plants, particularly along the western margins of their distribution in the eastern U.S.

Most milkweed species contain a variety of highly emetic and toxic cardenolides which have an effect similar to digitalis on the vertebrate heart. In contrast to most other milkweed herbivores examined, the monarch has been found to effectively sequester these compounds in their tissues. Only the lygaeid bugs, *Oncopeltus* and *Lygaeus*, appear more efficient on an insect weight basis (Duffey, 1977; Isman et al., 1977; Blum, 1981; Cohen, 1985). Most research has also shown that monarch larvae are able to avoid the toxic effects of cardenolides (Erickson, 1973; Dixon et al., 1978) although Cohen (1985) has recently found a significant negative correlation between wing length and cardenolide concentration in wild Florida adult males and females. Brower et al. (1982, 1984a,b) have analyzed the cardenolide contents of monarchs reared on three specific host-plants in California, *A. eriocarpa*, *A. speciosa*, and *A. californica*. They found that larvae selectively store the more polar cardenolides found in their host-plants and apparently convert some of the less polar forms to more polar forms. In addition, they have successfully established that monarchs reared on these three species each possess a distinctive thin-layer chromatography (TLC) pattern of cardenolides that is reflective of the pattern found in its host-plant species, supporting their hypothesis that TLC "fingerprints" could be developed to identify the host-plant origins of wild-caught monarchs.

Adult monarchs rich in cardenolides have been shown to be both emetic and distasteful to blue jays and other bird species, suggesting that they are defended chemically against many potential vertebrate predators (Brower et al., 1975, 1982). However, many adult monarchs contain little or no cardenolide in their tissues. Brower et al. (1972) and Brower and Moffitt (1974) found a broad spectrum of gross cardenolide content in populations of eastern U.S. monarchs, indicating some butterflies were palatable, some subpalatable, and some highly toxic. Fink and Brower (1981) found that only 10% of the butterflies they examined at the Mexican overwintering site contained more than one emetic dose of cardenolide and that a high percentage contained little or no cardenolide. Although Fink and Brower (1981) suggest that a large proportion of these butterflies fed on *A. syriaca*, *A. speciosa*, or other low-potency cardenolide-poor milkweeds, no cardenolide fingerprint profiles or pairwise concentration analyses have yet been published for monarchs feeding on milkweeds in eastern North America.

In this paper we present the first analysis in a series of investigations of

monarch remigration and recolonization along the western margins of their distribution in the eastern United States. Our purposes are to establish the timing and extent of host-plant utilization by monarchs as they remigrate into temperate North America, establish the quantitative variation in cardenolide contents of butterflies as they relate to their host-plants and to develop thin-layer chromatography cardenolide fingerprints of adult monarchs for milkweed species commonly utilized during recolonization. We now report on the cardenolide characteristics of monarch butterflies reared as larvae on *Asclepias viridis* Walt. in northwestern Louisiana.

METHODS AND MATERIALS

Geographic and Ecological Distribution of Asclepias viridis. *Asclepias viridis* Walt., a relatively common milkweed of the subgenus Asclepiodora, has a widespread distribution in the southeastern United States. It naturally occurs in glades, prairies, dry hillsides, and pine barrens. The species may be found as far south and east as the Florida Keys, with populations extending northward along the Appalachians to Ohio and westward across the southern United States to central Texas, Oklahoma, Kansas, and southern Nebraska. Figure 1 illustrates the known distribution of *A. viridis*.

Along the western boundary of its distribution *A. viridis* is often abundant in open fields and pastures, and often occurs in close sympatry with *Asclepias asperula* subsp. *capricornu* (Woods.) Woods., a morphologically similar yet distinct milkweed of the same subgenus. Although they largely remain distinct, individuals with intermediate vegetative and floral morphology may occur, suggesting occasional hybridization between the two species (Lynch, personal observation). In northwestern Louisiana *A. asperula* is absent but *A. viridis* is often abundant in pastures and openings and margins of piney woods. Other milkweed species do occur in this region but are only occasional and produce less biomass. East of the Mississippi River, *A. viridis* becomes increasingly less abundant and *A. humistrata* Walt., *A. tuberosa* L., and to a lesser extent *A. tomentosa* Ell. produce the principal milkweed biomass in the southeastern United States.

Location, Methods, and Dates of Sample Collections. During the spring of 1981, immature monarchs (*Danaus plexippus* L.) were collected from milkweeds in Caddo Parish, Louisiana, at a site south of Shreveport, 0.5 miles north of Wallace Lake on Wallace Lake Road. The site consists of a series of open, lightly grazed fields covered with both native and introduced grasses and forbs. The fields are surrounded by piney woods dominated by shortleaf and loblolly pines (*Pinus echinata* Mill. and *P. taeda* L.), sweet gum (*Liquidambar styraciflua* L.), sugarberry (*Celtis laevigata* Willd.), honeylocust (*Gleditsia triacanthos* L.), and several species of oak (*Quercus* spp.)

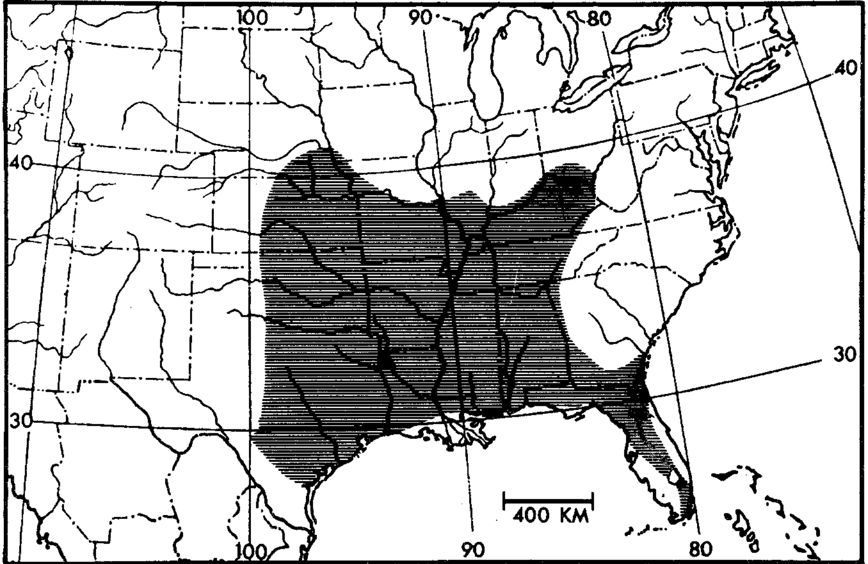

FIG. 1. The known geographic distribution of the milkweed *Asclepias viridis* Walt. in the United States (after Woodson, 1954). The triangle locates the northwest Louisiana population analyzed in this study. See text for discussion of relative abundance.

Collections of individually matched plant–butterfly pairs generally followed the procedures established in Brower et al. (1982, 1984a,b) with some minor revisions. Fifth-instar larvae were collected along with a sample of leaves from their individual host-plant. A portion of this material was placed with the larva into an 8-oz clear plastic food container (Sweetheart Plastics No. 40, Wilmington, Maryland), and the remaining leaves were placed in one-pint freezer bags and frozen for later chemical analysis.

Within three days each fifth-instar larva had spun a silken pad on the lid of the container, attached itself to the pad by its cremaster, and hung upside down to shed its last larval skin. Failure to hang free invariably resulted in a distorted chrysalid and either terminated further development or resulted in a badly disfigured adult. After pupation, the remaining leaves in the container were discarded, and a Whatman No. 1 filter paper was placed on the bottom to reduce humidity and to soak up fluids evacuated during emergence. The containers were stored at room temperature until emergence. Deposition of pigments into the adult monarch exoskeleton generally occurred the evening and night prior to emergence. Upon darkening of the chrysalids, small cylinders of metal window screen were sandwiched between the lid and bottom of the container, creating an open air cage in which the emerging adults could hang to expand and dry their wings.

Approximately 12 h after emergence, each butterfly was placed in a glassine envelope and frozen for later measurement and chemical analysis. Each plant–butterfly pair was provided with a collection number (S.P. Lynch, unpublished field catalog). A single digit alphabetical suffix was used to identify more than one butterfly collected from the same host-plant.

In addition to searching for and collecting wild fifth-instar larvae, several small larvae were bagged with nylon net bags and later collected as chrysalids or as late fifth-instar larvae. The rearing of the butterflies and the treatment of plant material following collection was identical for both bagged and unbagged material. Any chrysalids collected from bags were removed along with their silken pads which were then taped to the lid of the container. In all, 60 pairwise butterflies, 31 females and 29 males, were analyzed from 48 individual plants.

Determination of Total Cardenolide Content. The individual plant and butterfly samples were prepared and extracted with 95% ethanol as described by Brower et al. (1972). Spectrophotometric determinations were performed at 626 nm with a Gilford 280 single-beam UV–visible spectrophotometer at 20.0°C or ambient temperature (22°C), following the 2,2′,4,4′-tetranitrodiphenyl (TNDP) method of Brower et al. (1972, 1975) and Brower and Moffitt (1974). Those absorbances obtained at ambient temperature were determined in stoppered 1-cm polystyrene cuvettes (Kartell, matched within 0.005 A) whereas absorbances at 20.0°C were determined in matched glass cuvettes in a temperature-controlled cuvette holder. A minor change in dilution of the cuvette contents was necessary to minimize precipitation. The sample cell contained 0.5 ml of plant or butterfly extract, 0.8 ml of 95% ethanol, 1.0 ml of 0.15% TNDP in 95% ethanol, and 0.2 ml of 0.1 N sodium hydroxide in 50:50 water–95% ethanol. The reference cell contained the same except for the replacement of the 1.0 ml TNDP solution with 1.0 ml 95% ethanol. Freshly prepared TNDP solutions were used and stored in a refrigerator for no longer than 10 days. In the absence of the molar absorptivities of the TNDP complex of the *A. viridis* cardenolides, estimates were obtained using a digitoxin standard and reported in μg (equivalent to digitoxin) per 0.1 g dry weight (Roeske et al., 1976; Brower et al., 1982).

Thin-layer Chromatography (TLC). Pigments and other interfering substances were removed in a lead acetate precipitation procedure (Nelson et al., 1981; Brower et al., 1982) which utilized 6 ml of the *A. viridis* extract or 3 ml of the butterfly extract. After cleanup, the evaporated residue was dissolved in a calculated amount of chloroform in order to deliver 50, 75, 100, 125, or 150 μg/20 μl of solution.

Twenty microliters of the plant and butterfly samples were spotted with Drummond Wiretrol 5-μl pipets onto a heat-activated 20 × 20-cm TLC plate (0.25 mm, EM Science 5765 Silica Gel 60 F-254), providing six plant–butterfly pairs and five digitoxin and digitoxigenin standards (Sigma Chemical Co.) per plate. Development was in preequilibrated filter paper-lined glass chambers in

chloroform–methanol–formamide (CMF system, 90:6:1 by volume) four times or in ethyl acetate–methanol (EM system, 97:3 by volume) two times (Brower et al., 1982). The plates were visualized by spraying with 20 ml of 0.4% TNDP in toluene followed by 20 ml of 10% potassium hydroxide in 50% aqueous methanol. The plates were photographed within 1 min using Kodachrome 25 film and a Pentax Spotmatic 35-mm camera equipped with a Pentax ringstrobe. Pooled samples of both plants and butterflies were also run spotting approximately 100 μg of cardenolide in each position.

Four plates were run in the CMF solvent system. Plates 2 and 4 each contained six female plant–butterfly pairs, two pairs each selected from the low, middle, and high ranges of plant cardenolide concentration based on spectroassay results. Similarly, plates 1 and 3 were spotted with male plant–butterfly pairs except for one middle range female pair which was erroneously included on plate 1. This resulted in a total of 13 female and 11 male plant–butterfly pairs analyzed by the CMF system. Concentration ranges were intentionally placed in different regions on each plate to reduce or eliminate error due to plate position. The two standards, digitoxin and digitoxigenin, were run as a mixture in the outermost positions and in the central position of each plate, and each standard was run independently, immediately flanking the central channel. In a similar manner, two plates of pooled plant and butterfly extracts were chromatographed in adjacent positions, one plate in CMF and the other in EM. Because of poorer resolution of individual spots, no individual pairwise plates were run using the EM system (see Results section).

The digitoxin migration values from each origin were calculated by drawing a line from the outer digitoxin spots to the central spot and measuring from the origin to the line intersect. The distance of the concentrated centers of each spot from the origin was measured to the nearest 0.5 mm and later converted to an $R_{\text{digitoxin}}$ (R_d) value by dividing the spot migration distance by the calculated digitoxin migration distance. In addition to quantifying the relative positions of the various spots, comparisons of TLC patterns were also based on the mean and standard deviations of each spot's intensity value (SI) as well as each spot's probability of occurrence PO. Spot intensity was determined using a subjective but consistent visual 1–5 scale with 1 being the lightest and 5 the darkest.

Statistical Analyses. Statistical analyses were made on an IBM 3330 computer housed at Louisiana State University in Baton Rouge utilizing SAS statistical software, release 82.2 (SAS, 1982). We analyzed dry weights, wing and body measurements, cardenolide concentrations, and total cardenolide content of the butterflies, as well as the cardenolide concentrations of the plants. Using the Kolmogorov D statistic, only the cardenolide concentration data for plants were not normal ($P = 0.03$, HO $= P < 0.05$). A $\log_{10}$ transformation produced a normalized distribution for the plant concentrations ($P = 0.10$), and the fit to the model was improved in each case.

Two-step regressions were used to fit least-squares estimates to models

comparing cardenolide concentrations and total cardenolide contents of individual butterflies (Y, the dependent variable) to the cardenolide concentrations of the individual plants which the larvae had eaten (X, the independent variable). The first step considered the overall relation of Y to X, the calculated r^2, the slope, and the intercept; and the second step analyzed the dependence of the butterfly cardenolide concentration, butterfly dry weight, and the total cardenolide per butterfly on the cardenolide concentration of the plants (total and by sex).

Two way analyses of variance (ANOVA) on the TLC data were used to determine the influence of sex and plate number and their interaction on both R_d values and on SI values. Brower et al. (1982, 1984a,b) have pointed out that the reliability of cardenolide fingerprinting depends upon a high degree of correlations between the R_d values of the respective cardenolides in the plants and butterflies. Although we realize that our TLC spot comparison methodology largely predetermines the correspondence of the respective plant and butterfly spots for each plant–butterfly pair, the SAS linear regression program allows for the simultaneous testing of the dependence of the R_d values or the SI values for all butterfly spots on the corresponding values of their respective plant spots as affected by plate variation or sex in the entire sample.

RESULTS

Measurements of Gross Cardenolide Content. We found *A. viridis* host-plants to be rich in cardenolides. Each of the 60 plants used in this study contained measurable amounts of cardenolide, with concentrations ranging from 95 to 432 μg/0.1 g dry weight. The mean concentration for all plants measured was 245 μg/0.1 g dry weight with a standard deviation of 69.8 μg (Table 1). The pattern of variation is shown in Figure 2. The curves over the histograms for both plants and butterflies display the limits of the expected normal distributions calculated by the z statistic (Steel and Torrie, 1960). The overall dis-

TABLE 1. CARDENOLIDE CONCENTRATIONS OF MONARCH BUTTERFLIES AND THEIR HOST-PLANT MILKWEED, *Asclepias viridis* WALT.[a]

		Plant material			Butterfly material		
	N	Mean	SD	Range	Mean	SD	Range
Males	29	229.62	64.64	95–432	286.67	98.59	73–482
Females	31	259.21	72.46	130–432	384.76	89.29	216–591
Total	60	244.91	69.82	95–432	337.35	105.41	73–591

[a] Data are μg (equivalent to digitoxin) per 0.1 g dry wt of butterfly or plant material.

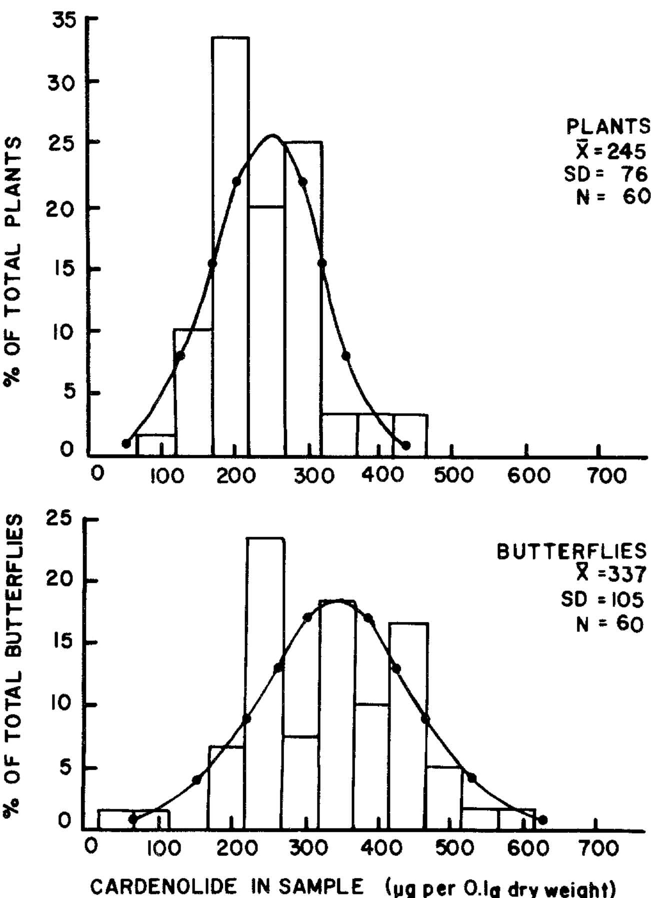

FIG. 2. Frequency distributions of the gross cardenolide content of 60 *Asclepias viridis* plants and 60 monarch butterflies reared thereon. Concentrations are expressed as μg/0.1 g dry weight, equivalent to digitoxin. The width of each bar is 50 μg. The curves above the histograms are the normal distributions calculated by the z statistic (Steel and Torrie, 1960).

tribution for the plants was positively skewed so that the majority of plants contained less than the mean concentration of cardenolide (moment of skewness = 0.79; mean and median, respectively, 244.9 and 235.0 μg; $D = 0.121$, $P < 0.03$). The $\log_{10}$-transformed data, however, did not depart significantly from normality (moment of skewness = −0.32; mean and median = 5.46; $D = 0.104$, $P > 0.10$). Further statistical analyses involving plant concentrations were therefore based on the $\log_{10}$-transformed data.

Butterflies reared on *A. viridis* were also found to be cardenolide-rich, with an even greater mean of 337 μg cardenolide/0.1 g dry weight. The range of cardenolide concentrations in the butterflies, 73–591 μg/0.1 g dry weight, was also greater than the range in the plant samples (Table 1). The pattern of variation in cardenolide concentration in the butteflies (Figure 2) was normally distributed (N = 60; mean and median, 337.3 and 341.0 μg, respectively; D = 0.075, $P > 0.15$).

Although Table 1 shows differences in the mean cardenolide concentrations of plants fed on by male vs. female butterflies, an analysis of the variance indicated that the difference in the plants according to the sex of the butterfly was not significant ($P < 0.36$). However, differences in the means for cardenolide concentrations in male and female butterflies were significant ($F = 16.35$, $P < 0.0002$; $r^2 = 0.22$). The grand variances of the cardenolide concentrations of the plants upon which male and female butterflies were reared were not significantly different (for the s^2 female/s^2 male, $F = 1.26$ with 28 and 30 *df*, $P = 0.54$) nor were the grand variances of the cardenolide concentrations between the male and female butterflies ($F = 1.22$ with 28 and 30 *df*, $P = 0.590$).

The mean cardenolide concentration in the butterflies was 92.4 μg (1.4 times) greater than that of their respective plants, but the overall range of variation in the butterflies (SD = 105.4) was also greater than that in the plants (SD = 69.8) so that the lowest (73 μg/0.1 g) and the highest (591 μg/0.1 g) concentrations were found in the butterflies. Figure 3 illustrates butterfly concentrations (Y, the dependent variable) plotted against plant concentrations (X, the independent variable) for all 60 plant–butterfly pairs. The main regression model (Table 2A) confirmed the logarithmic relationship $Y = 401.961 \log_{10} x = 616.021$ ($r = 0.476$; $P < 0.0001$). Both the slope ($P < 0.0001$) and the intercept ($P < 0.0101$) were significantly different from 0.

In a second set of regressions comparing plant and butterfly cardenolide concentrations by the sex of the butterfly (Table 2B) we found that while the overall model was highly significant ($P < 0.0001$) neither plant concentration, nor sex, nor their interaction were significant using type II sums of squares statistics. By running separate regressions on the sexes, we found that while in both sexes there was a positive correlation between butterfly concentration and plant concentration, in the females it was significant ($P < 0.0023$), while in the males it was not ($P > 0.0647$).

Table 3 lists the dry weights and total cardenolide content of the butterflies. The dry weights were normally distributed (mean and median, respectively = 0.178 and 0.181 g; $D = 0.088$, $P > 0.15$) with the average male butterfly being slightly heavier (0.185 g) than the female (0.172 g). The total cardenolide contents were also normally distributed (mean and median, respectively = 591 and 603 μg; $D = 0.090$, $P > 0.15$). The variances for the two sexes did not differ significantly for either dry weight ($F = 1.17$; $df = 28, 30$; $P = 0.67$) or total cardenolide ($F = 1.15$; $df = 28, 30$; $P = 0.70$). Analyses of the variances

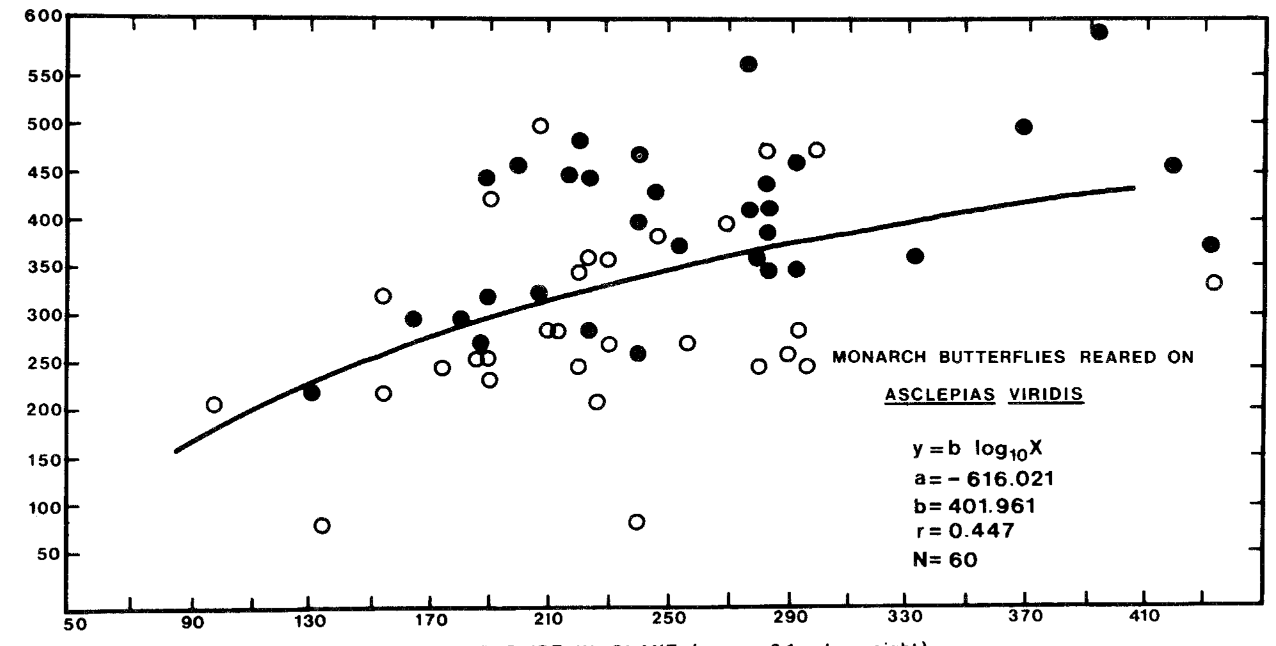

FIG. 3. Cardenolide concentrations of monarch butterflies (Y axis) as a function of the cardenolide concentrations of their larval food plant, *Asclepias viridis* Walt. (X axis). Each of the 60 data points represents one plant-butterfly pair. Open circles are males and solid circles are females. The line is derived from the regression equation $Y = b(\log_{10}X) + a$.

TABLE 2. LINEAR REGRESSION ANALYSES OF CARDENOLIDE CONCENTRATIONS[a]

Source of variation	*df*	SS	MS	*F*	*P*
A. Overall regression (r^2 = 0.476)					
Plant concentration	1	148378	148378	16.97	<0.0001
Error	58	507168	8744		
Corrected total	59	655546			
Estimated value of parameters			SE	*T* for H=O	*P*
a = y intercept = −616.021			231.76	−2.658	<0.0101
b = slope = 404.961			97.58	4.119	<0.0001
Equation for the line: $Y = 401.961\ (\log_{10} x) - 616.021$					
B. Butterfly concentrations on plant concentrations by sex					
Model (R^2 = 0.372)	3	243733	81244	11.05	<0.0001
Error	56	411813	7354		
Corrected total	59	655546			
Log plant conc	1	25548		3.20	<0.0789
Sex	1	1899		0.26	<0.6133
Log plant X sex	1	3533		0.48	<0.4911
Females (r^2 = 0.279)	1	66684	66684	11.21	<0.0023
Error	29	172510	5949		
Corrected total	30	239194			
Males (r^2 = 0.121)	1	32880	32880	3.71	<0.0647
Error	27	239303	8863		
Corrected total	28	272183			

[a] Micrograms per 0.1 g dry wt. in butterflies (Y = dependent variable) vs. cardenolide concentrations ($\log_{10}$ μg/0.1 g dry wt) in their respective plants, according to function $Y = b(\log_{10} x) + a$.

TABLE 3. SUMMARY OF DRY WEIGHTS AND TOTAL CARDENOLIDE CONTENT IN BUTTERFLIES REARED ON *Asclepias viridis* WALT.

		Dry weight (g)			Total cardenolide (μg)		
	N	Mean	SD	Range	Mean	SD	Range
Males	29	0.185	0.026	0.126–0.232	523	179	146–990
Females	31	0.172	0.028	0.110–0.212	655	167	411–973
Both	60	0.178	0.027	0.110–0.232	591	184	146–990

indicated that the mean dry weights of the two sexes were not significantly different ($F = 3.62$, $P < 0.0621$) but that the mean total cardenolide content in the female butterflies (655 μg) was significantly greater ($F = 8.81$, $P < 0.0043$) than in the males (523 μg).

A regression analysis was run to determine the relationship of the butterfly dry weights (Y) to the $\log_{10}$ cardenolide concentrations of the plants (X). The overall model indicated no significant correlation ($r^2 = 0.023$; $F = 1.333$, $P > 0.253$) as did models with either sex. The dry weights of the butterflies were similarly compared to the cardenolide concentrations of the butterflies. The main regression model indicated a significant negative correlation between butterfly dry weight and cardenolide concentration ($Y = -0.94x + 2099.11$; $r^2 = 0.13$; $P < 0.0047$). However, the second regression model, which also included sex and the log plant concentration as covariates, indicated no overall significant correlation ($R^2 = 0.189$; $F = 1.73$, $P < 0.1218$) and that, using type II sums of squares statistics, none of the variables or their interactions were significant predictors of butterfly dry weight (all P values > 0.26).

The same linear regression model as those comparing butterfly cardenolide concentrations and dry weights was run to relate total cardenolide in the butterflies to the $\log_{10}$ plant concentrations. We found butterfly total cardenolide is highly dependent on the $\log_{10}$ plant concentration ($r^2 = 0.43$; $F = 13.37$, $P < 0.0006$) and that both sexes individually showed similar dependence. Based on the equation $Y = 637.33(\log_{10} x) - 920.32$, the overall model predicts that butterflies which fed on plants containing 95 μg/0.1 g cardenolide (the lowest plant concentration in our study) would contain approximately 340 μg, those which fed on 245-μg plants (the mean plant concentration) would contain 602 μg, and those which fed on 432-μg plants (the highest plant concentration in our study) would contain 759 μg. The second regression for total cardenolide indicated that neither the log plant concentration nor the sex of the butterflies nor their interaction were significant predictors when using types II or IV partial sums of squares statistics.

TLC Cardenolide Profiles of Plants and Butterflies. Although TLC plates were run in two separate solvent systems, only those plates run in the chloroform–methanol–formamide (CMF) system were analyzed quantitatively. As found by Brower et al. (1982, 1984a,b) with three California milkweeds, the ethyl acetate–methanol (EM) system resolved fewer cardenolides in *A. viridis* than did the CMF system, and the individual spots were larger and less well defined. While the EM system may eventually provide information on the identity of individual cardenolide compounds by confirmation with known standards and may prove useful in their isolation and purification, the CMF system has proven more valuable in the quantitative analyses of individual cardenolides and in the establishment of TLC "fingerprint" profiles. For these reasons the remaining discussion will be concerned entirely with the CMF profiles.

Figure 4 is a photograph of portions of two plates run in the CMF system.

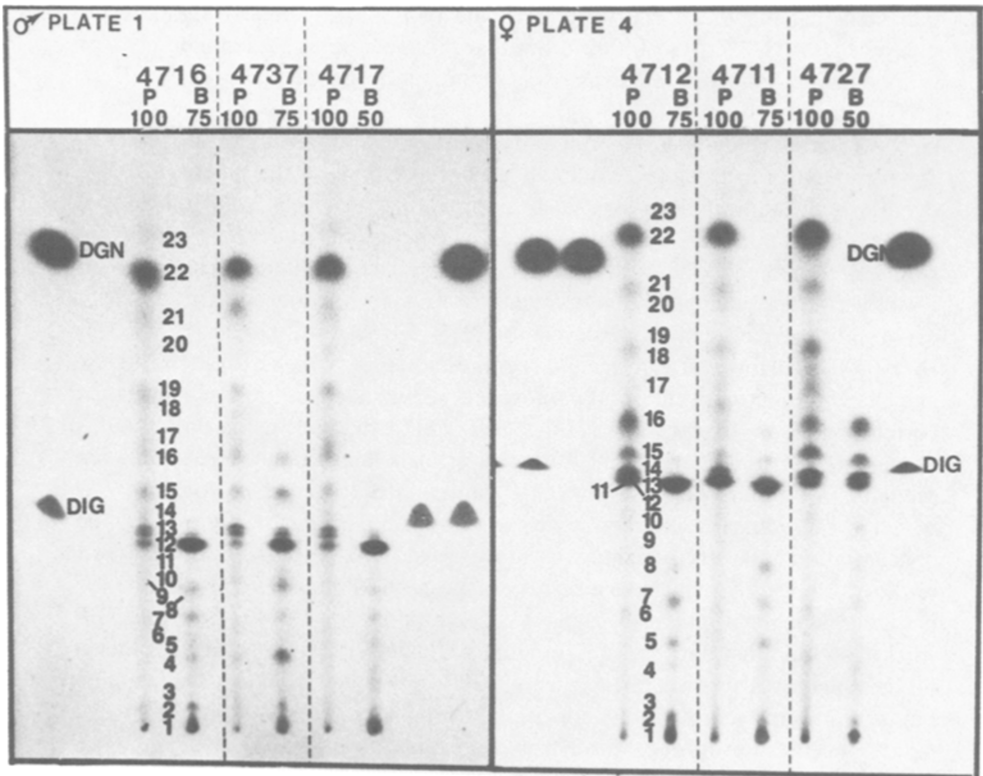

FIG. 4 Photographic reproduction of the thin-layer chromatographic profiles of the cardenolides present in monarch butterflies and their host-plant, *Asclepias viridis* Walt. Represented are six plant–butterfly pairs run in the chloroform–methanol–formamide system including three males (Plate 1: Nos. 4716, 4737, and 4717) and three females (Plate 4: Nos. 4712, 4711, and 4727). Twenty-three individual spots were resolved, including 21 in the plants (all except spots 2 and 3) and 15 in the butterflies (all except spots 6 and 17–23). Also included are two standards, digitoxin (DIG) and digitoxigenin (DGN), which were run on each plate. Several spots with low color intensity are visualized poorly through photographic reproduction (but see the generalized drawing in Figure 5). See text for discussion of possible identities of individual spots.

Represented are six butterfly–plant pairs including three female butterflies and three male butterflies. The number sequence of the individual spots is in the channel between the first plant–butterfly pair and each standard is identified. Headings for each pair include the research number (top), identity as either plant (P) or butterfly (B), and the approximate amount of cardenolide spotted in μg (lowest figures). Table 4 summarizes the means and standard deviations for both $R_{\text{digitoxin}}$ (R_d) and spot intensity (SI) values for all the spots in the 24

TABLE 4. SUMMARY OF MEANS AND STANDARD DEVIATIONS FOR $R_{digitoxin}$ (R_d) VALUES AND SPOT INTENSITIES (SI'S) FOR 23 CARDENOLIDES AND PROBABILITY OF THEIR OCCURRENCE IN 24 PAIRED PLANTS AND BUTTERFLIES[a]

	Means				Standard Deviations							
	R_d		SI		R_d		SI		Probability to spot		Subsample sizes	
Spot No.	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly
23	2.19		1.35		0.21		0.59		0.83		20	
22	2.05		4.25		0.17		1.11		1.00		24	
21	1.91		1.54		0.15		0.66		1.00		24	
20	1.73		1.16		0.12		0.38		0.79		19	
19	1.54		1.71		0.10		0.69		1.00		24	
18	1.42		1.13		0.11		0.46		0.96		23	
17	1.30		1.30		0.09		0.56		0.92		22	
16	1.23	1.21	1.73	1.19	0.07	0.06	0.83	0.68	0.96	0.83	23	20
15	1.09	1.06	1.86	1.45	0.05	0.04	0.71	0.74	1.00	0.92	24	22
14	0.99	0.97	1.00	1.00	0.04	0.05	0.00	0.00	0.67	0.17	16	4
13	0.91	0.91	2.13	1.46	0.05	0.05	0.82	0.78	1.00	0.50	24	12
12	0.87	0.86	2.04	2.00	0.07	0.08	0.62	0.72	1.00	1.00	24	24
11	0.83	0.83	2.88	3.92	0.07	0.08	0.90	1.02	1.00	1.00	24	24
10	0.75	0.77	1.00	1.00	0.06	0.08	0.00	0.00	0.46	0.38	11	9
9	0.71	0.70	1.00	1.00					0.04	0.04	1	1
8	0.67	0.61	1.00	1.38	0.04	0.04	0.00	0.71	0.29	0.96	7	23
7	0.50	0.49	1.00	1.29	0.01	0.04	0.00	0.55	0.13	1.00	3	24
6	0.45		1.00		0.02		0.00		0.92		22	
5	0.35	0.36	1.00	1.23		0.02		0.53	0.04	0.92	1	22
4	0.29	0.29	1.00	1.30	0.03	0.03	0.00	0.70	0.29	0.96	7	23
3		0.17		1.00		0.01		0.00		0.13		3
2		0.08		1.35		0.02		0.71		0.96		23
1	0.02	0.03	1.00	1.13	0.00	0.01	0.00	0.35	0.21	0.63	5	15

[a] Data are based on chromatograms of 24 butterflies and the corresponding plants on which they were reared (chloroform–methanol–formamide system).

plant and butterfly samples analyzed by TLC. Also included in Table 4 is the probability of occurrence of each spot (PO) based on the proportion of plants or butterflies in which each spot occurred and the subsample size or number of times each spot was detected.

The mean migration distance for the 12 digitoxin references (three on each plate) was 57.3 mm with a range of 47.0–67.5 mm and a SD of 6.8 mm. The 12 corresponding digitoxigenin references had a mean migration distance of 115.9 mm with a range of 110.0–120.0 mm and a SD of 3.3. The mean R_d value for digitoxigenin in the 12 reference channels was 2.05 with a range of 1.74–2.43 and a SD of 0.22.

Relatively weak spots were occasionally absent or difficult to discern in individual plants or butterflies, yet the standard deviations of the R_d values are low, indicating the resolution of the cardenolides is good. In general, the overall SIs were lower than expected for both the plants and butterflies. The apparently lower concentration in the cleaned extracts and on the plates may be due to cardenolide loss during the cleanup procedure. It is also possible that noncardenolide substances, present in the crude extract may have produced a false-positive response in the spectroassay and were either removed in the lead acetate cleanup or moved with the solvent front to the leading edge of the plate.

Figure 5 represents the mean cardenolide profile for the 24 plants and butterflies utilized in the TLC analysis. The relative positions and darkness of the spots are calculated from the mean R_d and SI values presented in Table 4. The shapes and sizes of the spots are drawn from a representative TLC plate and then generalized in the figure. The mean R_d values for the 12 digitoxin and digitoxigenin standards are also indicated as dotted lines across the profile. In all, 23 spots were resolved in the CMF system. Twenty-one spots were discerned in the plants and 15 in the butterflies. Spots 2 and 3 were not present in any of the plants while spots 6 and 17–23 were absent in the butterflies.

In the plants there were seven spots (Nos. 1, 4, 5, and 7–10) which were detected in less than 50% of the samples ($PO < 0.50$), although we found these spots consistently present in pooled samples spotted with an estimated 100 μg of cardenolide. This left 14 spots present in the majority of individual profiles and useful as diagnostic characters (Nos. 6, and 11–23). Nine spots (43%) had an $R_d > 1.0$ (above digitoxin) while 12 spots (57%) had an $R_d < 1.0$. Only spot 23 was consistently above digitoxigenin, although spot 22 occasionally migrated slightly above digitoxigenin's position and had a mean R_d value essentially the same as the standard's (2.05). Spots 11–13, 15, 19, 21, and 22 were present in all 24 plant channels and generally had the highest spot intensities.

In the butterflies, four spots had a $PO < 0.50$ (Nos. 3, 9, 10, and 14) leaving 11 as diagnostic. Only two spots had an $R_d > 1.0$ (13%, spots 15 and 16), while the remaining spots (87%) were below digitoxin. The butterflies only stored cardenolides within the lowest 55% of the R_d range of cardenolides pres-

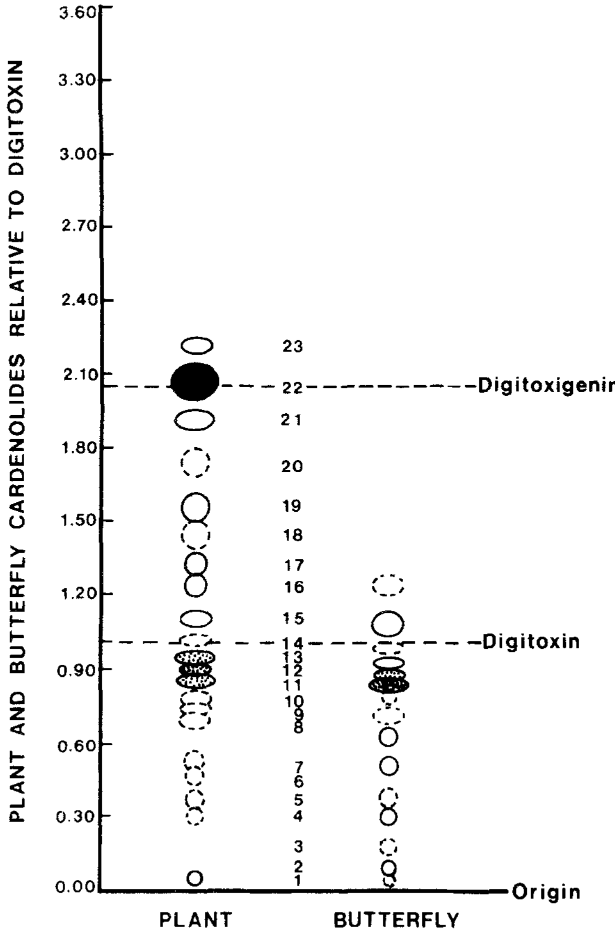

FIG. 5 The mean cardenolide profiles for 24 monarch butterflies and their individual *Asclepias viridis* host-plants based on the chloroform-methanol-formamide TLC system. Shown are the mean $R_{\text{digitoxin}}$ and spot intensity values for 21 plant and 15 butterfly cardenolide spots. Spot intensities are represented as follows: 1.00 < 1.25, dotted circle; 1.25 < 2.00 closed circle; 2.00 < 3.00, light stipple; 3.00 < 4.00, dark stipple; 4.00-5.00, black circle. The mean $R_{\text{digitoxin}}$ value for digitoxigenin was 2.05.

ent in the plants. Only spots 7, 11, and 12 were observed in all 24 butterflies, although several spots (Nos. 2, 4, 5, 8, 15, and 16) were present in 83–96% of the samples. Butterfly spots 1, 4, 5, 7, 8, and 11, all with an $R_d < 0.85$, have significantly greater mean SIs than do their corresponding plant spots, while butterfly spots 13, 15, and 16, with an $R_d > 0.90$, have significantly lower mean SIs than their corresponding plant spots. In addition, spots 2 and 3

are present only in the butterflies. These data indicate sequestration is greater for cardenolides represented by spots below an R_d value of approximately 0.85.

Several regression analyses were run to determine the dependence of butterfly R_ds and SIs on the corresponding plant R_ds. Because of the low *PO*s of several of the 16 spots in common, only 133 of a possible 384 (24 plant–butterfly pairs × 16) spot-pairs were present and used in this analysis. Our initial regression model examined the dependency of the butterfly R_ds on the plant R_ds alone. As expected, we found a high degree of correlation ($F = 17138$, $P < 0.0001$; $r^2 = 0.992$). This near one-to-one relationship of the paired plant–butterfly R_d values is indicated by the predicted regression equation $Y = 0.9784x + 0.0011$.

A problem arose in our analysis because of plate to plate variation in the R_d values and because the sexes (with one exception) were separated onto different plates. Statistics from a multiple regression model including both sex and plate effects as independent covariates with the plant R_ds could be misleading due to the production of singular matrices and to problems of interpretation resulting from the high degree of overlap in the variation. We ran several preliminary regressions, therefore, to determine the individual and additive effects of sex and plate number on the dependence of the butterfly R_ds on the plant R_ds. The addition of sex only to the model did not significantly increase the correlation ($F = 2.04$, $P < 0.156$), whereas the addition of plate only did ($F = 3.97$, $P < 0.010$), suggesting that any differences attributable to sex are too small to be separated from the effects of plate-to-plate variation.

For the above reasons, our second regression model contained only the effects of the plant R_ds, the plate number, and their interaction. Using type II SS statistics, the only significant predictors of the butterfly R_ds in this model are the plant R_ds ($F = 17160$, $P < 0.001$) since both plate and their interaction are insignificant ($P < 0.87$ and 0.67, respectively). Duncan's option indicated that plate 2 (all females) and plate 3 (all males) were not significantly different while plate 1 (five males and 1 female) and plate 4 (all females) were each significantly different from the other three plates. The experimental variable created by differential migration rates on the plates for separate TLC runs is difficult to control, but fortunately, although statistically significant, its magnitude is not great. In subsequent studies we strongly suggest that both sexes be run on each plate so that the differences attributable to sex and plates can be separated more clearly.

Similar regression analyses were run for the spot intensities without the difficulty of plate-to-plate variation found with the R_d values. Again, our initial regression model examined the dependency of the butterfly SIs on the plant SIs alone. This regression indicated a significant correlation ($F = 65.37$, $P < 0.0001$; $r^2 = 0.333$) and produced the equation for the line $Y = 0.7767x + 0.49$. In preliminary regression models neither sex ($F = 0.30$, $P < 0.5834$; $R^2 = 0.334$) nor plate ($F = 0.52$, $P < 0.6760$; $R^2 = 0.341$) contributed signifi-

cantly to the model. To remain consistent with our treatment of the R_d values, our second regression contained only the effects of the plant SIs, the plate number, and their interaction. Again, only the plant SIs contributed significantly to the model ($F = 57.82$, $P < 0.0001$), and the R^2 (0.352) was only slightly greater than the r^2 (0.333) of the effect of the plant SIs alone. Duncan's option indicated that plate 3 (all males) had a significantly lower mean SI than plate 4 (all females) but that none of the other plates or their combinations were significantly different.

DISCUSSION

General Field Observations during Sample Collections. For those monarch butterflies returning to temperate North America through Texas and Louisiana from their overwintering sites in Michoacan, Mexico, *Asclepias viridis* and the more narrowly distributed *A. asperula* subsp. *capricornu* (Woods.) Woods. probably represent the first abundant and widespread milkweed they encounter. It is likely, therefore, that a large number if not the majority of second-generation monarchs that continue the summer reproductive cycle of the monarch utilize these two species as their larval food plant. In western and central Florida, *A. viridis* is more localized and Brower (1961, 1962) and Cohen and Brower (1982) report that *A. humistrata* Walt. is more heavily utilized by the incoming monarchs. Both *A. viridis* and *A. humistrata* occur in Mississippi and Alabama, although the former species becomes increasingly more widespread and prevalent from western Florida to eastern Louisiana.

Other milkweed species in our region are either less common (e.g., *A. amplexicaulis* Sm., *A. longifolia* Michx., *A. michauxii* Dcne., *A. variegata* L.) or do not begin their development until after most migratory monarchs have passed northward (e.g., *A. tuberosa* L., *A. viridiflora* Raf., *A. perennis* Walt.). Lynch, Martin, and Riley (unpublished data) have observed large instar larvae on *A. amplexicaulis*, *A. longifolia*, and *A. viridiflora*, but have not observed successful feeding by monarchs on other species in Louisiana or eastern Texas.

In our area the first monarchs arrive in late March and numerous instars may be found on *A. viridis* by early April. First-, second-, and early third-instar larvae usually feed on floral parts, remaining hidden in the more compact preanthesis inflorescences. Later instar larvae usually feed on the leaves. We suggest that positional feeding preferences may be related to avoidance of predation and parasitism by other arthropods. Spiders, ants, and tachinid flies were observed to take a heavy toll of small larvae, and only a small percentage reached the fourth and fifth instar stages (Lynch and Martin, manuscript in preparation). Cohen and Brower (1982, and personal communication) report similarly low survivorships by monarchs feeding on Florida milkweeds.

By removing crab spiders from the plants and bagging only first- and second-instar larvae, spider predation was virtually eliminated and tachinid para-

sitism was rare. Ant predation, however, continued to be a problem. Bagging provided the further advantage of knowing without question that larvae ate only on the individual plants from which they were collected. In contrast to western U.S. monarchs which nearly always form their chrysalids upon their host plant (Brower et al., 1982, 1984a,b), no chrysalids were found on unbagged plants. Even bagged monarchs tended to spin their pads on the nylon net bags rather than on the leaves of the plant. By carefully selecting larvae from widely spaced plants where extensive feeding was apparent, we are confident that even unbagged plant–butterfly pairs were true to our pairwise analyses.

Quantitative Variation of Plant and Butterfly Cardenolides. Wide variation occurred in the cardenolide concentrations of both *A. viridis* plants and their respective butterflies. The positive logarithmic relationship we observed between individual plant and butterfly concentrations (Figure 3) is consistent with the observations of Seiber et al. (1980) that monarchs sequester cardenolides more efficiently when present in low concentrations, with uptake generally conforming to an inverse logarithmic relationship with increasing concentrations. Duffey et al. (1978) have suggested that cardenolides in *Oncopeltus*, a common milkweed bug, are sequestered in the dorsolateral spaces through a physical process involving a high concentration of nonpolar emulsion-phase droplets dispersed in the aqueous space fluid and that the amount sequestered is proportional to free hemolymph concentrations.

While the mechanisms for cardenolide uptake in monarchs are unknown, patterns of cardenolide uptake observed in all monarch–milkweed pairwise analyses to data could fit a physical uptake model. In the 60 *A. viridis* butterflies examined, both the mean (337 μg/0.1 g) and the range of cardenolide concentrations (73–591 μg) were greater than those in their host plants (245 μg/0.1 g and 95–432 μg). Similar patterns were found by Brower et al. (1984a,b) in *A. californica* and *A. speciosa* butterfly–plant pairs. Both species have a lower mean cardenolide concentration than *A. viridis*. However, in *A. eriocarpa*, a species with a significantly higher cardenolide content, the butterflies had a lower mean concentration (318 μg/0.1 g) and a narrower concentration range (136–606 μg) than their host plants (421 μg/0.1 g and 102–919 μg) (Brower et al., 1982). If host-plant concentrations approach or exceed the physical limits of uptake by the butterflies, mean concentrations and variation may be largely restricted by relatively fixed physical limitations on cardenolide absorption. Variation in relatively low host-plant concentrations, however, would compound any other individual variations present. The overall result would be a narrower range of cardenolide concentrations in monarchs than in their host milkweeds since rapid uptake would occur at low concentrations and uptake at high concentrations would be slowed.

The higher mean concentration of cardenolide we observed in female butterflies compared to male butterflies reared on *A. viridis* has also been demonstrated in butterflies reared on several other *Asclepias* species (Brower and Gla-

zier, 1975; Brower et al., 1975, 1982, 1984b) and in wild-caught adults from summer breeding populations in the eastern United States (Brower et al., 1972, Brower and Moffitt, 1974). The higher mean concentrations in female monarchs may simply reflect differences in the reproductive morphology between males and females. Brower (1984) cites work of Thomashow (unpublished) in which cardenolides were found in all life stages of monarchs reared on *A. curassavica*. Of particular interest, eggs were found to contain higher levels of cardenolide (0.6% by dry weight) than adults (0.4%). Since a female may lay as many as 100 (Erickson, 1973) to 400 eggs (Urquhart, 1960), as much as 97–388 μg of cardenolide could be present in the female reproductive tract. In the laboratory, Dixon et al. (1978) found that monarch females that had laid all their eggs were less emetic to pigeons than freshly eclosed females.

The female butterflies, while lighter than the males, also fed on plants containing a greater mean cardenolide concentration. The regression analyses showed that while there was a significant negative correlation between butterfly dry weight and concentration, when the plant concentrations and sex were added, neither the model nor any interactions were significant. No evidence, therefore, is provided by the dry weight data for a metabolic cost for either cardenolide ingestion or storage.

Qualitative Analyses of Plant and Butterfly Cardenolides. Comparisons were made of TLC R_f values of *A. viridis* cardenolides in both the CMF and EM solvent systems with desglucosyrioside, labriformin, and labriformidin standards run in our laboratory and with values published in Seiber et al. (1983) for 30 *Asclepias* cardenolides and reference standards. The coincidence of R_f values strongly indicates that spots 10 and 14 in the CMF system are calotropagenin and calotropin, respectively. Calactin may also be present at spot 14. Spots 19, 20, 21, and 22 appear to be uscharin, uscharidin, uzarigenin, and vorusharin, respectively, although only uscharidin is well confirmed in the EM system. All these cardenolides are members of a series derived from the genin calotropagenin (Bruschweiler et al., 1969; Seiber et al., 1983, 1984). Marty and Krieger (1984) and Brower et al. (1984b) cite evidence that calactin and calotropin are the principle cardenolides stored when monarchs feed on plants containing calotropagenin glycosides and that the 3′-keto glycoside uscharidin and its 3′-spiro derivatives uscharin and vorushcarin are not stored by monarchs but are metabolized by feeding larvae to the isomeric 3′-OH derivatives calactin and calotropin. Spot 15 appears to be desglucosyrioside, a 3′-OH derivative of the epoxy cardenolides labriformidin and labriformin known to occur in *A. labriformis* and *A. eriocarpa* of the *Asclepias* series roseae (Brower et al., 1982). Neither labriformin nor labriformidin were detected in either the CMF or EM systems.

Although unique, the general TLC patterns of *A. viridis* plant and butterfly cardenolides conform to those observed by Brower et al. (1982, 1984a,b) with *A. eriocarpa*, *A. speciosa*, and *A. californica*. Since larval metabolites are more

polar than their parent cardenolides, monarch profiles are distinct from the plants, favoring cardenolides with lower R_f values. The more polar metabolites desglucosyrioside, calactin, and calotropin, along with other low R_f cardenolides, produce the cardenolide profile of freshly emerged adults. While the general storage pattern appears consistent for those milkweed species examined so far and while the calotropagenin series occurs in several other species of *Asclepias*, and in *Calotropis*, *Gomphocarpus*, and *Pergularia* as well, similarities in certain cardenolides or even entire series do not preclude the possiblity of distinguishing among cardenolide patterns for butterflies reared on different milkweeds.

If remigrating monarch do, in fact, oviposit predominately on *A. viridis* as they reenter the temperate United States through Louisiana and Texas, the majority of late spring and early summer breeding monarchs seen in the northern midwestern and central United States would possess the *A. viridis* TLC profile. As additional monarch–milkweed pairwise analyses are completed, fingerprints of individuals from the spring and summer breeding populations can be compared with those from fall emigrating and Mexican overwintering populations with increasing accuracy, providing key data to our understanding of the monarch butterfly's remarkable life history. Determinations of the structural identity of individual cardenolides will further clarify ecological predictions based on these TLC profiles and may provide valuable insight into the infrageneric relationships of the more than 108 North American species of *Asclepias*.

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MUSK DEER (*Moschus moschiferus*): Reinvestigation of Main Lipid Components from Preputial Gland Secretion

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Abstract—The qualitative and quantitative composition of the principal lipid constituents of Siberian musk deer (*Moschus moschiferus*) preputial gland secretion, main odor carriers and potential precursors of odorous substances, was investigated by means of high-performance liquid chromatography. Free fatty acids and phenols (10%), waxes (38%), and steroids (38%) were found to be the main groups of the secretion lipids. Cholestanol (I), cholesterol (II), androsterone (III), Δ^4-3α-hydroxy-17-ketoandrostene (IV), 5β, 3α-hydroxy-17-ketoandrostane (V), 5α, 3β, 17α-dihydroxyandrostane (VI), 5β, 3α, 17β-dihydroxyandrostane (VII), and 5β, 3α, 17α-dihydroxyandrostane (VIII) were isolated from the steroid fraction and their structures confirmed by IR, PMR, and mass spectra. 3-Methylpentadecanone (muscone) was not identified among the secretion lipids. Preputial gland secretion stimulated sex behavior of musk deer females.

Key Words—Musk deer, *Moschus moschiferus,* preputial gland, chemical composition, secretion, androgens, odor, androsterone, steroids, muscone, pheromone.

INTRODUCTION

The musk deer (*Moschus moschiferus*) has a preputial gland, which is found only in sexually mature males. Its secretion has a specific musk odor, and therefore the secretion's chemical constituents can be involved in chemical communication, encoding at least the information about sex and maturity. Earlier,

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muscone (macrocyclic ketone, the principal odoriferous component of musk deer) (Ruzicka, 1926), a host of macrocyclic molecules (Mookherjee and Ledig, 1970), waxes and steroids (Do et al., 1975), muscopyridine (Schinz et al., 1946), and hydroxymuscopyridines (Yu and Das, 1983) were identified as components of preputial gland secretion. Muscone is claimed to be the sexual attractant for musk deer females (Mookherjee and Wilson, 1982).

The solving of chemical ecology problems for every species requires knowledge of the qualitative and quantitative composition of the compounds excreted by an animal, and their informative significance. The musk pouch composition of Siberia-dwelling musk deer has not been so far investigated by means of standard analytical techniques, and whether it is similar to that of other populations or species, and if so, to what extent, is not yet clear. Such knowledge would contribute to elucidation of the taxonomic status of the species under study and of related species. Furthermore, the standardized and quantitative analysis of skin gland secretions will be of importance in estimating the effect of chemical substances on animal interrelations.

To investigate the qualitative and quantitative composition of the main lipids (present at concentrations more than 0.5%) from preputial gland secretion, we employed high-performance liquid chromatography (HPLC), the structure of compounds isolated being confirmed by their infrared (IR), proton magnetic resonance (PMR), and mass spectral data and by comparison of the physicochemical properties with the authentic samples.

METHODS AND MATERIALS

Biological Material. The samples of the musk pouches were obtained from the animals from the area southeast of the Baykal Lake, in Siberia. This population geographically and taxonomically should be defined as *Moschus moschiferus*. The specimens from 18 individuals were obtained in the period from October to February (1979–1980), which coincided with the rutting season. The musk pouches were removed from killed animals and then air-dried by a standard method, i.e., it was exposed to open air temperatures, not exceeding 40°C, during one week. The largest pouches were obtained from adult musk deer (older than 2 years), whose preputial glands are developed to a much greater extent than those of younger sexually mature animals.

Behavioral Observations. The behavioral observations were carried out with 10 breeding musk deer females, kept in open air cages, Moscow Region, between 1976 and 1984. Five individuals were captured in Siberia, and five were born in captivity.

Chemicals. Solvents used in extraction and HPLC were of reaction grade and distilled before use. The samples of free fatty acids, their methyl esters and *p*-, *m*- and *o*-cresols were obtained from Chem. Service (West Chester, Pennsylvania). The samples of I–III, V, VII, and Δ^4-3, 17-diketoandrostene were

purchased by Sigma Chemical Company (St. Louis, Missouri). The sample of rac-muscone was the gift of Dr. G. Ohloff (Firmenich SA, Geneva Switzerland).

Instrumentation. Analytical gas chromatography (GC) was performed with a Pye Unicam 104 gas chromatograph (FID) using a 2.1-m × 4-mm glass column with 3% SE-30. Gas chromatograms of methyl esters of fatty acids were obtained at isothermal conditions (205°) or temperature programming: 110° (10 min), 10°/min to 230°; chromatograms of the alcohol fraction (after waxes fraction saponification) were obtained at isothermal (214°) or programmed [85° (5 min), 10°/min to 230°] conditions.

Analytical HPLC was carried out using a DuPont 8800 multisolvent liquid chromatograph equipped with a high-sensitivity refractive index (RI) detector (Erma Optical Works, Japan, model ERC-7510), two solvent degassers (ERC-3320, Erma Optical Works), UV spectrophotometric detector (DuPont) and a 3380A reporting integrator (Hewlett Packard; the accuracy of integrating was approximately 5%). It was necessary to install the additional home-made magnetic mixing chamber (volume about 6 ml) on the high-pressure line of the chromatographic pump in order to provide stability of the RI detector at the highest sensitivities. DuPont packed analytical columns (Zorbax Sil and Zorbax ODS, 0.46 × 25 cm) were used for normal and reversed-phase HPLC.

Preparative HPLC was performed using a DuPont 830 preparative liquid chromatograph equipped with a Chrompack-packed preparative column (2.24 × 25 cm, containing Lichrosorb SI 60, 7 mkm) and DuPont RI detector (a Nupro needle valve was employed to divide the flow in a ratio of approximately 1:20; the smaller part of the flow was directed to RI detector).

Mass spectrometry was carried out using an LKB 9000 instrument (70 eV). IR spectra were measured on a Beckman Acculab VI spectrophotometer (5% carbon tetrachloride solutions). PMR spectra were obtained using a Brucker 250 MHz spectrometer ($CDCl_3$, tetramethylsilane internal standard).

Mobile Phases for HPLC. The mobile phases were: normal phase preparative HPLC, hexane–ethyl acetate, 6:4; normal phase analytical HPLC, hexane–chloroform, 1:3 (A), hexane–ethyl acetate, 4:6 (B); reversed-phase analytical HPLC, isopropanol–acetonitrile, 1:1 (C), methanol–acetonitrile–water, 4:3:3 (D).

Extraction of Secretion Lipids. The preputial gland secretion from five animals (dark-red substance, 60 g) was extracted with 0.5 liters of diethyl ether in a Soxhlet apparatus, solvent was removed in vacuo, residue (red oil having a distinctive odor, 10% of secretion weight) was dissolved in 150 ml of ether, and an equal amount of 0.3 M NaOH solution added. From the organic layer, the fraction of neutral compounds was obtained, and from water solution, after acidification to pH 2 and ethyl acetate extraction, the fraction of free fatty acids and phenolic compounds (10% of extract weight) was isolated.

Separation of Fraction of Neutral Compounds. This fraction, obtained in

the general extraction procedure and forming ~90% of the extract weight, was applied to the conventional column with 60 g of silica gel, elution with a mixture of hexane–ether (95:5) followed by ether gave a waxy fraction (38% of the extract weight) and a fraction of steroids (38%).

RESULTS

Analysis of Free Fatty Acids and Phenolic Compounds Fraction. The composition of this fraction was analyzed by GC of the methyl esters (after CH_2N_2 treatment). Aliphatic acids C_{10}–C_{24} (C_{16}–C_{24} predominating) and phenylacetic acid were found to be the main constituents of the "acidic" fraction.

Using an Zorbax Sil HPLC column, operated in analytical and semipreparative mode, *p*-cresol was isolated and identified in the secretion extract (mobile phase A, pressure 68 bar, flow 1 ml/min, UV (254 nm) detector, retention time was 6.3 min, and coincided with authentic sample (separation factor for *p*- and *m*-cresols was 1.048) (Schabron et al., 1979). The content of *p*-cresol in the secretion extract was 0.5%.

Analysis of Waxes Fraction. Waxes were saponified with methanolic alkali (Kates, 1972), and the reaction mixture was treated with hexane. From the organic layer, the fraction of the alcohols was obtained, and from the water–methanolic solution, the fraction of acids. Aliphatic alcohols C_{14}–C_{18} (unsaturated and iso-compounds predominating) and cholesterol were identified by GC and HPLC in the alcohols fraction, and fatty acids C_{10}–C_{24} (C_{16}–C_{24} predominating) in the acids fraction.

Investigation of Steroid Fraction. The fraction of steroid compounds was separated using a preparative HPLC on a Lichrosorb SI 60 column (Figure 1). The mixture of unresolved V and VI was subjected to the additional separation by reversed-phase HPLC (semipreparative mode, up to 10 mg of the mixture) on a Zorbax ODS column (mobile phase D, see Table 1). The following steroids were found to present in the preputial gland secretion (homogeneity of the individual components confirmed by normal and reversed-phase analytical HPLC, retention data given in Table 1, PMR data and melting points in Table 2):

For compounds I–III, V, and VII (Scheme 1), IR, mass spectra, and retention in HPLC were identical to those of the authentic samples.

For compound IV, IR: 3520(OH), 1740(C=O), 1650(C=C), 1080(O—CC=C) cm^{-1}; mass spectrum: 288(M^+, 0.8%), 270(M^+—H_2O, 0.25), 234(0.5), 218(0.9), 149(1.2), 120(1.8), 119(1.6), 118(1.9), 117(1.1), 105(4.4), 87(10.3), 86(3.8), 85(64.9), 84(4.3), 83(100).

For compound VI, IR: 3500–3200(OH), 1040(C—O) cm^{-1}; mass spectrum: 292(M^+, 20%), 277(8), 274(10), 259(9), 233(18), 215(22), 201(6), 185(6), 174(10), 166(19), 165(18), 157(14), 155(13), 149(37), 148(24), 147(18), 133(14), 131(15), 122(16), 121(21), 120(23), 119(22), 118(21),

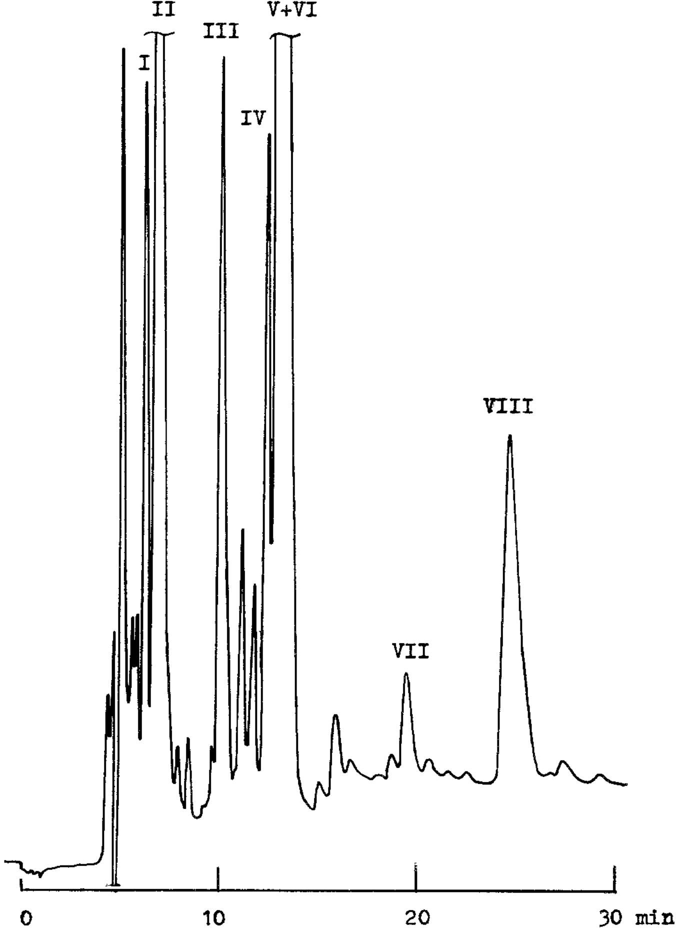

FIG. 1. Preparative HPLC separation of the steroid fraction. Pressure 55 bar, flow rate 25 ml/min, RI detector (attenuation 2×10^{-3} RI units/full scale). Sample: 300 mg.

117(22), 108(17), 107(25), 105(22), 95(23), 93(22), 87(31), 86(24), 85(72), 84(52), 83(100).

For compound VII, IR: 3500–3200(OH), 1040(C—O) cm^{-1}; mass spectrum: 292(M^+, 7%), 274($M^+ - H_2O$, 0.9), 256(87), 241(47), 230(40), 215(100), 149(40), 147(40), 95(60), 93(60), 81(73); diacetate 380(M^+).

3-Methylpentadecanone (Muscone). All attempts to reveal the presence of muscone in secretion lipids (detection limit was ~0.05%) failed.

Other components of the secretion lipids (their content is less than 0.5%) remained unidentified. The composition of secretion constituents, which can be distilled in vacuo, is being investigated at the present time.

TABLE 1. RETENTION DATA AND CONTENT OF STEROIDS FROM PREPUTIAL GLAND SECRETION

	Retention time (min)		
	Zorbax Sil mobile phase B, pressure 103 bar, flow 1.5 ml/min, 20°C	Zorbax ODS mobile phase D, pressure 95 bar flow 1 ml/min, 45°C	% in secretion lipids
I		16.8[a]	1.8
II		14.1[a]	7.0
III	4.6	12.4	3.0
IV	5.5	10.1	3.4
V	5.8	11.5	9.5
VI	6.2	10.1	3.0
VII	8.6	9.6	1.0
VIII	10.9	18.3	3.1

[a] Mobile phase C, pressure 66 bar, flow 1 ml/min, 20°C, RI detector

DISCUSSION

The preputial musk gland is located in the abdominal region near the penis, and its duct opens near the urethra (Sokolov, 1982). According to our observations, by the time the gland becomes active (10–18 months), young musk deer have attained sexual maturity, and the secretion structure changes. The amount of secretion increases, moisture content decreases, and a specific musk odor appears, detectable as early as in 5-month-old males. It has recently been found that musk secretion is androgen-dependent: testosterone administration induces an increase of musk secretion up to 114% (Wang and Huang, 1980). As previously believed, the biological significance of the gland secretion is to attract the females to males during the rutting season, to mark territory (Cherkasov, 1884; Flerov, 1952; Tarasov, 1960) and also to mark covered females (Shaposhnikov, 1956).

Observations of musk deer females olfactory behavior showed that before and during the rutting season (October–March) all mature females (in contrast to males) react to the preputial gland secretion, left in marking the female's territory, by showing specific reactions: nondirectional, fast, and prolonged running behavior, which testifies to the strong excitement of the animal. From two to three weeks before the rutting season, the females, when contacting with male, sniffed the preputial gland region. Before this prerutting period the male, approaching the female closer than 6–7 m, induces an aggressive behavior. Thus, the odor of preputial gland secretion and male urine (which normally

TABLE 2. PMR SPECTRAL DATA[a] AND MELTING POINTS OF STEROIDS FROM MASK DEER PREPUTIAL GLAND SECRETION

			3H		17H				Melting point	
	$CH_{3(19)}$	$CH_{3(18)}$	eq	ax	eq	ax	$COCH_2$	Δ^4-H	°C	°C, lit.
I				3.58					141–142	142
				m						
II									146–148	147–148
III	0.82	0.88	4.05				2.25		182–184	183
			J = 2–3 Hz							
IV	1.01	0.88	4.09							
			J = 2 Hz				2.28	5.49	195–198	196–199[b]
								J = 4.8 Hz		
V	0.91	0.82		3.6			2.2		152–153	152–153
VI	0.82	0.65		3.6	3.73				217–218	212–214[c]
					d, J = 8 Hz					
VII	0.93	0.71		3.56		3.82			220	224–230[c]
VIII	0.94	0.65		3.62	3.73				228–230	232–233[c]
					d, J = 8 Hz					

[a] Chemical shifts are in ppm; eq, equatorial; ax, axial; m, multiplet; d, doublet.
[b] Paradisi et al. (1977).
[c] Nambara and Takahashi (1970).

HO H I

HO II

O HO H III

O HO IV

O HO H V

OH HO H VI

OH HO H VII

OH HO H VIII

SCHEME 1.

acquires a musk smell during rutting) probably reduces the aggressive reaction of females and stimulates sexual behavior.

The first stage of our study of the possible role of secretion chemicals in musk deer ecology and ethology consisted in the investigation of the secretion main lipids (down to 0.5%) by means of modern preparative and analytical methods of isolation and identification of the chemical structure. Simple lipids constitute the bulk of odoriferous compounds, and lipid components of other types may serve as potential precusors of those compounds.

The secretion of the preputial gland was extracted by ether to isolate the great bulk of odoriferous components. Free fatty acids and phenolics were separated by alkaline solution treatment and, after rapid conventional silica gel chromatography and preparative HPLC, the qualitative and quantitative composition of lipid extract was determined.

It was found that waxes, esters of cholesterol, and aliphatic long-chain alcohols and fatty acids account for the 38% of the extract weight, a value by far greater than the one previously reported for Nepal musk deer (17%, Do et al., 1975). The waxes can serve as inert material, prolonging the action of odoriferous components, and also as the source of free fatty acids (for example, in bacterial transformation), as indicated by the similar composition of free and bound (in waxes) fatty acids.

Steroid compounds are the second large group of secretion lipid components (38%), which agrees with previously reported data (46%, Do et al., 1975). These are cholestanol, cholesterol, and a number of the androstane derivatives. It was the most complicated part of our research to isolate, purify, and identify the components from this group. Various modes of HPLC, widely employed recently in chemistry, biology, and medicine, were used in this study. Normal-phase analytical HPLC conditions of the steroid fraction (Table 1, Zorbax Sil column) were adjusted for the preparative HPLC. Complete separation was achieved, however, only after reversed-phase HPLC. A number of reversed phase HPLC methods were developed recently to separate androgens (Sunde and Lindmo, 1982; Lin and Heftmann, 1982). We used the triple mixture of methanol, acetonitrile, and water in HPLC on Zorbax ODS column (Table 1) to verify the homogeneity of components, isolated by preparative HPLC and/or to separate by semipreparative HPLC the mixture of compounds which could not be separated with an adsorption HPLC. Isolated compounds were characterized by their spectral data and by direct comparison of chromatographic retention on HPLC along with the authentic samples available.

The composition of lipids from the Siberian musk deer preputial gland secretion only partially coincides with data reported by Do et al. (1975) (see Table 3). The waxes, steroids, and muscone account for 86% of secretion extract for Nepal musk deer, whereas waxes and steroids from the Siberian musk deer constitute 76% and muscone is totally absent. Of the steroids, both Nepal

TABLE 3. CHEMICAL CONSTITUENTS OF MUSK DEER PREPUTIAL GLAND SECRETION (% OF EXTRACT WEIGHT)

	Nepal musk deer (Do et al., 1975)	Siberian musk deer (our results)
Waxes	17	38
Muscone	22.6	0[b]
Aliphatic alcohols	2.8	0[b]
Acids and phenols	—[a]	10
Steroids (total)	46.4	38
I	—[a]	1.8
II	7.7	7.0
5α-13,17-Diketoandrostane	1.3	0[b]
5β-3,17-Diketoandrostane	1.3	0[b]
Δ^4-3,17-Diketoandrostene	1.7	0[b]
$\Delta^{4,6}$-3,17-Diketoandrostadiene	1.7	0[b]
III	3.1	3.0
IV	—[a]	3.4
Δ^5-3β-Hydroxy-17-ketoandrostene	1.2	0[b]
Epiandrosterone	0.7	0[b]
V	5.6	9.5
VI	5.3	3.0
VII	1.4	1.0
VIII	6.3	3.1

[a] Data are absent.
[b] Compound is either absent or its concentration is lower than 0.5%.

and Siberian musk deer contain, in amounts greater than 0.5%, II, III, and V–VIII. We failed to reveal the presence of 5α- and 5β,3,17-diketoandrostanes, Δ^4-3,17-diketoandrostene, $\Delta^{4,6}$-3,17-diketoandrostadiene, and Δ^5-3β-hydroxy-17-ketoandrostene at concentrations reported for Nepal musk deer. Compounds I and IV have not been described by Do et al. (1975).

The structure of IV was established as follows. The molecular weight was determined to be 288 (mass spectrum). From IR and PMR data the presence of one hydroxyl (3520 cm^{-1}; δ 4.09, 1H), one carbonyl (1740 cm^{-1}; δ 2.28, 2H), and trisubstituted double bond (1650 cm^{-1}; δ 5.49, 1H, doublet, $J = 4.8$ Hz), adjacent to a methine proton, was deduced. The allylic arrangement of hydroxy group is shown by converting the signal from olefinic proton to a singlet and reducing the multiplicity of methine proton signal in double resonance experiments. Chemical shift and multiplicity of the signal from proton, geminal to the hydroxy group at C_3 (δ 4.09, $J = 2$–3 Hz), pointed to the equatorial position of this proton, and, correspondingly, to the 3α orientation of hydroxyl (this signal in PMR spectra of 3-hydroxy-steroids, having the equatorial hydroxy group, has δ 3.6 ppm) (see Table 2 and Williams and Bhacca, 1964). PMR

spectral data and the melting point value coincided with those reported previously (Paradisi et al., 1977). Oxidation of compound IV by pyridinium chlorochromate gave Δ^4-3, 17-diketoandrostene, identical to the authentic sample. In addition, successive treatment of *O*-tetrahydropyranyl-protected testosterone by lithium aluminum hydride, acetic anhydride, HCl, pyridinium chlorochromate, and methanolic alkali gave Δ^4-3β-hydroxy-17-ketoandrostene, which was not identical to IV in chromatographic mobility at HPLC.

It is not yet known which components of preputial gland secretion (separately and/or in combination) act as stimuli. It seems possible that monofunctional chemicals of medium molecular weight could be the candidates to mammalian pheromones, because their volatility allows them to reach olfactory receptors at above-threshold concentrations. From this point of view, free fatty acids, *p*-cresol, and other similar compounds could be pheromone components, and bifunctional steroids (present in the secretion at rather high concentrations) may perform the androgen functions and/or stimulate the accessory olfactory system. It is not clear, however, whether the concentration of bifunctional steroids (relatively polar molecules) in vapor phase over secretion is sufficient to excite the main olfactory receptors. Data concerning the odor of bi- and polyfunctional steroids and available at present, are not very reliable, because no special checks for purity or the absence of odoriferous impurities have been taken. Due to these considerations, we investigated the possibility of androsterone (III), one of the components of preputial gland secretion having an odor in humans, and we found that this compound really has a santal odor. It is known that Δ^{16}-5α, 3α-hydroxyandrostene, one of the boar pheromone components (Reed et al., 1974), causes two types of odor, urinous and santal, in humans (Ohloff et al., 1983).

The same sample of III was subjected to successive purification by normal and reversed phase HPLC, and the following mobile-phase fractions were investigated olfactorally by panelists sensitive to santal odor: the fraction before the peak, containing the peak, and after the peak of III. It was found that the only fractions having santal odor were those containing androsterone (III). The purification of androsterone carried out by means of such fundamentally different (from the chemical point of view) techniques as normal and reversed-phase HPLC, practically guarantees the absence of the impurities, which can distort the results of olfactory analysis. Thus, bifunctional steroids at normal conditions can be volatile enough to excite the olfactory receptors. We believe it possible that it is androsterone (III), having both the odor and androgenic activity, that is most likely the sex pheromone of musk deer males. The functional role of separate lipid components from preputial gland secretion and their combinations will be the subject of further research.

The difference in chemical composition of Siberian musk deer preputial gland secretion, found in our research and reported previously (Ruzicka, 1926; Mookherjee and Ledig, 1970 and, in particular, Do et al. 1975) (see Table 3),

cannot be explained by the different analytical techniques employed. Moreover, we carried out additional experiments to determine the presence of secretion components (i.e., muscone and Δ^5-3β-hydroxy-17-ketoandrostene) reported earlier. Most likely, these differences are due to dissimilarities in the taxonomy and ecology of the subjects. One of the latest manuals on mammalian taxonomy (Honachi et al., 1982) splits *Moschus* into four geographically separated species. Based on this manual, we studied the individuals of *Moschus moschiferus*, whereas the musk pouches, investigated by the South Korean group (Do et al., 1975), were obtained from Nepal musk deer, which should be assigned to *M. chrysogaster*. Thus the differences in the chemical composition of preputial gland secretion, at least in this case, are presumably interspecific. But, in spite of the inferences drawn from musk deer taxonomy, one should not exclude the possibility of some differences in chemical constituents of the secretion arising from the dissimilar ecology of the population under study (including feeding specialization). The knowledge of variability of mammalian gland secretions, including skin glands, is one of the still obscure and intriguing fields of chemical ecology.

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INTERRUPTION OF RESPONSE OF *Dendroctonus brevicomis*[1] TO ATTRACTIVE PHEROMONE BY RELEASE OF PHEROMONE AT SEVERAL RATES AND SPACINGS[2]

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Abstract—The number of western pine beetles, *Dendroctonus brevicomis*, trapped at the center of three 90 × 90-m plots was reduced during a 16-hr period after surrounding an attractive source of *exo*-brevicomin, frontalin, and myrcene with 16, 48, or 168 sources of the same attractant. Compounds were released from the center of the plot at 1 mg/16 hr/compound, and from the surrounding sources at 4–280 mg/16 hr/compound. About half of these amounts was released between 1530 hr and 1930 hr, the period of peak beetle flight. No treatment differences were apparent in reduction of catch within the range of release rates and spacings tested. When compounds were released continuously from 168 stations for 17 days, catch at the center of a plot was generally lower than catch before or after this period, but fluctuated daily. More beetles were caught on traps hung on ponderosa pines within a plot and the number of these traps catching beetles was greater when compounds were released from all stations than from only the center station. Beetles caught on traps were attracted into the plots from the surrounding forest, but appeared to be dispersed within the plot when compounds were released from many stations. *D. brevicomis* attacked at least 91 trees in the plots, of which 25 were killed during two summers.

Key Words—*Dendroctonus brevicomis*, Coleoptera, Scolytidae, bark beetle, western pine beetle, pheromone, interruption, behavior, release device.

[1] Coleoptera: Scolytidae.
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INTRODUCTION

The response of the western pine beetle *Dendroctonus brevicomis* LeConte to the attractive mixture of racemic *exo*-brevicomin (E), racemic frontalin (F), and myrcene (M) and to an attractive bolt cut from a tree being colonized by *D. brevicomis* was interrupted in 90 × 90-m plots by surrounding the source of attraction with 48 stations releasing EFM at 2 mg/24 hr/compound (Tilden et al., 1981). Beetles attracted from the surrounding forest, however, attacked and killed trees in the plots. Richerson et al. (1980) disrupted mass attack of host trees by the southern pine beetle *D. frontalis* Zimmerman by baiting other trees previously killed by the beetle with the attractive mixture of frontalin and alpha-pinene. The effects of varying the attractant release rate or the spacing between release devices were not examined in these studies.

The orientation of male moths to female moths in agricultural fields was interrupted by permeating the air with a synthetic pheromone (Shorey et al., 1972) or an attractive compound (McLaughlin et al., 1972) at several release rates and spacings. Shorey et al. (1972) speculated that the amount of pheromone released was more important than the distribution of release points in disrupting male orientation.

The tests described below were performed to see if aggregation of *D. brevicomis* at a single source of attractant could be interrupted by surrounding it with other sources of attractant released at several rates from variously spaced release devices. This design was based on our assumption that lowered catch at a single trap might be indicative of a treatment's potential to interrupt aggregation at trees in large plots. We anticipated that beetles attracted from the surrounding forest might attack and kill trees in our small plots (Tilden et al., 1981). We also assumed, however, that such tree mortality might not occur in large plots because beetles would be dispersed rather than concentrated, the treatment would be released over a period of many days instead of a few hours, and edges of large plots would be away from host trees.

METHODS AND MATERIALS

The tests were performed during August and September 1974 and July to October 1975, near Bass Lake and Cedar Valley, Madera County, California (1100–1300 m elevation). Each year, three 90 × 90-m plots (Tilden et al., 1981) at least 3.2 km apart were installed in stands of ponderosa pine, *Pinus ponderosa* Dougl. ex Laws., where similar numbers of *D. brevicomis* were caught at traps baited with EFM during a three-week period preceding the tests. No trees under attack or with emerging brood were within 200 m of the plots. Two plots were in the same locations both years; the third plot was in a different location each year.

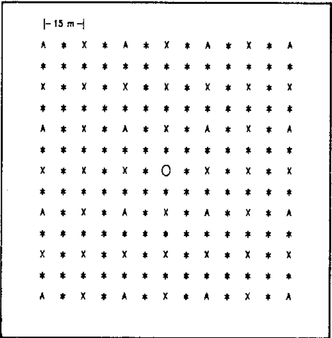

FIG. 1. Plot in which *exo*-brevicomin, frontalin, and myrcene were released at three spacings surrounding a source of the same compounds released at the plot center (0). A = 16 release stations 30 m apart; A and X = 48 release stations 15 m apart; A, X, and * = 168 release stations 7.5 m apart.

Each plot consisted of 168 pheromone release stations 7.5 m apart surrounding a center station (Figure 1). The EFM mixture was released from the center station inside a cylindrical hardware-cloth sticky trap placed 1.5 m above ground on a steel pipe (Bedard and Browne, 1969). The compounds evaporated at about 1 mg/16 hr/compound from glass tubes inside an inverted glass salt shaker covered with aluminum foil (Tilden et al., 1981). EFM attractant was also released at some or all of the remaining 168 stations from devices attached to the top of wooden stakes, 1 cm × 4 cm × 1.5 m, in three grid patterns (Figure 1): 13 × 13 (168 stakes 7.5 m apart); 7 × 7 (48 stakes 15 m apart); and 4 × 4 (16 stakes 30 m apart). Total EFM attractant released from stations surrounding the center trap was divided among the number of stations in each test. When using fewer than 168 stations, release devices were attached to the 16 or 48 wooden stakes in nearly equal numbers.

Each plot plus an additional 15-m-wide strip around its perimeter was divided into 64 15 × 15-m blocks (Figure 1). One unbaited 30.5 × 61-cm hardware-cloth sticky trap was hung 1.5 m above ground on each of 64 ponderosa pines >25 cm DBH per plot to monitor *D. brevicomis* visitation. One tree from each 15 × 15-m block was chosen; but if a block had no trees, two or more trees from an adjacent block were chosen. All traps were coated with melted Stikem Special®.

Each test ran a sequence of two days per plot. EFM attractant was released from the center trap only on day 1 (control) and from the center trap plus surrounding stations on day 2 (treatment). No compounds were released in a plot on the next two days so that attracted beetles could disperse. This procedure was followed once per plot for each treatment, for a total of three replicates. We assumed that most variation in catch between day 1 and day 2 would be

due to the treatment, although daily catch at a source of attractant is variable (Bedard et al., 1980; Tilden et al., 1983). Interaction could also occur between days. Beetles caught on day 1 could not be caught on day 2, or beetles attracted and not caught on day 1 would be available to be caught on day 2. We felt that a single source of attractant (control) on the day preceding treatment would introduce less bias than if treatment preceded control.

Compounds were released in a plot for about 16 hr, from 1530 hr of one day until 0730 hr the next morning, but most beetle flight occurred during the afternoon and early evening. A treatment was presented in only one of the three plots each day to minimize the number of release devices needed. *D. brevicomis* were picked from all traps every morning, after removing compounds from the plots, and placed in labeled vials of solvent (Chevron-325®) for later counting. Also, every ponderosa pine in and within 30 m of the plots was examined for signs of attack by *D. brevicomis*.

We planned to release the compounds at three rates tenfold apart in 1974 and at two rates tenfold apart in 1975. Tests were randomly assigned to plots and days. In 1974, however, the 1× and 10× treatments were completed before the 100× treatments were begun because we felt that trees would most likely be attacked during the latter treatments.

Three types of devices were used to release EFM from the stations surrounding the center trap (Table 1). One device was made from 1-μl glass pipets (Drummond Microcaps®) 32 mm long × 0.20 mm ID, filled to about half their capacity. Pipets containing E had both ends open; those containing F had one end flame-sealed. Pipets containing M initially had both ends open, but were later flame-sealed at one end because of excessive loss of material. Two pipets each of E and F and one (both ends open) or two (one end sealed) of M were attached to a small card with a piece of transparent tape and laid horizontally inside the lid of a perforated aluminum tea ball 5 cm maximum diam. × 6 cm high to provide protection from sun and wind. The small chain and hook normally attached to the lid of the tea ball was attached instead to the apex of its

TABLE 1. DEVICES USED TO RELEASE *exo*-BREVICOMIN, FRONTALIN, AND MYRCENE IN 90 × 90 m PLOTS FROM TREATMENT STATIONS[a]

Planned relative rate (year)	No. treatment stations	Device[b]
1× (1975)	16, 48, 168	Glass pipet
1× (1974)	16	Polyethylene capsule
10× (1974, 1975)	16, 48, 168	Polyethylene capsule
100× (1974)	16, 48, 168	Glass tube

[a] Bass Lake and Cedar Valley, Madera County, California.
[b] See text for description of devices.

body, so that it hung with the lid down from the top of a wooden stake. A total of 336 pipets of each compound or 168 of M with both ends open were used for the 1× rate in 1975.

The second device, a polyethylene capsule containing about 25 mg of compound, was made by plugging both ends of a 15-mm-long piece of 3.17 mm ID × 6.35 mm OD polyethylene tubing with a 5-mm-long piece of 3.17 mm diam. polyethylene rod. The ends of the capsule were heat-sealed to prevent leakage. The release rate was regulated by enclosing each capsule in a tightly fitting sleeve cut from thin-walled Teflon® tubing. Pheromone diffused through the polyethylene and evaporated from the surface of the capsule through one or two holes cut in the Teflon sleeve: for E, one 6-mm-diam. hole; for F, one 3-mm-diam. hole; and for M, two 6-mm-diam. holes diametrically opposed. One capsule of each compound was placed inside the lid of an aluminum tea ball. Sixteen capsules of each compound were used for the 1× rate in 1974; 168 capsules of each compound were used for the 10× rate in 1974 and 1975 (Table 1).

The third device, glass tubes placed inside an inverted salt shaker, was the same device which released EFM from the center trap. A total of 336 tubes of each compound were used for the 100× rate in 1974.

We measured daily pheromone loss from 10 treatment devices placed randomly in a plot, with no two devices at the same release station on the same day. These devices were marked for identification and were included in the total number of devices for each treatment. We also measured release rates of EFM from the glass tubes at the center of a plot. The amount of material lost from glass tubes and polyethylene capsules was measured gravimetrically. The amount of material lost from pipets was measured with a micrometer eyepiece in a microscope.

To estimate pheromone release rates during the period of peak beetle flight, the weight loss of EFM from polyethylene capsules and glass tubes was measured on four consecutive days in 1974 in an area separate from the test plots. One glass tube of each compound was in each of nine salt shakers, and two polyethylene capsules were in each of nine tea balls. Each release device was hung from the top of a 1.5-m-high wooden stake. Weight loss was measured daily after 4-hr exposure (1530–1930 hr, the period of greatest beetle flight) and after subsequent 12-hr exposure (1930–0730 hr).

Following the 16-hr tests in 1974, a long-term test was conducted in two of the plots. In each plot, EFM attractant was released continuously for 24 days from the trap at the plot center. Compounds evaporated from release devices—the glass tubes used in earlier tests—at about 2 mg/24 hr. From the fourth through twentieth days, EFM attractant was also released from the other 168 stations in each plot, from 1-μl glass pipets; two each of E and F, and four of M with one end sealed. This was essentially the 1× treatment used during the 16-hr tests. Pipets were removed from the 168 treatment stations on the morning

TABLE 2. DAILY CATCH OF *Dendroctonus brevicomis* IN THREE 90 × 90-m PLOTS WHERE SOURCE OF ATTRACTANT[a] WAS ALONE (DAY 1) OR SURROUNDED (DAY 2) BY GRID OF TREATMENT STATIONS RELEASING ATTRACTANT AT SEVERAL RATES AND DENSITIES[b]

	Catch			
	At center		At trees[c]	
Plot	Day 1	Day 2	Day 1	Day 2
1×[d] at 16 stations, 1974				
A	54	9	3 (2)	35 (14)
B	38	15	2 (2)	10 (7)
C	49	9	48 (12)[e]	33 (15)[e]
1× at 16 stations, 1975				
A	69	13	23 (1)[e]	64 (8)[e]
B	242	21	13 (3)	64 (15)[e]
D	16	3	1 (1)	20 (11)
1× at 48 stations, 1975				
A	169	22	6 (2)	16 (11)
B	22	4	1 (1)	15 (8)
D	15	4	3 (1)	26 (12)
1× at 168 stations, 1975				
A	115	6	2 (2)	2 (2)
B	319	15	19 (5)	27 (13)[e]
D	12	3	0 (0)	50 (12)
10× at 16 stations, 1974				
A	88	2	6 (4)	51 (18)
B	20	4	5 (5)	127 (14)[e]
C	215	2	30 (7)[e]	183 (19)[e]
10× at 48 stations, 1974				
A	118	1	15 (5)[e]	70 (23)[e]
B	63	0	4 (3)	99 (25)
C	36	1	5 (5)	62 (17)[e]
10× at 168 stations, 1974				
A	124	4	5 (3)	139 (17)[e]
B	40	2	6 (4)	90 (16)
C	27	1	1 (1)	113 (26)
10× at 16 stations, 1975				
A	224	1	2 (2)	7 (5)
B	232	5	15 (5)	164 (16)[e]
D	6	0	0 (0)	42 (14)

TABLE 2. Continued

Plot	Catch			
	At center		At trees[c]	
	Day 1	Day 2	Day 1	Day 2
10× at 48 stations, 1975				
A	68	2	0 (0)	34 (14)
B	53	0	1 (1)	38 (19)
D	26	0	1 (1)	11 (10)
10× at 168 stations, 1975				
A	141	5	5 (1)	96 (19)[e]
B	21	3	43 (8)	46 (17)[e]
D	1	0	0 (0)	48 (14)
100× at 16 stations, 1974				
A	8	0	8 (3)[e]	96 (26)[e]
B	26	1	5 (5)	65 (18)[e]
C	56	2	41 (10)[e]	151 (29)[e]
100× at 48 stations, 1974				
A	10	1	17 (5)[e]	101 (32)[e]
B	24	2	48 (8)[e]	400 (24)[e]
C	73	1	8 (3)	117 (19)[e]
100× at 168 stations, 1974				
A	70	1	5 (3)	151 (31)[e]
B	24	5	11 (3)	322 (28)[e]
C	27	0	143 (12)[e]	316 (37)[e]

[a] *exo*-Brevicomin, frontalin, and myrcene released from plot center at ca. 1 mg/16 hr/compound.
[b] Bass Lake and Cedar Valley, Madera County, California, August 3 to September 25, 1974, and July 17 to August 10, 1975.
[c] One unbaited trap 1.5 m above ground on each of 64 trees per plot. Number in parentheses is number of trees on whch *D. brevicomis* were trapped.
[d] Planned relative release rate of *exo*-brevicomin, frontalin, myrcene per plot. See Table 3 for estimated release rates. Treatment released at stations which were 30 m apart (16 stations), 15 m apart (48 stations), or 7.5 m apart (168 stations).
[e] At least one tree with a trap was attacked by *D. brevicomis*.

of the twenty-first day. Each morning, we picked *D. brevicomis* from all traps and examined ponderosa pines in and near the plots for signs of attack.

Interruption of aggregation at the center trap was evaluated by comparing the number of beetles caught there when treatment was absent with the number caught when treatment was present. For the 16-hr tests, catch on the first day (treatment absent) was compared with catch on the second day (treatment pres-

ent). For the long-term test, catch on the first three and last four days was compared with catch on the fourth through twentieth days. We did not conduct formal statistical evaluations of the results, since the several factors—days, plots, and treatments—were confounded in the design, and the experiment was not extensive enough to incorporate adjustments for these factors into an analytical model. Trees attacked by beetles attracted into the plots were also a confounding factor.

RESULTS

For each pair of treatment and control days during the 16-hr tests, fewer *D. brevicomis* were caught on the trap at the plot center when it was surrounded by 16, 48, or 168 sources of attractant (day 2) than when it was not (day 1) (Table 2). The mean numbers of *D. brevicomis* caught at the center trap on control days (day 1) and on treatment days (day 2) were as follows (numbers in parentheses are standard deviation and number of days):

	1974	1975
Control	56.7 (48.3, 21)	97.0 (100.0, 18)
1×	11.0 (3.5, 3)	10.1 (7.8, 9)
10×	1.9 (1.4, 9)	1.8 (2.1, 9)
100×	1.4 (1.5, 9)	

More *D. brevicomis* were trapped on trees—in every case but two—when a treatment was in a plot than when it was not (Table 2). Beetles were trapped on more trees—in every case but one—when treatment was in a plot than when it was not (Table 2, numbers in parentheses). On days when one or more of the trees with traps were attacked by *D. brevicomis*, most of the trapped beetles were caught on these trees.

During the long-term test, August to September 1975, catch at the plot center fluctuated daily, but was generally lower during the 17 treatment days than on the three days before and four days after treatment in both plots (Figures 2 and 3). The mean number of *D. brevicomis* caught at the center trap on control and treatment days was as follows (standard deviation in parentheses):

	Plot A	Plot B
7 control days	41.0 (11.9)	31.7 (11.0)
17 treatment days	15.9 (8.7)	10.9 (9.1)

Total catch on the 64 trees with traps in each plot was low prior to treatment, then peaked during the middle of the treatment period. The number of *D. brevicomis* trapped on trees declined after treatment was removed in plot A

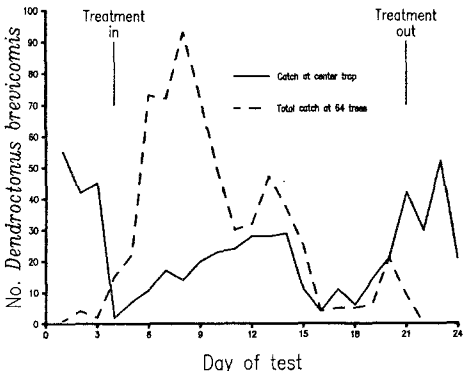

FIG. 2. Catch of *Dendroctonus brevicomis* at two types of traps in a 0.81-hectare plot in which *exo*-brevicomin, frontalin, and myrcene were released from a center trap for 24 days and from 168 treatment stations surrounding the center trap on days 4–20. Plot A, August 26 to September 18, 1975.

(Figure 2). None of these trees was successfully attacked. The number of *D. brevicomis* trapped on trees increased after treatment was removed in plot B (Figure 3), since one of these trees was successfully attacked by *D. brevicomis* and caught most of the beetles.

Several pines in the plots were attacked, and some were killed by *D. brevicomis* during the tests. A tree was counted as attacked if one or more pitch tubes were seen. A tree was counted as killed if subsequent examination revealed successful egg galleries and faded foliage. In 1974, 64 trees in the three

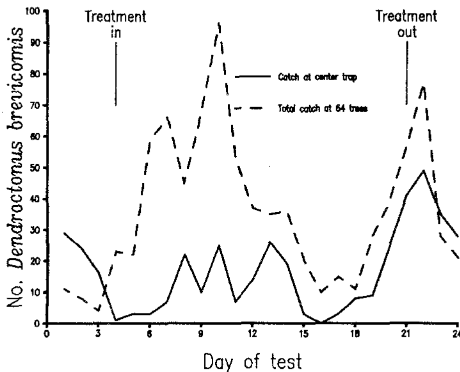

FIG. 3. Catch of *Dendroctonus brevicomis* at two types of traps in a 0.81-hectare plot in which *exo*-brevicomin, frontalin, and myrcene were released from a center trap for 24 days and from 168 treatment stations surrounding the center trap on days 4–20. Plot B, August 26 to September 18, 1975.

TABLE 3. MEAN AMOUNTS[a] OF *exo*-BREVICOMIN (E), FRONTALIN (F), AND MYRCENE (M) RELEASED PER 16 HR FROM 10 DEVICES EXPOSED AT TREATMENT STATIONS[b]

Device[c] (year)	Days[d]	E (mg)	F (mg)	M (mg)
Polyethylene capsule (1974)	20	0.25 (0.19)	0.22 (0.19)	0.43 (0.30)
Polyethylene capsule (1975)	9	0.34 (0.25)	0.28 (0.20)	0.60 (0.32)
Glass tube (1974)	9	0.68 (0.13)	0.71 (0.14)	0.84 (0.20)
Glass pipet (1975)	9	0.033 (0.025)	0.024 (0.015)	0.108 (0.073)[e] 0.011 (0.0003)[f]

[a] Standard deviations in parentheses.
[b] Bass Lake and Cedar Valley, Madera County, California, August 3 to September 25, 1974, and July 17 to August 10, 1975.
[c] See text for description of devices.
[d] Number of days on which weight loss of 10 devices was measured.
[e] Five days for M pipets with both ends open.
[f] Four days for M pipets with one end open.

plots were attacked and 17 of these were killed. In 1975, during the 16-hr tests, 20 trees in the three plots were attacked and six of these were killed. During the long-term test, seven trees were attacked in the two plots and two of these were killed.

Results of the daily measurements of material lost from 10 devices exposed at treatment stations (Table 3) were used to estimate the total amounts of EFM that were released in a plot (Table 4). Release rates of compounds from these devices varied daily as much as 100%, and partitioning of variance components showed that most of this was probably due to variation in environmental conditions rather than individual devices. Tenfold differences in release rates between treatments were planned, but this succeeded only for the 1× and 10× treatments in 1974. About fivefold differences occurred between the 10× and 100× treatments in 1974 and between the 1× and 10× treatments in 1975 (Table 4). Results of the weight loss measurements made of the polyethylene capsules and glass tubes in an area separate from the plots (Table 5) indicated that about half the amount of EFM was released during the four-hour period (1530–1930 hr), corresponding to the period of greatest beetle flight.

DISCUSSION

We speculate that fewer *D. brevicomis* were caught at the center trap on days when treatment was present than when it was not because aggregation at

TABLE 4. TOTAL AMOUNTS OF *exo*-BREVICOMIN (E), FRONTALIN (F), AND MYRCENE (M) ESTIMATED TO HAVE BEEN RELEASED PER 90 × 90-m PLOT PER 16 HR[a]

Year	Planned rate	Treatment stations[b]			Plot center[c]		
		E (mg)	F (mg)	M (mg)	E (mg)	F (mg)	M (mg)
1974	1×	4.00 (3.04)	3.52 (3.04)	6.88 (4.80)	1.10 (0.36)	1.12 (0.26)	1.49 (0.38)
1974	10×	42.00 (31.92)	36.96 (31.92)	72.24 (50.40)	1.10 (0.36)	1.12 (0.26)	1.49 (0.38)
1974	100×	228.48 (43.68)	238.56 (47.04)	282.24 (67.20)	0.90 (0.26)	0.86 (0.24)	1.18 (0.19)
1975	1×	11.09 (8.40)	8.06 (5.04)	18.14[d] (12.26) 3.70[e] (0.10)	0.80 (0.29)	0.90 (0.36)	0.97 (0.40)
1975	10×	57.12 (42.00)	47.04 (33.60)	100.80 (53.76)	0.80 (0.29)	0.90 (0.36)	0.97 (0.40)

[a]EFM released on two consecutive days for each test: from plot center on day 1 and from plot center plus surrounding 16, 48, or 168 treatment stations on day 2. Bass Lake and Cedar Valley, Madera County, California, August 3 to September 25, 1974, and July 17 to August 10, 1975.
[b]Values are means (standard deviations) calculated from daily measurements of 10 devices (Table 3). See text for description of devices.
[c]Values are means (standard deviations) of 24 (1× and 10× tests, 1974), 18 (100× tests, 1974), or 33 (1975) daily measurements.
[d]Pipet with both ends open.
[e]Pipet with one end open.

TABLE 5. MEAN WEIGHT LOSSES[a] OF *exo*-BREVICOMIN (E), FRONTALIN (F), AND MYRCENE (M) FROM POLYETHYLENE CAPSULES AND GLASS TUBES MEASURED IN THE FIELD AFTER TWO PERIODS[b]

Device	Time (hr)	E (mg)	F (mg)	M (mg)
Polyethylene capsule	1530–1930	0.11 (0.06)	0.08 (0.06)	0.10 (0.07)
	1930–0730	0.11 (0.06)	0.09 (0.06)	0.17 (0.06)
Total		0.22 (0.08)	0.17 (0.09)	0.27 (0.11)
Glass tube	1530–1930	0.43 (0.12)	0.43 (0.12)	0.45 (0.14)
	1930–0730	0.41 (0.14)	0.42 (0.14)	0.58 (0.12)
Total		0.84 (0.16)	0.85 (0.12)	1.03 (0.13)

[a]Standard deviations in parentheses; nine of each device measured per day.
[b]September 7–11, 1974. See text for description of devices.

the center was interrupted by competing sources of attractant. Further study would be needed, however, to correlate reduction in catch at a baited trap with reduction in aggregation on trees low enough to protect them from mass attack. The greater catch at trees in a plot on treatment days—compared to nontreatment days—could be due to the increased level of pheromone attracting more beetles (Tilden et al., 1983) as well as the tendency for beetles to land on vertical silhouettes near sources of attractant (Bedard et al., unpublished observations). We have no measure of how many beetles may have landed on trees without traps or on portions of a tree not covered by the small trap.

During the one-day tests when attractants were released for 16 hr, beetles could disperse into the surrounding forest during the two-day hiatus in attractant release following treatment. Natural sources of attractant from trees under attack could, however, keep beetles in a plot and attract new beetles. During the long-term test, beetles probably did not disperse, and additional beetles could be attracted each day. In either case, with attractants released in a small area, beetles were concentrated instead of dispersed, which probably contributed to trees being attacked in the plots. This is a major problem in using a small plot to evaluate treatments to be applied over a large area. We also noticed that beetles sometimes bored into a tree behind the sticky trap, which may have increased the changes that the tree and neighboring trees would be successfully attacked and killed.

A lowered catch at the plot center occurred in our tests at total amounts of EFM of about 14–750 mg/16 hr/0.81 hectare (Table 4), which correspond to about 34–1850 mg/day/hectare. This assumes that the amount released in 24 hr is twice that released in 16 hr (Tilden, unpublished data). We speculate that many sources of attractant emitted over a large area at something near the lowest level tested might interrupt the response of *D. brevicomis* to initial attacks on ponderosa pines by dispersing the beetles over the treated area. Applying many devices releasing small amounts of attractant may also reduce the risk of producing sharp gradients in attractant, which could increase the probability of beetles attacking trees near the edges of areas of little or no attactant.

A large-scale interruption test would require aerial application of long-lasting chemical release devices (Furniss et al., 1981), which are not available for *D. brevicomis*. Important considerations in developing devices for deployment of attractants are their longevity, flatness of the emission decay curve, variation in release rates from individual devices due to environmental conditions, and the height above ground for deployment.

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EXTRACTIVES OF SEEDS OF THE MELIACEAE: EFFECTS ON *Spodoptera frugiperda* (J.E. Smith), *Acalymma vittatum* (F.), and *Artemia salina* Leach[1,2]

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Abstract—Hexane and ethanol extracts of seeds from 22 species of plants of the family Meliaceae from a number of countries were prepared. The extracts were submitted to antifeedant and toxicity bioassays utilizing fall armyworm [*Spodoptera frugiperda* (J.E. Smith)] (Lepidoptera: Noctuidae) larvae and striped cucumber beetle [*Acalymma vittatum* (F.)] (Coleoptera: Chrysomelidae) adults. Toxicity tests were also performed with brine shrimp, *Artemia salina* Leach. Feeding inhibition and mortality produced by some of these extracts were comparable to and, in certain cases, slightly greater than the effects produced by comparable neem (*Azadiracta indica* A. Juss.) seed preparations. Brine shrimp toxicity data do not extrapolate to insect activity, and vice versa.

Key Words—*Spodoptera frugiperda*, Lepidoptera, Noctuidae, *Acalymma vittatum*, Coleoptera, Chrysomelidae, brine shrimp, *Artemia salina*, insecticides, antifeedants, Meliaceae, neem, limonoids, azadiractin, seeds.

INTRODUCTION

While the search for natural pest control agents among plant "secondary metabolites" has extended into many different families of plants, the species currently receiving most worldwide attention is *Azadiracta indica* A. Juss. (neem

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tree, Meliaceae). Since Warthen (1979) summarized the literature describing the effects of neem preparations on various insects, many additional publications have appeared addressing various aspects of insect control using crude neem preparations as well as its principal active constituent, azadiractin. These reports encompass subjects ranging from effects on certain insects (Reed et al., 1982), to field trials on specific crops (Adhikary, 1984; Meisner et al., 1983; Mariappan and Saxena, 1983), to systemic-type greenhouse applications (Larew et al., 1985). A number of recent basic studies designed to show how these materials disrupt normal insect behavior and development have also been reported (Chiu et al., 1984; Ladd et al., 1984; Rembold, 1984; Zebitz, 1984; Saxena and Khan, 1985).

Although some 280 limonoids, including azadiractin, have been isolated from plants of the Meliaceae and identified (Taylor, 1984), new compounds from neem (Kubo et al., 1984) and certain other species (Nakatani et al., 1984, 1985) are still being discovered.

The molecular complexity of azadiractin precludes an economically feasible route of synthesis. In addition, the structure of this compound has been challenged and recent revisions proposed (Rembold, 1984; Bilton et al., 1985). Because of these factors, it seems likely that commercial development of products with these potent insect control properties will be based on neem tissues and extractives rather than on synthetic materials. Although current emphasis is on neem, other plants of the Meliaceae are widely distributed in tropical and subtropical areas of the world and may serve as sources of materials which are just as effective as those from neem. In addition, new compounds, perhaps simpler than azadiractin and therefore more amenable to chemical synthesis, may be found. If the suggestion made by Taylor (1984) that "indications are that insects may adapt to limonoids rather quickly" proves to be true, this approach may be even more meritorious.

Although the effects of neem seed and chinaberry (*Melia azedarach*) fruit extracts have recently been compared (Lee et al., 1985), the activity of other meliaceous plant materials compared to neem has not been investigated; hence, the current study was initiated to provide such a comparison. Effects of hexane and ethanol extracts of seeds from 21 Meliaceae species were compared to those of comparable neem seed extracts in bioassays utilizing the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), and the striped cucumber beetle (SCB), *Acalymma vittatum* (F.). Additional toxicity tests were done with brine shrimp (BS), *Artemia salina* Leach, to determine if this procedure (Meyer et al., 1982) might be useful as a screening technique.

METHODS AND MATERIALS

Extracts. Seeds (collected and identified by USDA botanists, Beltsville, Maryland) were ground to the consistency of commercial corn meal and were

Soxhlet extracted, first with *n*-hexane and then after drying, the meal, with 95% ethanol. Solvent was removed at 40°C on a rotary evaporator and the extracts were refrigerated.

FAW Two-Choice Antifeedant Bioassay. The procedure used was based on the leaf disk bioassay described by Kubo and Nakanishi (1977). Disposable Petri dishes (100 × 15 mm) were inverted, and a moistened filter paper disk large enough to cover the bottom was placed in each dish. Green bean leaf disks (1.0 cm diam.) were dipped for 5 sec either in a 5% (w/v) solution of the extract being tested or in solvent (control); ethanol extracts were redissolved in ethanol and hexane extracts in pentane. After allowing 5 min for solvent to evaporate, three sample-treated disks and three control disks were arranged alternately in each Petri dish around and about 0.5 cm inward from the circumference. Three FAW larvae that had been reared on pinto bean artificial diet (Shorey and Hale, 1965) for 7–11 days posteclosion and starved overnight were placed in the center of each Petri dish and were permitted to feed 2 hr (10- to 11-day-old larvae) or 3 hr (7- to 9-day-old larvae). Assays were conducted in darkness at 26–28°C and 55–65% relative humidity in three simultaneous replicates.

The percentage of each disk eaten was estimated visually, and feeding ratios, defined as percentages of extract-treated disks consumed/percentage of control disks consumed, were calculated. A feeding ratio of 1.00 means that equal quantities of treated and control leaf tissue were eaten (no deterrency), and a ratio of 0.25 or less was arbitrarily set as being indicative of strong feeding deterrency by that particular extract.

FAW No-Choice Toxicity Bioassay. Seed extracts were incorporated into pinto bean diet at the 1% (w/v) level. Ethanol extract (0.120 g), dissolved in 1 ml of ethanol, was placed in a shallow agate mortar, the solvent was allowed to evaporate, and the residue was mixed thoroughly with 12 cc of prepared diet. This mixture was packed into a 10-cc disposable hypodermic syringe barrel that had been cut off at the zero mark, and 1-cc plugs were extruded into each of 10 1-oz plastic cups.

Diet containing hexane extracts was prepared in the same manner except that a pentane solution of the sample was applied to the surface of 12 cc of diet spread out in the mortar and the solvent was allowed to evaporate prior to mixing. Control cups were set up identically using solvent only, and blank cups contained only diet that had not been exposed to either extract or solvent.

One 6-day-old FAW larva was introduced into each cup which was then capped with the usual paper lid, a layer of aluminum foil, and a plastic cap to minimize moisture loss. Bioassays were run at 26–28°C, 55–65% relative humidity, under a 16:8 light–dark cycle. Each larva was observed on day 4, 11, and 18, when the assay was terminated. Every group of 10 extracts (each in 10 replicates) was accompanied by three control sets and one blank set, also in 10 replicates.

BS Toxicity Bioassay. This bioassay was conducted basically as described

by Meyer et al. (1982), except that 4 mg of crude extract in either methanol or dichloromethane solution was applied to each paper disk and the disks were allowed to air dry. Mortality counts were made at 24 and 48 hr. Five replicates with 10 BS in each were done. If control deaths occurred, the bioassay was repeated.

SCB Two-Choice Leaf Disk Bioassays. The two-choice leaf disk antifeedant tests were performed with Burpee Hybrid cantaloupe leaves as reported by Reed et al. (1981, 1982). Appropriate quantities of the extracts were suspended in acetone, diluted with water containing 0.01% of Tween 20 to a sample concentration of 0.5% (w/v), and then emulsified with a Brinkman Polytron homogenizer. The 0.1% solutions were derived by dilution of the 0.5% solution. Leaf disks (2.0 cm diam.) were dipped in either the sample homogenate or a corresponding homogenate containing no sample (control disks), air dried, and then two of each type of disk were arranged alternately around 93-mm-diam. × 73-mm-deep polyethylene dishes. Five newly emerged female SCB, after being starved for 24 hr, were introduced into each chamber, and the chambers were covered with muslin; these covers were kept moist during the first 6 hr of the bioassay by misting them periodically with water. Tests were conducted in two replicates under ambient greenhouse conditions. Observations were made at 3, 6, and 22 hr to estimate visually the amount of leaf tissue consumed and to check for deaths.

Data are presented as a consumption index, which is defined as percentage of treated disks consumed × 100/(percentage of control disks consumed + percentage of treated disks consumed). A value of 50 indicates treated and untreated disks have been consumed in equal amounts; an extract that gives an index of 20 or less is considered highly deterrent in these bioassays.

SCB No-Choice Leaf Disk Bioassay: For the no-choice feeding study, extract samples were prepared (0.5% w/v solutions only), leaf disks (1.5 cm diam.) were treated and dried, and the insects used were as described for the two choice bioassay. Single treated disks were placed in individual 2-dram glass vials, and one SCB was introduced into each vial. Water was provided by a soaked 0.5-cm length of dental wick and the vials were stoppered with cotton plugs. The bioassays were done in 10 replicates under ambient greenhouse conditions; leaf consumption and mortality data were taken daily for three days. Data are presented as percent of leaf disks consumed.

RESULTS AND DISCUSSION

FAW Bioassays. Results of the FAW feeding experiments are presented in Table 1. Ethanol extracts of seeds from *Aglaia cordata*, *Azadiracta indica* (neem), *Dysoxylum malabaricum*, *D. reticulatum*, *D. spectabile*, *Melia dubia*, *Sandoricum koetjape*, *Swietenia mahogani*, *Trichilia connaroides*, *T. prieu-*

reana, and *T. roka* all exhibited comparable, high levels of activity in both bioassays. All of these extracts strongly inhibited feeding (feeding ratio <0.25) in the two-choice leaf disk bioassay and also resulted in high mortality (>80%) in the no-choice artificial diet bioassay. At the other extreme, ethanol extracts of *Aglaia odoratissima*, *M. azedarach*, *Toona sinensis*, and *Trichilia triphyllaria* had minimal antifeedant effects and produced no mortality over the time span of the experiment. However, of this group, only the insects on diets which contained *A. odoratissima* and *T. sinensis* extracts pupated normally.

Of the ethanol extracts giving intermediate results, those from *Chickrassia tabularis* and *D. binectariferum* produced moderately reduced feeding ratios of 0.52 and 0.36, but caused high mortalities of 100% and 90%, respectively, in the no-choice bioassay. Conversely, extracts of *Carapa procera*, *Lansium domesticum*, and *Swietenia macrophylla* seeds were highly deterrent (feeding ratios of <0.04), but produced <20% mortality when consumed in artificial diet. At termination of the bioassay, however, no insects in this group had pupated and all larvae were small.

Three of the hexane extracted seed oils, from *A. cordata*, *Chickrassia tabularis*, and *Sandoricum koetjape* were as potent as neem seed extract in both FAW tests. Very effective deterrency was achieved with oils from *Carapa procera*, *D. malabaricum*, *L. domesticum*, *M. dubia*, *Swietenia macrophylla*, and *S. mahogani*, but no more than 20% the test insects died in the no-choice bioassays. Overall pupation in this group of six bioassays was 80% compared to the controls at 18 days.

Oils resulting in intermediate mortality (30–60%) and low feeding ratios (<0.15) were from *D. binectariferum*, *D. reticulatum*, *M. toosendan*, and *T. prieureana*. In this case, only seven of the 23 survivors pupated.

Four seed oils, from *A. odoratissima*, *Toona sinensis*, *Trichilia connaroides*, and *T. triphyllaria*, had no apparent deleterious effects on the FAW larvae in either of the bioassays.

SCB Bioassays. Results of the SCB two-choice and no-choice leaf disk bioassays are given in Table 2. Two dose levels (leaf dipping solutions of 0.5% and 0.1%, w/v) of both the ethanol and hexane seed extracts were used. Data for the 3-hr observation of the two-choice test and for the two-day observation of the no-choice test were omitted because they increased the complexity of the table while contributing little to overall conclusions. However, certain unusual values for 3-hr observations are footnoted.

Only four of the ethanol extracts at the 0.5% level did not strongly inhibit feeding (consumption index <20) in the two-choice bioassay up to the 22 hr termination; they were from *L. domesticum*, *M. azedarach*, *Toona sinensis*, and *Trichilia triphyllaria* seeds. At the 0.1% level, only neem seed extract totally prevented feeding until 22 hr. However, other ethanol extracts that demonstrated good antifeedant properties against the SCB at this level were those from

TABLE 1. FALL ARMYWORM [*Spodoptera frugiperda* (J.E. SMITH)] AND BRINE SHRIMP (*Artemia salina* LEACH) BIOASSAY RESULTS FOR MELIACEAE SEED EXTRACTS

		Fall armyworm (FAW) bioassays				Brine shrimp (BS) bioassays			
		Two-choice test, feeding ratio[b]		No-choice test, % mortality		Mortality (%/24 hr)[c]		Mortality (%/48 hr)[c]	
Plant name	Seed origin[a]	Ethanol extract	Hexane extract	Ethanol extract	Hexane extract	Ethanol extract	Hexane extract	Ethanol extract	Hexane extract
Aglaia cordata Hiern.	T	0.16	0.10	100	100	100	46	—	96
A. odoratissima Blume	T	0.71	0.83	0	0	0	0	0	0
Azadiracta indica A. Juss.	I	0.00	0.22	80	100	0	0	18	0
Carapa procera DC.	G	0.02	0.14	0	0	0	0	0	0
Chickrassia tabularis A. Juss.	I	0.52	0.25	100	80	0	0	0	0
Dysoxylum binectariferum (Roxb.) Hook & Bedd.	I	0.36	0.14	90	40	100	0	—	52
D. malabaricum Bedd. ex C. DC.	I	0.08	0.05	100	0	100	0	—	0
D. reticulatum King	I	0.08	0.07	100	30	100	0	—	0

D. spectabile (Forst. F.) Hook F.	NZ	0.05	0.38	100	10	0	0	10	0
Lansium domesticum Corr.	T	0.03	0.02	0	20	0	0	0	0
Melia azedarach L.	P	0.71	0.83	0	30	0	0	0	14
M. burmanica Kerz.	I	0.14	0.75	60	20	100	0	—	0
M. dubia Cav.	I	0.01	0.21	100	10	96	0	100	10
M. toosendan Sieb. & Zucc.	I	0.09	0.09	60	60	0	0	0	0
Sandoricum koetjape (Burm. F.) Merr.	T	0.05	0.21	90	100	0	0	20	0
Swietenia macrophylla King	I	0.02	0.18	20	0	22	44	48	76
S. mahogani (L.) Jacq.	I	0.02	0.14	90	0	0	46	50	78
Toona sinensis A. Juss.	I	1.26	0.95	0	0	76	46	80	76
Trichilia connaroides (Wright & Am.)	I	0.00	0.73	80	0	100	0	—	0
T. prieureana A. Juss.	G	0.10	0.04	100	40	0	0	0	0
T. roka (Forsk.) Chiov.	G	0.00	0.31	100	0	100	0	—	0
T. triphyllaria C. DC.	U	0.54	1.15	0	0	0	0	12	0

[a] T = Thailand, I = India, G = Ghana, NZ = New Zealand, P = Pakistan, U = Uruguay.
[b] Feeding ratio = % treated disk consumed/% control disk consumed.
[c] Control mortality = 0%.

TABLE 2. STRIPED CUCUMBER BEETLE [*Acalymma vittatum* (F.)] FEEDING BIOASSAY RESULTS FOR MELIACEAE SEED EXTRACTS

Plant name	Two-choice test, consumption index[a,b]				No-choice test,[c] % consumed[d] (% mortality)	
	Ethanol extract		Hexane extract			
	0.5% Soln.	0.1% Soln.	0.5% Soln.	0.1% Soln.	Ethanol Extr.	Hexane Extr.
Aglaia cordata	0,0	0,13	0,0	11,27	8,29	6,18
A. odoratissima	0,0	17,14	68,55	39,58	25,58	54,85
Azadiracta indica	0,0	0,0	3,17	7,55	12,28	55,71
Carapa procera	0,4	6,9	33,48	69,50	20,80	—,—[e]
Chickrassia tabularis	0,0	18,15	0,0	0,31	15,38	6,26
Dysoxylum binectariferum	0,10	18,26	12,31	14,47	3,11	37,75
D. malabaricum	0,0	14,6	17,14	50,41[f]	5,34	28,87
D. reticulatum	0,12	10,36	52,51	85,65[g]	40,61(10)	6,77
D. spectabile	0,10	21,29	31,23	68,38[h]	15,64(20)	10,46
Lansium domesticum	0,24	5,44	7,36	26,43	7,18	41,75(10)
Melia azedarach	12,47	62,47[i]	19,48	41,51	16,54	61,75
M. burmanica	8,14	9,31	43,43	55,36[j]	6,25	18,37(30)

M. dubia	0,8	37,32	52,40	27,45	5,29	—,—[e]
M. toosendan	0,2	0,38	0,10	21,37	5,10	30,52(10)
Sandoricum koetjape	0,0	14,19	8,21	46,42	4,7	10,18(10)
Swietenia macrophylla	0,9	23,36	5,37	52,39	5,50	—,—[e]
S. mahogani	0,0	0,13	0,50	29,35	3,13	—,—[e]
Toona sinensis	54,28	19,43	27,41	69,56[k]	—,—[e]	—,—[e]
Trichilia connaroides	0,7	31,28	8,19	63,58[l]	25,51	30,73
T. prieureana	0,0	33,39	52,51	43,45	4,18	—,—[e]
T. roka	0,5	10,14	32,42	37,42	2,5	—,—[e]
T. triphyllaria	4,29	40,48	60,50	59,44	—,—[e]	—,—[e]

[a]Consumption index = % treated disk consumed × 100/(% control disk consumed + % treated disk consumed).
[b]Data shown are for 6,22 hr observations.
[c]0.5% solution used; control values = 31,74(0) (means of three sets of 10 replicates).
[d]Data shown are for 1,3 day observations.
[e]Experiments not done.
[f]3 hr = 0.
[g]3 hr = 81.
[h]3 hr = 69.
[i]3 hr = 68.
[j]3 hr = 58.
[k]3 hr = 71.
[l]3 hr = 90.

both *Aglaia* species, *Carapa procera*, *Chickrassia tabularis*, *D. malabaricum*, *Sandoricum koetjape*, *Swietenia mahogani*, and *T. roka*.

The hexane extracts, as a group, possessed considerably lower antifeedant activity with the SCB than did the ethanol extracts. Good feeding inhibition held to 22 hr in the 0.5% level two-choice bioassay for extracts of *A. cordata*, neem, *C. tabularis*, *D. malabaricum*, *M. toosendan*, and *T. connaroides* seeds. Activity observed with a number of other hexane extracts to 6 hr did not persist until 22 hr. At the 0.1% concentration, only *A. cordata*, neem, *C. tabularis*, and *D. binectariferum* inhibited feeding and then only to 6 hr; *A. cordata* and *C. tabularis* gave the lowest consumption indexes of this group.

The SCB no-choice leaf disk feeding bioassays were conducted using only 0.5% solutions of the ethanol and hexane seed extracts. Ethanol extracts from *D. binectariferum*, *L. domesticum*, *M. toosendan*, *Sandoricum koetjape*, *Swietenia mahogani*, *T. prieureana*, and *T. roka* gave good antifeedant activity up to the three-day limit of the test. All of these appeared more effective than neem extract in this bioassay.

High deterrency in the no-choice bioassay was provided by only two hexane extracts, those from *A. cordata* and *Sandoricum koetjape*, until three days, although *C. tabularis* extract allowed only 26% of the leaf disks to be consumed in three days.

Some insect mortality was observed in these SCB bioassays with two ethanol extracts and four hexane extracts; the greatest mortality (30%) was caused by the hexane extract of *M. burmanica*.

BS Bioassay. Results of the BS toxicity studies (Table 1) indicate that the ethanol extracts, with exception of those from the two *Swietenia* species, tend to kill most or all of the shrimp or to have little or no effect. Three *Dysoxylum* species ethanol extracts caused 100% mortality while that of *D. spectabile* caused none. Similarly, ethanol extracts of two of the four *Trichilia* species killed all of the organisms while those from the other two species were without effect.

Hexane extracts of *A. cordata*, *S. macrophylla*, *S. mahogani*, and *Toona sinensis* exhibited moderate toxicity at 24 hr and the values increased at 48 hr. In spite of the fact that the bioassay results are reproducible, the relative lack of activity observed with hexane extracts may be partially attributable to their reduced solubility in the aqueous system.

The data in Tables 1 and 2 also reveal that activity in the BS system cannot be extrapolated into similar effects on FAW or SCB, or vice versa. Hence, if one is searching for insect-active materials, this BS bioassay cannot be used as the sole screen. Once the presence of insect activity has been established in any particular extract, and if BS respond similarly, then in some cases this procedure might find limited use as a rapid, economical monitor for subsequent fractionation. However, appropriate insect bioassays would still be required to reliably

document that the BS toxic entities being concentrated produce the desired effect in the target insect.

SUMMARY

Some limonoids, isolated from a number of Meliaceae species listed in Table 1, have been investigated (Taylor, 1984), although not usually from an insect activity standpoint. Species of those listed in Table 1 that have received the greatest attention, in addition to neem, are *C. procera*, *M. azedarach* (chinaberry), and *S. macrophylla*. More recently, the properties and structures of trichilins, potent limonoid antifeedants from *T. roka* root bark, have been investigated (Nakatani et al., 1984, 1985). The ethanol extract of *T. roka* seeds, also is very potent, as seen in Tables 1 and 2.

Components of chinaberry (*M. azedarach*) fruit have been reported to possess antifeedant (Lavie et al., 1967) and insecticidal (Lee et al., 1985) properties; however, extracts from our seed sample were virtually devoid of activity in all bioassays. We feel that this anomaly is due to unknown characteristics inherent in the particular seed sample we received and that usually plant material from this species will show activity. Other extracts producing minimal overall effects in this group of bioassays were those of *A. odoratissima*, *Toona sinensis*, and *Trichilia triphyllaria*.

Azadiractin (and probably some other limonoids) have significant solubility in lipophylic solvents such as hexane, and this property probably accounts for the observed potent activity in both extracts of some seeds. However, little or no activity is detected in hexane extracts of other seeds that show strong ethanol-extractable activity; this variation may indicate the presence of types of active materials different from azadiractin, and these extracts warrant further investigation from this standpoint. Lack of stability of a number of limonoids (Taylor, 1984), particularly in solution (Zanno et al., 1975), should be borne in mind when comparing relative magnitudes of activities reported.

Thus, we have demonstrated that seed extracts of plants of the Meliaceae produce effects ranging from none to total inhibition of feeding in both the FAW and SCB and 100% mortality in the FAW. Although seeds are the subject of this communication, there is reason to expect that other plant parts would also be good sources of similar activity. A few extracts, notably those from *A. cordata*, *Chickrassia tabularis*, and *Sandoricum koetjape*, exhibit overall total activity in these bioassays exceeding that of neem seed extracts. Further biological testing is continuing with a number of these extracts; isolation and chemical identification of active components are also being initiated in our laboratory.

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KAIROMONE-MEDIATED HOST FINDING BY SPRUCE BUDWORM EGG PARASITE, *Trichogramma minutum*

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Abstract—The host-finding responses of the egg parasite, *Trichogramma minutum* Riley, reared from its natural host, *Choristoneura fumiferana* (Clemens) and a laboratory host, *Sitotroga cerealella* (Oliver), to hexane extracts of scales of these hosts were compared in laboratory experiments. When presented with *C. fumiferana* eggs on filter papers treated with hexane and hexane extract of grain moth scales, egg finding by *T. minutum* was random. When presented with budworm eggs on filter papers treated with hexane and hexane extract of budworm scales, *T. minutum* found significantly more eggs on extract-treated filter papers, indicating the presence of kairomones on the scales of spruce budworms. *T. minutum* responded to kairomone-treated patches with a localized search behavior characterized by intense antennal tapping, decreased locomotion and increased turning within the patch, and repeated returns to the patch. In the absence of successful host finding, this response slowly waned. No significant difference in behavior of *T. minutum* reared on either *C. fumiferana* or *S. cerealella* was observed.

Key Words—*Trichogramma minutum*, Hymenoptera, Trichogrammatidae, *Choristoneura fumiferana*, Lepidoptera, Tortricidae, moth scale, kairomone, mass rearing, parasitism.

INTRODUCTION

The most important insect pest in the spruce–fir forests of northeastern North America is the spruce budworm (SBW), *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). Outbreaks of this pest currently are treated with aerial applications of chemical insecticides or *Bacillus thuringiensis* Berl. (Bt). While both of these are effective, Bt is relatively expensive and alternatives of chemical insecticides are desirable in sensitive areas such as parklands and

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aquatic environments. As such areas may contain tree stands of considerable value, other methods are being explored to provide protection against outbreaks of SBW. The naturally occurring egg parasite of SBW, *Trichogramma minutum* Riley (TM) (Hymenoptera: Trichogrammatidae), is being evaluated as an inundative control agent and is most economically reared on eggs of the facultative host, the Angoumois grain moth (AGM), *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelichiidae) (Houseweart et al., 1984; Smith, 1984).

During preliminary field trials with TM in Ontario, Smith (1984) observed that parasites reared in the facultative host appeared to parasitize fewer spruce budworm eggs after release than did their "wild" progeny. One of several possible explanations for this observation is that extended rearing on AGM reduced the parasites' ability to locate natural hosts in the wild. Kairomones have repeatedly been shown to act as critical cues in host finding (Lewis et al., 1976), and previous studies have demonstrated that kairomones on moth scales play an important role in guiding *Trichogramma* spp. to their hosts in agroecosystems (Lewis et al., 1971, 1972; Nordlund et al., 1981). Although it has been suggested that extended rearing on an alternate host can influence host acceptance or the host preference of a parasite (Narayanan and Subba Rao, 1955; Taylor and Stern, 1971), little is known of the host's influence on kairomone utilization by a parasite during host finding. The possibility that extended rearing on AGM may impair the host finding ability of TM could influence the choice of hosts on which the parasite is reared for use in inundative releases against SBW. The objectives of this study were to determine if *T. minutum* uses scale kairomones while searching for SBW hosts, to determine the behavioral response of TM to these kairomones, and to determine if this behavioral response is affected by extended rearing on the facultative host, AGM.

METHODS AND MATERIALS

Cultures of Trichogramma minutum. The colony of the TM strain used in this study was established at the Biological Control Laboratory, University of Guelph, from parasitized eggs of SBW collected near Hearst, Ontario, in 1983, and was reared for 21 generations on AGM. Subsequently, two smaller experimental cultures were established from this parent colony; one was reared continuously on AGM and the other was reared continuously on SBW. Eggs of AGM were obtained from the Biological Control Laboratory at the University of Guelph, and eggs of SBW were obtained from the Great Lakes Forest Research Centre (GLFRC), Sault Ste. Marie, Ontario. All eggs were refrigerated until exposed to parasites. Eggs were glued with wallpaper paste to strips of white construction paper (2 × 8 cm), killed by exposure to ultraviolet light (254 nm) for 30 to 40 min, then exposed to adult TM in corked glass vials for 24 hr. Parasitized egg cards were then transferred to clean glass vials and placed

in a growth room at 25 ± 1°C and 16:8 hr light–dark. Relative humidity was maintained at 75% by keeping the vials over saturated NaCl solutions in covered clear plastic vegetable crispers (Winston and Bates, 1960). Under these conditions, adult parasites emerged 10–12 days after host eggs were parasitized. All parasites used in the experiments were 0- to 24-hr-old females.

Scale Extracts. Scales of SBW were collected from air filters at GLFRC and scales of AGM were collected from an egg/scale separator in the Biological Control Laboratory. Weighed scales were placed in a coarse glass fritted funnel and washed with 5 ml of hexane/100 mg of scales. The washings were concentrated under reduced pressure and stored at −20°C. Solutions of 10 μg scales/ml were prepared from these stock solutions by dilution with hexane, as needed.

Bioassays. Filter papers (1.5 × 1.5 cm Whatman No. 1) were treated with 25 μl of hexane alone (control) or one of the hexane extracts (treatment) for use in bioassays. After air-drying for 30 min, an egg of SBW was attached to each filter paper at its center with shellac gel. Both a treated and a control filter paper were placed in a 5.5-cm-diameter plastic Petri dish with a tight-fitting lid. An AGM-reared or SBW-reared TM was placed on the Petri dish lid and allowed to search until it made contact with one of the eggs. The choice of egg found by the parasite was recorded, and the filter papers and TM were replaced. Parasites that flew before finding an egg or remained inactive for 15 min were discarded.

The experiment was repeated a minimum of 60 times for each of the four combinations of filter paper treatment and host of rearing. Data collected on different days were pooled after appropriate tests for homogeneity. The hypothesis that eggs finding was independent of filter paper treatment was tested for each of the four host of rearing/extract combinations using an adjusted chi-square test. The hypothesis that the responses of SBW-reared and AGM-reared TM did not differ was tested for each of the scale extracts using an interaction chi-square test.

Behavioral response experiments were conducted on 11-cm-diameter filter papers inscribed in pencil with three concentric circles of 5, 7, and 10 cm diameter. The area within the inner circle was treated with 400 μl of either hexane (control) or SBW extract (treatment) to form a patch. The area between the two inner circles was designated the patch boundary, and the area between the two circles was designated the arena. A single female TM, reared in either AGM or SBW, was released in the center of the patch and the total time spent by the parasite in each of the three regions before first leaving the arena was recorded. In addition, the number of times that TM left and returned to the patch before going beyond the patch boundary was recorded as patch reentries. Parasites that flew before leaving the arena or remained inactive for 15 sec were discarded.

The experiment was repeated nine times for each of the four hosts of rearing/patch treatment combinations. The data were arranged and analyzed as a three-factor (host of rearing/patch treatment/filter paper region) completely ran-

dom design experiment after applying a log $(X + 1)$ transformation to stabilized variance. Hypotheses tested were that there was no interaction between factors, no difference in response due to host of rearing, no difference in response due to patch treatment, and no difference in response between the three filter paper regions. Mean numbers of patch reentries for each of the four host of rearing/ patch treatment combinations were calculated and compared.

RESULTS

Free-Choice Experiments. When presented with eggs attached to hexane-treated and AGM scale extract-treated filter papers, both AGM-reared and SBW-reared TM found eggs randomly (Table 1). However, when presented with eggs attached to hexane-treated and SBW scale extract-treated filter papers, both AGM-reared and SBW-reared TM found significantly more eggs ($P < 0.05$) on the extract-treated filter papers (Table 1). The proportion of eggs found on extract-treated filter papers by AGM-reared TM was not significantly different from that found by SBW-reared TM ($\chi^2 = 1.26$, 1 df).

The parasites continuously tapped the substrate with their antennae. Travel by TM was relatively fast and with few turns until it made first physical contact with the SBW scale extract-treated filter paper. When this happened, the TM would usually pause briefly, tapping the filter paper intently with its antennae. Then it would begin an investigation of the filter paper that was characterized by considerably reduced speed and frequent pauses and turns. Although a similar response was not observed on the hexane-treated AGM scale-treated filter papers, TM passing within 2–3 mm of a SBW egg would pause, turn, and slowly advance towards the egg while TM passing slightly farther away would not.

Behavioral Response Experiments. The times spent per unit area for AGM-reared and SBW-reared TM were combined when preliminary analysis showed

TABLE 1. FILTER PAPER TREATMENT OF EGG FIRST FOUND BY *Trichogramma minutum* FEMALES IN FREE-CHOICE EXPERIMENTS

TM host of rearing	N	Distribution of first-found hosts[a]		
		Hexane	AGM extract	SBW extract
AGM	131	60	71	—
SBW	96	45	51	—
AGM	100	37	—	63*
SBW	60	17	—	43**

[a]Distribution significantly different from random by χ^2 test at $P \leq 0.05$* or $P \leq 0.01$**.

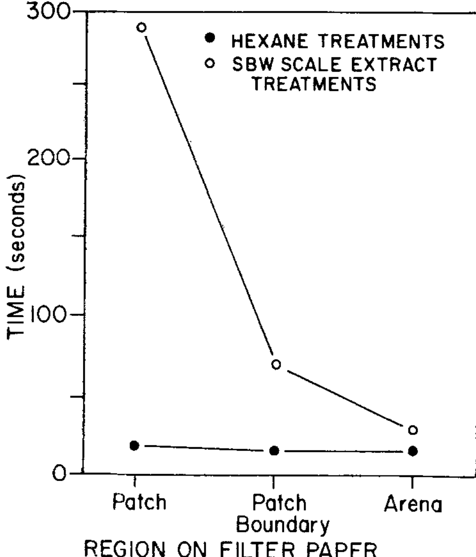

FIG. 1. Mean times spent on filter paper regions by *T. minutum* females released on hexane-treated and spruce budworm scale extract-treated patches. Each mean calculated after log $(X + 1)$ transformation of raw data $(N = 18)$.

that they did not differ significantly. Analysis of variance of the transformed data showed that, while both the patch treatment and filter paper region appeared to influence the parasites' movements, a significant interaction ($P < 0.001$) existed between these two factors.

Examination of simple effects revealed that treatment of the patch with SBW scale extract resulted in significantly more ($P < 0.001$) time being spent in each of the regions compared to the control (Figure 1). In addition, on the SBW scale extract-treated filter paper, times spent in each region differed significantly ($P < 0.001$), with the most time being spent in the patch and the least time being spent in the arena. In contrast, on hexane-treated filter papers, times spent in the boundary and the arena did not differ significantly and time spent in the patch was only slightly greater ($P < 0.05$), a difference that can be attributed to the slightly larger area of the patch. On extract-treated filter papers, AGM-reared TM made 5.0 ± 3.5 patch reentries and SBW-reared TM made 4.9 ± 2.5 patch reentries (Figure 2). No patch reentries were observed on hexane-treated filter papers.

As in the free-choice experiments, it was observed that TM traveled more slowly and with more frequent pauses and turns on SBW scale extract-treated patches than on other surfaces. Observations under a dissecting microscope revealed that TM on extract-treated patches also examined the substrate much

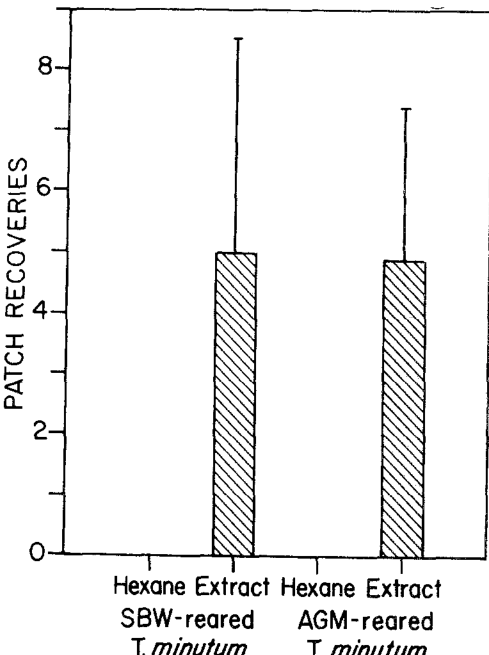

FIG. 2. Mean number of patch recoveries by Angoumois grain moth-reared and spruce budworm-reared *T. minutum* on hexane-treated and spruce budworm scale extract-treated filter papers. (SD = standard deviation; $N = 9$ for each treatment).

more intensely with their antennae than TM on hexane-treated patches. Furthermore, the response on the extract-treated patches was not uniform with time. Travel was slowest, and pauses and turns were most frequent, immediately after contacting the treated patch. As time spent on the patch increased, turns and pauses became less frequent and speed increased, with the result being that an area of increasing size was searched. At first, when a TM wandered off the patch it would stop and return to the patch, but eventually it abandoned the patch completely (i.e., left the arena). Tracings of the movements of AGM-reared and SBW-reared TM on filter papers with hexane-treated and SBW scale extract-treated patches are illustrated in Figure 3.

DISCUSSION

The chemical orientation of insect parasites to their environment during host selection has been extensively studied, and the process has been divided into a number of steps, including host habitat finding, host finding, and host acceptance (Vinson, 1976). In the host-finding step, a parasite can greatly in-

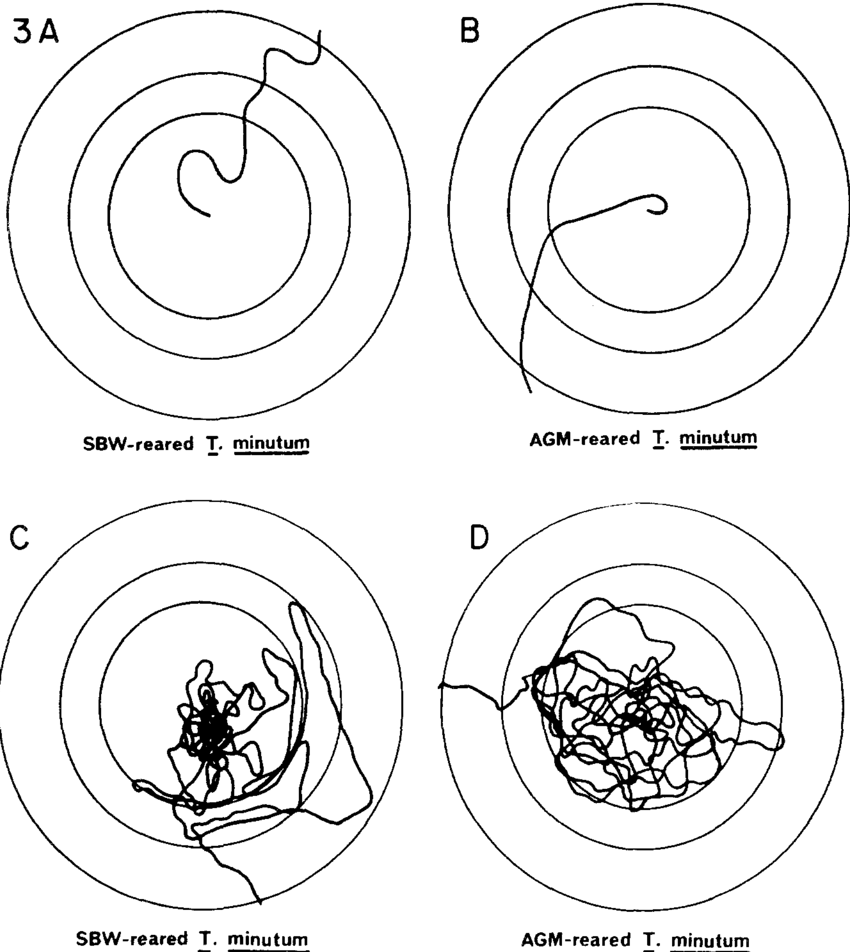

FIG. 3. Tracing of responses of SBW-reared and AGM-reared *T. minutum* to hexane and scale extract-treated filter papers. (A) Response of SBW-reared to hexane, (B) response of AGM-reared to hexane, (C) response of SBW-reared to SBW scale extract, (D) response of AGM-reared to SBW scale extract.

crease the efficiency of its search if it can identify and concentrate its efforts in those sites most likely to contain hosts. Our study suggests that TM uses SBW scale kairomones for this purpose.

Naturally occurring eggs of SBW would often be accompanied by a patch of scales left by the ovipositing moth, thus the behavioral response exhibited by *T. minutum* upon encountering kairomone patches of scale extract can be expected to substantially improve the parasite's searching efficiency. The localized nature of the response allows the parasite to concentrate its search in

small areas likely to harbor hosts. At the same time, the tendency for the intensity of the response to wane allows the parasite to quickly delimit and search the area of the patch, and ultimately allows it to escape unproductive patches. Similar reduction of response has been reported by Waage (1978) in studies on the arrestment behavior of *Nemeritis canescens* (Grav) responding to contact kairomones produced by *Plodia interpunctella* (Hübner). Therefore, such behaviors may be common among parasite species.

T. minutum continuously taps the substrate with its antennae and only shows a behavioral response to the scale extract of SBW after making physical antennal contact with the treated filter paper. This suggests that the kairomone of SBW is a contact chemical that is detected with receptors on the parasite's antennae. Vinson (1976) discussed the importance of contact chemicals in both host location (finding) and host selection (acceptance) and reviewed their use by several parasites. The behavior exhibited by TM in response to the scale extract of SBW is similar to that reported for *Trichogramma pretiosum* (Riley) on patches of scale extract of the corn earworm, *Heliothis zea* (Boddie) (Beevers et al., 1981), as well as for a number of other parasitoids that use contact kairomones. Bell and Tobin (1982) suggest that reduced locomotion and increased turning within patches, accompanied by large abrupt turns at the patch edge, serve to arrest an animal's motion and lock it into a patch.

In the absence of the kairomone of SBW, TM turns towards eggs on hexane-treated filter papers only if it passes very near them. This is similar to the observation of Laing (1937), who found that sight was the sense by which *Trichogramma evanescens* Westwood perceived hosts over short distances. Together with the observation that *T. minutum* would frequently search a kairomone-treated filter paper only to leave without finding the attached egg, this suggests that TM relies on sight to find hosts once it is within a kairomone patch. If this is so, the increased rate of turning observed within kairomone patches would increase the probability of passing within sight of a host.

Differences have been noted between TM reared on more than one host, but whether these are due to host influence or to strain differences is sometimes unclear. Southard et al. (1982) found that the Maine strain of TM reared in AGM were significantly smaller than those reared in SBW. Houseweart et al. (1983) showed that the Maine strain of TM had lower daily progeny production when reared in AGM eggs, but that total progeny production for parasites reared on AGM and SBW eggs was the same, mainly due to differences in longevity. Houseweart et al. (1984) found that the Maine strain of TM reared on AGM significantly out-performed a strain from California originally collected from *Cydia pomonella* (Walsh) and subsequently reared on AGM. This difference was probably due to the strain of TM, not due to a host influence.

There have been suggestions that extended rearing on an alternate host can change the host preference of a parasite. Narayanan and Subba Rao (1955) reported that *Microbracon gelechiae* Ashmead, bred on gram flour moth for

seven years, developed into a distinct race which showed preference for its new host over its natural host, the potato tuber worm, *Gnorimoschema operculella* (Zeller). Unfortunately, they did not compare these data with parasites reared continuously on the natural host. Taylor and Stern (1971) reported a complete reversal of host preference by *Trichogramma semifumatum* (Perkins) reared for 100 generations on AGM, but their interpretation was based on data comparing parasites from what were probably two strains of quite different origin. Reliable interpretation of the influence of the host-selection behavior of parasites can only be made if parasites of one defined strain are reared on different hosts under otherwise identical conditions.

Our experiments show that utilization of SBW kairomone by a defined strain of TM was not different between parasites reared on SBW and parasites reared on AGM. Furthermore, data for these experiments were homogeneous over the 7–10 generations that the parasites were reared on each host, indicating that there was no cumulative influence of the host over this period. Based on these conclusions, the observed difference in levels of parasitism of SBW between generations of TM and their progeny after their release cannot be attributed to an influence of the host on scale kairomone utilization. Other possible explanations are that "wild" parasites are more vigorous than laboratory-reared parasites or simply that there were more parasites in the progeny generation than in the released generation. Laboratory rearing of TM on AGM does not appear to alter the ability of TM to utilize kairomones of SBW.

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ALDRIN EPOXIDASE ACTIVITY AND CYTOCHROME P-450 CONTENT OF SAWFLY LARVAE, *Pergagrapta polita* Leach (Hymenoptera: Pergidae) FEEDING ON TWO *Eucalyptus* SPECIES

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Abstract—Aldrin epoxidase and cytochrome P-450 levels were determined in sawfly larvae, *Pergagrapta polita* Leach. Of the tissues examined the anterior portion of the midgut had the highest levels of aldrin epoxidase activity and cytochrome P-450 content, 3.56 nmol dieldrin produced/min/mg protein and 1.28 nmol/mg protein, respectively. No significant differences in aldrin epoxidase activities were observed between groups of larvae representing the last three larval instars and between larvae feeding on two eucalypt species.

Key Words—Sawfly larvae, *Pergagrapta polita*, Hymenoptera, Pergidae, *Eucalyptus* spp., monooxygenases, aldrin epoxidase, cytochrome P-450, allelochemicals, induction, polyphagy.

INTRODUCTION

Monooxygenases convert lipophilic foreign compounds to more polar products that can be excreted easily (Kulkarni and Hodgson, 1980). These enzymes are also used by insects in the detoxification of secondary plant compounds, or allelochemicals, many of which are toxic (Brattsten, 1983).

In Hymenoptera, the honeybee, *Apis mellifera* L., is very susceptible to insecticides (Metcalf and Fukuto, 1965), and Metcalf et al. (1966) suggested that monooxygenases may not even be present among Hymenoptera. Gilbert and Wilkinson (1974) and Yu et al. (1984), however, showed there was some activity in honeybees, while Croft and Mullia (1984) showed that an ectoparasitic hymenopterous species also had some activity. In addition, Krieger et al. (1970) reported monooxygenase levels in a sawfly, *Macremphytus varianus* (Norton).

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In general, pergid (sawfly) larvae are monophagous feeders, i.e., they feed on hosts contained within one plant family. In Australia, a number of sawflies feed on *Eucalyptus* spp. (Riek, 1970) which have numerous allelochemicals, especially monoterpenes (Morrow and Fox, 1980). Such compounds have been shown to induce monooxygenases in lepidopterous larvae (Brattsten et al., 1977; Moldenke et al., 1983). Also in lepidopterous larvae, it was shown by Krieger et al. (1971) that polyphagous feeders had much higher monooxygenase activity than monophagous species.

It was therefore of interest to study the monooxygenase system of a monophagous hymenopterous species feeding on hosts containing monoterpenes. The species used was *Pergagrapta polita* Leach, chosen because of its large size and local availability. The activity of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) dependent aldrin epoxidase in various tissues was used as an index of monooxygenase activity. Aldrin was chosen as the substrate as it has been used widely in comparative metabolic studies.

METHODS AND MATERIALS

Insects. Larvae of *P. polita* were collected in September and October 1983 from *Eucalyptus globulus* and *E. parramattensis* growing in the grounds of Macquarie University, Sydney, New South Wales, Australia. Larvae were brought into the laboratory and used immediately.

Chemicals. Glucose-6-phosphate (G6-P), G6-P dehydrogenase, NADP (Boehringer Mannheim), and analytical grade aldrin and dieldrin (Shell Chemical Company, Australia) were purchased. Other chemicals and solvents were of analytical grade.

Enzyme Preparations. Larvae (in groups of one or more, depending on size), were decapitated and the posterior tip of the abdomen removed with scissors. The gut was dissected out, incised longitudinally, freed of its contents, and the various gut portions rinsed in ice-cold 0.1 M phosphate buffer, pH 7.5 (this was the buffer used throughout the study). Fat body was obtained after removal of the gut and slitting the body wall longitudinally. Carcass was also used and is defined as the tissues of the body midsection minus fat body and gut, i.e., essentially integument and muscle. Tissues were ground in a glass tube with ice-cold buffer using a motor-driven Teflon pestle. The homogenate was filtered through cheesecloth and was known as crude homogenate.

Microsomal pellets were prepared by centrifuging crude homogenates for 15 min at 4°C, at 10,000*g*, in an MSE centrifuge. The resulting supernatants were centrifuged for 60 min at 4°C, at 100,000*g*, in an IEC ultracentrifuge. Each microsomal pellet was surface-rinsed and resuspended in buffer.

The protein content of preparations was determined by the method of Lowry et al. (1951) before assays were undertaken.

Enzyme Assays. The aldrin epoxidase assay used was similar to that of Yu

(1982) and Rose (1984). The 5-ml incubation mixture contained buffer, 1.1 μmol NADP, 12.1 μmol G6-P, 2 units of G6-P dehydrogenase, either crude homogenate containing 2 ± 0.2 mg protein homogenate or microsomal suspension containing 1 ± 0.1 mg protein, and 274 nmol of aldrin in 0.1 ml methyl cellosolve to initiate the reaction. The incubations were carried out with shaking at 35°C for 15 min. These conditions were optimal with respect to midgut preparations and were used for all tissues. The reaction was stopped with 2 ml of 3 N HCl. Hexane extracts (10 ml) of the reacted homogenates were then analyzed by electron gas chromatography using a Varian model 3700 equipped with a ^{63}Ni electron capture detector. The glass column, 2.0 m long × 2 mm inside diameter was packed with 5% OV-210 on chromosorb WHP 80-100 mesh. The retention time of dieldrin was 2.9 min under the following conditions: injector temperature 210°C, column temperature 181°C, detector temperature 230°C, N_2 flow rate 86 ml/min. The detection limit of dieldrin was 5 pg, and the amount of dieldrin was quantified by means of the peak height from standard solutions.

Cytochrome P-450 levels in the microsomal suspension were measured by the method of Omura and Sato (1964). Spectra were recorded on a Varian Superscan 3 spectrophotometer.

Some results were analyzed stastically. In such cases data were log transformed and examined by analysis of variance.

RESULTS

Midguts of sawfly larvae consist of anterior and posterior portions separated by gastric cecae (Maxwell, 1955). Aldrin epoxidase activities of crude homogenates are shown in Table 1. Activity was highest in the anterior portion of the midgut. Fat body homogenates were less active than midgut preparations

TABLE 1. ALDRIN EPOXIDASE ACTIVITY OF CRUDE HOMOGENATES OF DIFFERENT TISSUES

	Picomoles dieldrin/min[a]		
Tissue	Per mg protein	Per mg individual	*N*
Fat body	114.5 ± 25.0	0.079 ± 0.028	4
Foregut	1.8 ± 1.0	0.0005 ± 0.0004	2
Anterior midgut	1667.0 ± 136.0	2.286 ± 0.517	4
Posterior midgut	338.4 ± 60.7	0.581 ± 0.155	4
Hindgut	10.1 ± 1.4	0.0022 ± 0.0008	2
Carcass[b]	1.7 ± 0.9	0.0060 ± 0.0029	2

[a] Mean ± SD of *N* homogenates.
[b] Tissues of body midsection minus fat body and gut.

TABLE 2. ALDRIN EPOXIDASE ACTIVITY OF CRUDE AND MICROSOMAL HOMOGENATES AND CYTOCHROME P-450 CONTENT IN MIDGUT PREPARATIONS

	Crude homogenate		Microsomes			
	Picomoles dieldrin/min[a]		Picomoles dieldrin/min[a]		Cytochrome P-450 content[a]	
Tissue	Per mg protein	Per mg individual	Per mg protein	Per mg individual	(nmol/mg protein)[a]	N
Total midgut	1102 ± 61	4.685 ± 0.302	1698 ± 490	1.064 ± 0.296	0.727 ± 0.258	3
Anterior midgut	1915 ± 215	3.537 ± 0.560	3562 ± 154	1.304 ± 0.220	1.277 ± 0.354	2
Posterior midgut	635 ± 163	1.043 ± 0.252	1400 ± 433	0.292 ± 0.101	0.292 ± 0.012	2

[a] Mean ± SD of N homogenates.

TABLE 3. ALDRIN EPOXIDASE ACTIVITY OF CRUDE MIDGUT HOMOGENATES FROM VARIOUSLY AGED LARVAE

Weight of individuals (mg ± SD)	Width of head capsule[a] (mm ± SD)	Picomoles dieldrin/min[b]	
		Per mg protein	Per mg individual
1011 ± 5	3.81 ± 0.21	1164 ± 181	4.829 ± 1.222
1442 ± 38	4.45 ± 0.13	1137 ± 157	3.462 ± 0.891
2356 ± 17	4.95 ± 0.14	1247 ± 210	4.283 ± 1.270

[a] Distance between the outer edges of the head capsule at the point where three arms of the epicranial suture intersect.
[b] Mean ± SD of three homogenates.

and both the fore- and hindgut had only negligible activity. The remainder of this study deals with midgut preparations only.

Aldrin epoxidase activities of crude homogenates and microsomes of total midgut and of the two midgut portions are shown in Table 2. On a per milligram protein basis, microsomal activity increased from 1.5- to 2.2-fold over crude homogenates, and on a per milligram individual basis, activity in crude homogenates was 2.7- to 4.4-fold higher than in microsomes. Cytochrome P-450 was greatest in the anterior midgut portion.

The relationship between the age of larvae and aldrin epoxidase activity was investigated (Table 3). Groups of larvae collected from the same host tree were sorted into similar sized individuals before assays were undertaken. The life cycle of this species is unknown, but with another related species, *Perga dorsalis* Leach, when the head capsule widths are plotted against the various instars, a straight line results (A.D. Clift, unpublished observations). Using this criterion for *P. polita*, it would appear that the final three instars were represented in Table 3. An analysis of the data revealed no significant diffences in activities among the groups either on a milligram protein basis or milligram individual basis ($P > 0.05$).

P. polita larvae were collected on three occasions from different trees of *E. parramattensis* and on one occasion from two different trees on *E. globulus*. Analysis showed no significant differences in aldrin epoxidase activities (Table 4) of crude midgut homogenates ($P > 0.05$).

DISCUSSION

Previous monooxygenase studies with larval Hymenoptera have shown that species have only low to moderate activity when compared with other insect species. Whole-body homogenates were used to determine aldrin epoxidase levels in both drone honeybee larvae (Gilbert and Wilkinson, 1974) and in larvae

TABLE 4. ALDRIN EPOXIDASE ACTIVITY OF CRUDE MIDGUT HOMOGENATES OF LARVAE COLLECTED FROM TWO *Eucalyptus* SPECIES

	Picomoles dieldrin/min[a]		
Host	Per mg protein	Per mg individual	*N*
Eucalyptus	1175 ± 348	2.926 ± 2.918	3
parramattensis	1247 ± 210	4.283 ± 1.270	3
	1102 ± 61	4.685 ± 0.302	3
Eucalyptus	925 ± 280	3.046 + 0.998	4
globulus	1074 ± 122	3.291 ± 0.500	4

[a]Mean ± SD of *N* homogenates.

of a braconid parasite, *Oncophanes americanus* (Weed) (Croft and Mullin, 1984). Aldrin epoxidase activities were 231 and 0.6 pmol dieldrin/min/mg protein, respectively. Krieger et al. (1970) studied various tissues in a sawfly, *Macremphytus varianus*, and found that microsomes prepared from a gut homogenate yielded an aldrin epoxidase activity of 263 pmol dieldrin/min/mg protein. They found that homogenates prepared from head capsule, fat body, and skeletal tissues were inactive.

My study shows that all tissues examined exhibited aldrin epoxidase activity, with midgut and fat body preparations having the highest activities (Table 1). These results are similar to a number of lepidopterous larvae where gut preparations have higher activities than fat body (e.g., Krieger and Wilkinson, 1969; Krieger et al., 1976; Rose, 1984). Carcass activities of lepidopterous larvae are higher than those reported here (Farnsworth et al., 1981; Moldenke et al., 1983). This study also shows that the anterior part of the midgut has considerably higher activity than the posterior part. The significance of this result is unknown at this stage. The aldrin epoxidase activity recorded for anterior midgut microsomes is 13.5-fold greater than that of gut microsomes of *M. varianus* (Krieger et al., 1970). This value of 3.56 nmol dieldrin/min/mg protein (Table 2) is comparable to the highest activities reported in only a few lepidopterous larvae (e.g., Yu et al., 1979; Farnsworth et al., 1981).

The cytochrome P-450 value of 0.73 nmol/mg protein for total midgut (Table 2) was greater than that found by Krieger et al. (1970) for *M. varianus* (0.23–0.35). The value for anterior midgut, 1.28, is quite high compared to most lepidopterous larvae which have gut values generally below 0.5 nmol/mg protein (Wilkinson and Brattsen, 1972). This value is also higher than that found in the liver of a number of vertebrate species (Hodgson, 1985).

Monooxygenase activity of final instar larvae of Lepidoptera invariably have higher activity than earlier instars (e.g., Krieger and Wilkinson, 1969;

Berry et al., 1980) and so too with drone honeybee larvae (Gilbert and Wilkinson, 1974). With *P. polita*, however, no significant differences were observed in aldrin epoxidase activities between the final three instars either on a milligram protein or milligram individual basis (Table 3). It cannot be concluded from these data that there are no differences in activities between these instars until age profiles are undertaken. In some lepidopterous species, large variations in activity may occur within a single instar (Wilkinson and Brattsen, 1972), a possibility not examined in this study.

Midgut homogenates of *P. polita* larvae collected from *E. parramattensis* and *E. globulus* had similar levels of aldrin epoxidase activity (Table 4). Marty et al. (1982) found that there were no differences in aldrin epoxidase levels of the monarch butterfly, *Danaus plexippus* (L.) which had fed on four different species of *Asclepias*. Yu (1982), however, found that with another lepidopterous species, *Spodoptera frugiperda* (Smith), larvae fed immature tops of leaves from 7-week-old corn plants contained only one half the activity compared with those fed on mature leaves from plants of the same age.

The high activity of aldrin epoxidase in the midgut of *P. polita* is most likely due to the presence of monoterpenes in eucalypts (Morrow et al., 1976). A variety of monoterpenes have been shown by Moldenke et al. (1983) to induce aldrin epoxidase in a lepidopterous species. There are, however, other allelochemicals present in eucalypts (Morrow and Fox, 1980), and their effects on monooxygenase activities of *P. polita* are unknown.

It is widely acknowledged that in lepidopterous larvae, monooxygenases act as an effective biochemical defense against allelochemicals (Brattsten, 1979; Ahmad, 1982; Scriber, 1984; Terriere, 1984). The gut is the first organ to encounter dietary allelochemicals, so high monooxygenase activity in this tissue is essential for effective detoxification of toxic allelochemicals before they enter the hemolymph. This explanation appears to fit the results obtained with *P. polita* which consumes a wide variety of allelochemicals. This species, however, is monophagous and does not fit the widely held hypothesis of Krieger et al. (1971) that polyphagous feeders have much higher activities than monophagous feeders. The activity obtained in this species is similar to larval Lepidoptera which feed on hosts containing monoterpenes regardless of whether the larvae are polyphagous or not (Rose, 1985). Additional research will be required, however, to resolve whether aldrin epoxidase activity typifies generalized monooxygenase activity.

Also, whether monoterpenes are being detoxified by *P. polita* is unknown. It is possible, as Gould (1984) has suggested, that induction could occur in the absence of detoxification. For example, Morrow and Fox (1980) found that beetles feeding on leaves did not detoxify the essential oils of eucalypts. While no detailed studies have been undertaken with metabolism of eucalypt leaves by sawfly larvae, Morrow et al. (1976) showed that, chromatographically, midgut and fecal pellet extracts were much less complex than leaf extracts. This

suggests that sawflies are capable of metabolizing essential oils. Brattsten (1983) has shown indirectly the involvement of monooxygenases in the metabolism of a number of monoterpenes by *Spodoptera eridania* Cramer, a lepidopterous species. This issue of metabolism of monoterpenes by monooxygenases in sawfly larvae is being investigated in my laboratory.

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LINAMARIN AND HISTAMINE IN THE DEFENSE OF ADULT *Zygaena filipendulae*

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Abstract—We determined the protective values of histamine and linamarin to an aposematic moth, *Zygaena filipendulae*. Using ion-exchange resin techniques, we found that the mean histamine concentrations in the wings were 0.061 ± 0.047 μg/mg and 0.013 ± 0.0051 in the moths' bodies (totals: 0.586 μg and 2.921 μg, respectively, all wet weights). Average HCN evolution (mainly from the bitter cyanogen linamarin) from the wings was 0.049 ± 0.41 μg/mg (0.426 μg/ml of linamarin could produce this amount of HCN) and 0.029 ± 0.0026 μg/mg HCN (0.281 μg/mg linamarin) evolved from the bodies (total linamarin 4.09 μg and 61.258 μg, respectively, all wet weights). Therefore, higher concentrations of toxicants were found in the part of the body most liable to initial attack. We found, in offering various toxic solutions to 10 common quails, that 0.1% linamarin (mean linamarin consumed equal to about 70% of the average total wing content) but 1.0% histamine (mean histamine consumed equal to that found in about 8.9 average wing sets) solutions significantly lowered drinking rates. However, combination solutions were still effectively aversive at 0.001% histamine plus 0.028% linamarin. This synergism would allow a moth under local abiotic or dietary stress to elaborate substantially less of one or both compounds than that normally synthesized. The implications to kin selection are discussed.

Key Words—*Zygaena filipendulae*, Lepidoptera, Zygaenidae, allomone, histamine, linamarin, cyanoglycosides, defense, synergism, avian insectivory, aposematic coloration.

INTRODUCTION

Zygaena filipendulae L. (Lepidoptera: Zygaenidae, the six-spotted Burnet moth) has a brilliant red and black aposematic coloration and is known to be unacceptable to a wide variety of vertebrate predators (Wiklund and Järvi, 1982;

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Evans et al., 1985). Larvae and imagines of *Zygaena* are cyanogenic (Jones et al., 1962), and the adults have histamine-like activity in their body tissues (Frazer and Rothschild, 1960). The sources of cyanide are the internally synthesized bitter cyanoglycosides, linamarin and lotaustralin (Davis and Nahrstedt, 1984). The major contributor is the former (Davis and Nahrstedt, 1979, 1981, 1984). Behavioral studies with avian insectivores have indicated gustatory sensitivity and aversion to bitter substances (Brower, 1969; Alcock, 1970; Lea and Turner, 1972). Since a defensive role of these glycosides has not been demonstrated (Davis and Nahrstedt, 1981), we were interested in determining the protective value of the cyanogens and histamine in the levels actually found in the insect.

Many noxious prey have their aversive quality most highly concentrated in areas of the body most likely to receive an initial predatory attack (e.g., monarch butterflies, Brower and Glazier, 1975; and chrysomelid beetles, Pasteels et al., 1978; *Ambystoma* salamanders, Di Giovanni and Brodie, 1981). Avian insectivores usually first peck at a moth's wings (Evans, 1983); therefore, an optimal defensive strategy is the allocation of greater concentrations of protective resources in the wings. An earlier test for histamine-like activity using the inaccurate rabbit-ileum bioassay had indicated that Lebanese *Z. filipendulae* had higher levels of histamine in the wings (D.L. Evans, personal communication). A greatly improved technique (Sekardi and Friedberg, 1981) has now made it possible for us to determine accurately the concentrations of the toxin in the wings and in the bodies. This has enabled us to evaluate the defensive roles of the natural concentrations of histamine and that of cyanogens by offering these chemicals in single solutions or in combinations to avian insectivores.

METHODS AND MATERIALS

The specimens of *Z. filipendulae* were collected from various locations along the valley of the Nahr Ibrahim, Lebanon, from mid-April to mid-June, 1984 and 1985. The wings and bodies of cooled (4°C) (freezing has the effect of substantially reducing histamine content), living Burnet moths were separated, weighed, and analyzed. Half the moths from each collection were allocated to the histamine determination and half to the cyanogen tests.

The histamine assays followed the techniques of Sekardi and Friedberg (1981), used successfully by other workers (Robinson-White and Beavan, 1982). This method is summarized in Figure 1. In brief, the recovery of histamine was determined by comparing the relative fluorescence intensity of histamine coupled directly with ophthaldialdehyde and the same samples after ethanol extraction and isolation on a cation-exchange resin. Relative recovery of authentic knowns was 86.50 ± 2.49%. The linearity of the relative fluorescence intensity to histamine concentration was checked by the addition of 0.5, 1.0, 2.0, and

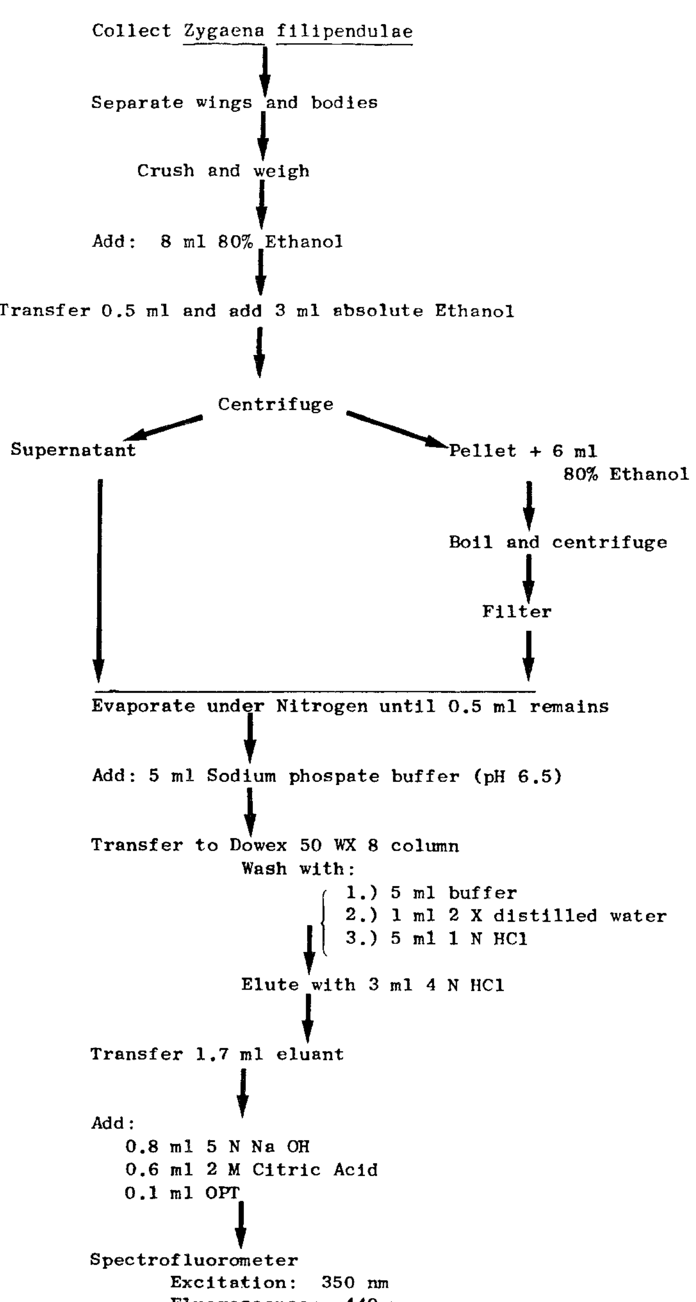

FIG. 1. Protocol for histamine determination in summary; see text for further details.

5.0 μg histamine dihydrochloride to moth tissues. Further confirmation of the results came from an earlier rabbit ileum test which indicated histamine-like activity in the same ranges as we found using the ion-exchange technique in the wings and in the bodies.

We used micro-diffusion procedures with colorimetric methods which were first coupled by Feldstein et al. (1954) to determine cyanide and, hence, cyanogen levels. This method is summarized in Figure 2 and is accurate to 1.0 ng. We checked our accuracy by means of known dish and tube standards and linearity of cyanide calibration curves. We obtained further confirmation by using a Fisher $^{-}$CN/$^{-}$I ion-selective electrode with portions of samples.

We used 10 hand-reared common quails, *Coturnix coturnix coturnix* L., to test various toxic solutions because quail tastes are better known than other easily maintained birds. In addition, low population densities of other avian insectivores in Lebanon make it difficult, practically and ethically, to experiment with other species. No quail died during these taste tests.

We used in our solutions commercially available linamarin and histamine hydrochloride. Davis and Nahrstedt (1979, 1981, 1984) have shown that linamarin and lotaustralin are the cyanogens in adult *Z. filipendulae.* We used only linamarin because: (1) it is usually present in substantially higher concentrations than lotaustralin and (2) lotaustralin is not available commercially and is difficult to isolate or to synthesize. Therefore, we allowed linamarin to act for both cyanogens, an approach also used by Witthohn and Naumann (1984).

In the solution test series, we allowed the 10 quails to choose to drink, in isolation, from two 25-ml bird fountains: one containing the toxic solution and the other distilled water (for further details, see Schuler, 1983). We adjusted for losses due to evaporation in each case. We used distilled water as an alternative rather than a sugar solution because in preliminary work we had found that there was no significant difference in the birds' choices between water and

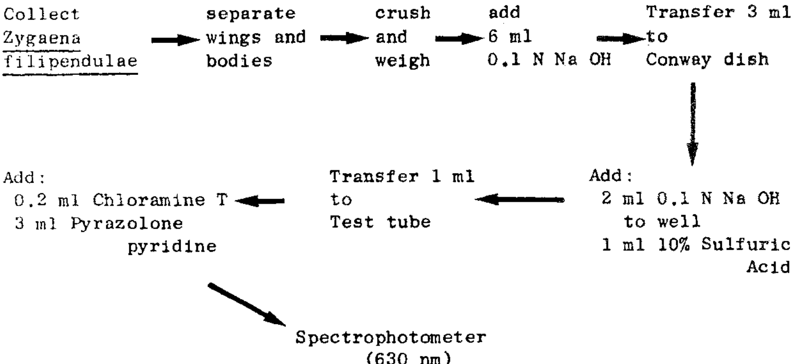

FIG. 2. Steps in cyanide quantification as outlined by Feldstein et al. (1954).

IM D-glucose ($P > 0.05$, Wilcoxon-Mann-Whitney U test; Sokal and Rohlf, 1980). The positions of test and alternative solutions were alternated to avoid the development of side preference (Evans, 1978).

It was necessary to use nonparametric statistical analyses between samples since there was significant skewness (m_3) and kurtosis (m_4) (Sokal and Rohlf, 1980).

RESULTS

The average *Zygaena filipendulae* body contained 2.92 μg of histamine and 1.632 μg HCN (61.258 μg linamarin equivalence), while the average set of wings contained 1.586 μg histamine and 0.422 μg HCN (equivalent to 4.09 μg linamarin) (all are wet weights). Insectivorous birds usually only hold unpalatable moths in their beaks rather than consuming them (Evans, 1983). Therefore, it seems to be more relevant to discuss concentrations likely to be tasted rather than contents of entire structures.

The mean concentrations of histamine and cyanogens (Table 1) were significantly different when comparing the wings versus the bodies (Wilcoxon-Mann-Whitney U tests, $P \gg 0.01$; Sokal and Rohlf, 1980). There was considerable intersample variation within the wings group and the bodies group with respect to toxin concentration. However, within some samples (each containing 6–10 moths), the histamine concentration was near the maximum but the cyanogen concentration at the minimum and vice versa. For example, a sample of wings from Ghazir, Lebanon, collected on April 18, 1985, produced 0.019 μg/mg HCN but had 0.11 μg/mg of histamine. A similar phenomenon could be demonstrated when comparing wing versus body concentrations.

We wished to determine the actual selective value to the moths of these concentrations of poisons. Merely finding something toxic in an animal does not prove that this phenomenon is useful in the creature's defense against predation. It is possible, for instance, that certain compounds are intermediates or products of some novel metabolic pathway and have no protective value whatever. Although the quails, on the average, drank lower amounts of the 1.0% histamine and the 0.1% linamarin solutions (there was a relatively high consumption of the water accompanying the 0.1% linamarin) (Table 2), a Kruskal-Wallis ANOVA test demonstrated no statistical significance ($P > 0.1$; Sokal and Rohlf, 1980) when comparing the various toxic solutions. However, Wilcoxon-Mann-Whitney U tests showed that there were significant differences between the mean amounts drunk from the poison-containing solutions and the pure water. An average concentration of linamarin in either the wings [0.462%, near the rat LD_{50} (Philbrick et al., 1977)] or the bodies (0.281%) might alone offer protection to the moths. However, no *Z. filipendulae* sample contained the

TABLE 1. ANALYSES OF HISTAMINE AND CYANOGENS IN *Zygaena fillipendulae* BODIES AND WINGS[a]

	Histamine[b]		HCN Detected[b]		Linamarin equivalence of HCN means[c]
	Means	Ranges	Means	Ranges	
Wings (samples mean 9.6 mg)	0.0610 ± 0.0470	undetectable[d]–0.120	0.044 ± 0.041	0.019–0.135	0.426
Bodies (samples mean 218.0 mg)	0.0134 ± 0.0051	0.0064–0.020	0.0029 ± 0.0026	0.005–0.0047	0.281

[a] Values given as μg/mg. Means (±SD) of six samples each.
[b] Relative recoveries with known authentic samples 86–100%.
[c] Lotaustralin is also found in *Z. filipendulae* but is not available commercially. Therefore, linamarin was the only cyanogen (found in the moth) which we could use in our bioassay (Table 2). NB: both cyanogens contain only one —CN radical.
[d] Only one sample was this low, the next lowest was 0.03 μg/mg.

TABLE 2. MEAN (±SD) MILLILITERS OF SOLUTION DRUNK FROM PAIRED 25-ml FOUNTAINS BY 10 ISOLATED ADULT (<72 days) COMMON QUAILS, *Coturnix coturnix coturnix*, WITHIN 2 HOURS[a]

Test solution fountain			
Solute(s) concentration(s) (%)	Means	Distilled water fountain (means)	Significance
Histamine			
0.001	4.64 ± 5.33	5.86 ± 4.05	NS
0.01	4.60 ± 3.10	3.54 ± 3.71	NS
0.1	3.59 ± 2.57	5.96 ± 6.36	NS
1.00	0.52 ± 0.39	6.54 ± 4.78	**
Linamarin			
0.0001	5.75 ± 3.74	5.75 ± 3.43	NS
0.001	5.75 ± 4.83	6.90 ± 4.59	NS
0.01	5.75 ± 4.42	6.30 ± 5.20	NS
0.04	4.70 ± 4.49	8.80 ± 5.75	NS
0.1	2.90 ± 1.63	8.35 ± 6.16	**
Histamine 0.006 + linamarin 0.04	3.60 ± 2.93	12.3 ± 8.40	*
Histamine 0.001 + linamarin 0.028	3.15 ± 2.82	6.25 ± 3.80	*
Histamine 0.0005 + linamarin 0.014	3.44 ± 1.90	5.30 ± 3.08	NS
Histamine 0.00025 + linamarin 0.007	4.35 ± 1.46	4.81 ± 2.34	NS

[a] We determined the significance using the Wilcoxon-Mann-Whitney U test (Sokal and Rohlf 1980): NS = no significance; * = 0.05; ** = 0.01. In the order of presentation to birds.

necessary 1.0% (Table 2) concentration of histamine. Significant feeding aversion could occur if a quail had eaten about 70% of the average (Table 2) total set of moth wings if linamarin were the only noxious compound present. On the other hand, feeding avoidance could only be achieved (if histamine occurred alone) when an average quail had eaten 8.9 sets of moth wings (Table 2).

Separate screening tests have also shown that 1.0% acetylcholine was the minimum aversive level, comparable to the toxic concentration of β-cyanoalanine (Ressler, 1962) found in *Z. trifolii* larvae (Witthohn and Naumann, 1984). By definition, not all samples have levels as high as the mean. However, many experimental feeding studies with several avian species (e.g., Wiklund and Järvi,

1982; Evans et al., 1985) have shown that *Z. filipendulae* are not usually handled roughly enough in birds' beaks to be killed and are rarely, if ever, eaten. Clearly, even moths with low cyanogen levels are protected.

We attempted to elucidate the role of histamine when linamarin levels were minimal. We began testing the quails' drinking rates of solutions with the highest nonsignificant linamarin solution (0.04%, Table 2) and the 0.006% mean histamine concentration actually found in the body samples. To our surprise, the birds were significantly more likely to drink distilled water ($P < 0.05$). We continued to reduce both concentrations in further trials. We next offered a solution with 10% of the mean concentration of body linamarin and with an amount of histamine equal to the lowest single solution previously offered. Significantly less of this combination toxic solution was consumed ($P = 0.05$). We diluted this latter solution by half and found no significant difference. A further dilution confirmed this latter result (Table 2).

All dosage curves were sharply sigmoidal (Table 2) with a clear threshold of aversion. The combination of both toxicants is present in substantially larger concentrations than that actually required to prevent further consumption by the birds. In a separate, ongoing set of learning experiments involving feeding on pastry worms, even the minimal histamine–linamarin combination here reported was significantly deterrent. The excessive amounts of defensive compounds usually present in the moths seem to be necessary so that the individual insects may powerfully deter feeding (and, thus, possibly instructing a predator capable of remembering the accompanying warning signal) and also be so repellent as to avoid rough handling which might lead to death or loss of reproductive potential. Hence, individual fitness is enhanced (see also Wiklund and Järvi, 1982; Evans et al., 1986).

DISCUSSION

The wings of *Z. filipendulae* usually contain a higher concentration of cyanogens and histamine than do the bodies. These concentrations are more than sufficient to induce taste aversion. Therefore, the part of the body most likely to be attacked first by insectivorous birds (Evans, 1983) has the greatest concentration of protective compounds. Evans et al. (1985) had found that naive quails did pick up the moths by the wings but then dropped the insects without further damaging the prey (see also Wiklund and Järvi, 1982).

The effect of the combination of the two poisons was greater than that of the sum of each toxicant by itself, i.e., synergism. Histamine is normally very rapidly cleared from avian circulatory systems and, in any case, gut microflora convert this compound to the inactive, *N*-acetyl histamine (Robinson-White and Beavan, 1982). Possibly, the released HCN blocks one or more of these processes. It is possible that other ratios and concentrations of these two toxins

would also be effectively deterrent. Local abiotic or dietary factors or metabolic turnover might make it impossible for an individual to elaborate or to maintain a large amount of one of the poisons throughout imaginal life. By having at least two (there are probably others; Rothschild et al., 1970; Jones et al., 1962) deterrent toxins which can act synergistically, the moth is efficiently defended even if relatively low levels of protective compounds are synthesized. Binary systems of chemical defenses have been noted before, and it has been suggested that the allelochemicals would be directed at different predatory types (Järvi et al., 1981). While this may be generally true, and operant in *Z. filipendulae* as well, it is obvious that this double system can allow for lower, effective poison levels. This powerful feeding deterrent allows for the selection of aposematism without necessarily relying on kin.

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SEX PHEROMONE BLEND DISCRIMINATION BY MALE MOTHS FROM *E* AND *Z* STRAINS OF EUROPEAN CORN BORER

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Abstract—Sex pheromone behavioral responses were analyzed in a flight tunnel with European corn borer, *Ostrinia nubilalis* (Hübner), males from three distinct populations. Males from a bivoltine and a univoltine biotype using a 97.8:2.2 blend (*Z* strains) of (*Z*)- and (*E*)-11-tetradecenyl acetate were assayed with treatments containing 0, 0.5, 1, and 3% of the *E* isomer. Males from neither population oriented in the plume to the 100% *Z* treatment, but bivoltine males oriented and flew to the source to the other three treatments, whereas univoltine males oriented and flew to the source only to the 1% and 3% *E* treatments. Males from a bivoltine biotype using a 1:99 blend of *Z/E* isomers (*E* strain) were assayed with sources containing 0, 0.5, 1, and 3% of the *Z* isomer. Males did not orient to the 0% *Z* source, but oriented and flew to the source to the other three treatments. In addition to using opposite geometric isomers for the main pheromone component, the bivoltine *E* strain differed from the bivoltine *Z* strain by producing and responding better to 1% of the minor component and by storing 3 times more pheromone in the female glands. Contrary to previous reports, the blend of two pheromone components is significantly better than the main component alone in eliciting oriented flight and close-range behavior with males from all three populations of European corn borer.

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

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INTRODUCTION

The European corn borer (ECB), *Ostrinia nubilalis* (Hübner), is a cosmopolitan species from Europe that was introduced into North America on at least two and probably three separate occasions during the early 20th century (Caffrey and Worthley, 1927). The populations presently established in New York have distinct voltinism (Eckenrode et al., 1983) and pheromone blend characteristics. The initial infestations in New York were univoltine (Hervey and Carruth, 1939), with a bivoltine biotype initially introduced in eastern Massachusetts, spreading into New York and heavily infesting Long Island and the Hudson River Valley by 1939. The history of these biotypes in New York is outlined in Roelofs et al. (1985) and the references therein. In addition to voltinism differences, ECB populations have been found that utilize two distinct sex pheromone communication systems: a *Z* strain using a 97:3 mix of (*Z*)- and (*E*)-11-tetradecenyl acetates (*Z*11-14:OAc and *E*11-14:OAc) (Klun et al., 1973), and an *E* strain using a 4:96 blend of (*Z/E*11-14:OAcs (Kochansky et al., 1975). Roelofs et al. (1985) describe several New York populations including a bivoltine *Z*, a univoltine *Z*, and a bivoltine *E*, as well as mixed populations.

Trapping studies have demonstrated that the greatest capture of *Z* strain ECBs occurs using a 97:3 blend of *Z*11-14:OAc and *E*11-14:OAc (Klun et al., 1973, 1979; Klun and cooperators, 1975, Cardé et al., 1975). Since some trap capture also occurs with *Z*11-14:OAc alone, Webster and Cardé (1984) have suggested that *E*11-14:OAc is not required for attraction of males in this strain. They reported the results of a study involving various concentrations of the 97:3 *Z/E*11-14:OAc blend and of *Z*11-14:OAc alone. Behavioral analysis of upwind walking, wing fanning, and clasper extrusion of *Z* strain ECB males in orientation tube olfactometers with these treatments did not reveal a significant difference between the blend and the *Z*11-14:OAc alone. Although this study supported the conclusion of Chapman et al. (1975a,b) that precopulatory behavior in the *Z* strain could be elicited in the absence of the *E* isomer component, Webster and Cardé did suggest that flight-tunnel bioassays should be conducted to determine the precise behavioral responses.

In this paper we report the results of flight-tunnel studies that show the importance of the natural pheromone blend of the three ECB populations compared to the single major component and also describe important differences in behavior between the univoltine Z and bivoltine Z population described by Roelofs et al. (1985). In this paper, populations differing in voltinism are referred to as biotypes, whereas those differing in pheromone blends are referred to as strains.

METHODS AND MATERIALS

Rearing. The univoltine *Z* culture was started from field-collected larvae, pupae, and adults from corn stubble near Paris, New York, during July 1984.

The bivoltine *Z* culture was started from field-collected larvae, pupae, and adults from corn stubble near Eden, New York, during August 1984. The bivoltine *E* culture was started from adults emerging from corn stubble collected near Geneva, New York, during May 1985. The culture techniques and wheat-germ diet were the same as described in Roelofs et al. (1985). Larvae were reared at 30°C with a 16-hr photophase, and the adults were kept in a fluctuating temperature regime (27°–18°C) with a 16-hr photophase.

Pheromone Blend Sources. The *E*11–14 : OAc was TLC-purified material and the *Z*11–14 : OAc was purchased from Dr. S. Voerman at The Institute for Pesticide Research, The Netherlands. Purity of the starting materials and composition of the blends were determined by capillary GLC with a 30-m Supelcowax 10 column (bonded Carbowax 20 M, Supelco Inc.), 0.32 mm ID, using temperature programming from 80°C to 180°C at 10°/min. Both the *E*11–14 : OAc and *Z*11–14 : OAc are >98.6% pure by GLC analysis and both contain <0.05% of the opposite isomer. Blends were made in concentrations of 1 mg/ml using redistilled Skelly B as solvent. An appropriate amount of solution was then applied to 5 × 9-mm rubber stoppers (Arthur H. Thomas Company, catalog No. 8753-D22) so that the final amount of each blend was 30 μg. The 30-μg dosage was chosen after preliminary studies indicated that it was the lowest concentration to elicit maximum behavioral responses. Each stopper was allowed to stand in a hood 2–4 hr before use in the flight tunnel.

Flight Tunnel. The flight tunnel was constructed of rectangular pieces of glass with aluminum framing. The outside dimensions of the tunnel were 183 cm (72 in.) long, 61 cm (24 in.) high, and 61 cm (24 in.) wide. The wind source was a 61-cm household fan supported by brackets 30 cm from the upwind end of the tunnel with a sleeve of heavy clear plastic sheeting connecting the fan to the aluminum and glass tunnel. A wooden frame the size of the tunnel opening and covered with cheesecloth was situated between the fan sleeve and the tunnel proper. The floor of the tunnel had a pattern consisting of alternating black and white stripes 9 cm wide. Diffuse light was supplied by eight 7.5-W, 120-V GE red bulb indicator lights suspended above the tunnel from wooden framing. Single sheets of chromatography paper between the lights and the tunnel ceiling served to diffuse the light so that the illumination was uniformly 11 lux at the tunnel floor.

The downwind end of the tunnel was covered with screening to prevent insects from escaping or being sucked into the 30-cm exhaust, which was positioned 15 cm outside the end of the tunnel. The wind speed during all flights was maintained at 0.25 m/sec. The front of the tunnel consisted of a pair of sliding glass doors that facilitated recapture of the insects. The rubber stopper lures were placed 30 cm inside the upwind end of the tunnel on a stand made of 0.95 cm ($\frac{3}{8}$ in.) copper tubing. The stand consisted of a coiled base with a removable vertical section. The small end of the rubber stopper was slipped into the opening in the copper tubing, allowing the larger end to remain exposed

to the air stream at a height of 12 cm from the tunnel floor. A screen cylinder 10 cm high and 6 cm in diameter was placed 30 cm inside the downwind end of the tunnel to serve as a release stand for the males.

Flight Tunnel Protocol. Individual insects were placed in 3-cm × 6-cm screen cylinders, which in turn were placed on the release stand with the open end of the cylinder upwind. The insects were tested individually in the flight tunnel and were allowed 30–60 sec to take flight. The amount of time required to initiate flight was recorded with a stop watch. The final behavioral response was recorded using the following sequence of criteria: NR, not responding; ACT, activation, rapid wing beating, and walking; TF, taking flight; OR, orientation flight in the pheromone plume; 30 cm, flight in the plume to within 30 cm of the pheromone source; TS, touching source, landing on the pheromone stand, and touching the rubber stopper; DIS, display, clasper extrusion with wings held vertically and abdomen waving slowly from side to side. All moths tested were either flown naively or, in one specific test, were flown first to a 100% *Z* pheromone source and then within 10–30 min reflown to a 3% *E* pheromone source. All flights were conducted at 18 ± 1°C, 3–5 hr into scotophase. All insects were aged 2–3 days after eclosion in the absence of females before being used in the bioassays. The flights were conducted from August 6 to October 7, 1985.

Analysis of Natural Sex Pheromone Blends. Female ECB sex pheromone glands were analyzed similarly to the techniques described in Roelofs et al. (1985), except that tips were pooled in the present study for isomer ratios and were analyzed on the Supercowax 10 column described above. Quantitation of pheromone in single glands was carried out by adding 5 ng of standard tridecenyl acetate in 10 μl of Skelly B to the tip extract with subsequent analysis by capillary GLC.

RESULTS

Z Strains. The 2-day-old male ECBs from the bivoltine *Z* strain and the univoltine *Z* strain were flown to a series of pheromone sources that varied in the percentage of the *E* isomer from 3% to 0%. Final behavior exhibited by each moth was recorded and is summarized in Table 1. We consider any moth that touches the pheromone source to have completed the behavioral response to the source and have lumped the insects with TS and DIS responses together in our analyses. Within each strain, there were significant differences in the numbers of individuals completing the behavioral sequence at various percentages of the E isomer. The K-sample binomial test for equal proportions (Marascuilo and McSweeney, 1977) yielded chi-square values of 63.17 for the univoltine population and 69.24 for the bivoltine population ($P < 0.01$). Post hoc multiple comparisons using the simultaneous $\sqrt{\chi^2}$ procedure to generate confi-

TABLE 1. FINAL BEHAVIORAL RESPONSE OF 2-DAY-OLD ADULT MALE *Ostrinia nubilalis* (*Z* STRAINS) TO 3%, 1%, 0.5%, OR 0% *E*11-14:OAc IN Z11-14:OAc[a]

	E in pheromone blend (%)			
Population	3	1	0.5	0
Bivoltine				
NR	0	1	1	2
ACT	0	0	1	2
TF	0	3	4	31
OR	0	0	0	0
30 cm	0	0	0	0
TS	0	2	1	1
DIS	30	26	23	6
Percent of sample touching source and/or displaying	100a	87a	80a	17b
Univoltine				
NR	1	2	6	8
ACT	0	0	0	2
TF	2	4	18	18
OR	0	2	0	2
30 cm	0	0	1	0
TS	0	0	1	0
DIS	27	22	4	2
Percent of sample touching source and/or displaying	90a	73a	17b	6b

[a] Percentages in the same row followed by the same letter are not significantly different at the 5% level using the post hoc multiple comparisons procedure of simultaneous $\sqrt{\chi^2}$ to generate confidence intervals for simple contrasts. NR = not responding; ACT = activation; TF = taking flight; OR = orientation flight in pheromone plume; 30 cm = flight in plume to within 30 cm from source; TS = touching source; DIS = display.

dence intervals for the simple contrasts (Marascuilo and McSweeney, 1977) show that in the bivoltine population the 0% *E* treatment has significantly ($P < 0.01$) fewer insects completing the behavior sequence than the other three treatments. The 3% *E*, 1% *E*, and 0.5% *E* treatments are not significantly different from each other according to this analysis. The univoltine population shows a different pattern with both the 0.5% *E* and 0% *E* sources eliciting significantly fewer responses than the 3% *E* and 1% *E* sources ($P < 0.01$).

Table 2 summarizes the amount of time required for the insects to take flight when presented with various pheromone blends. In the bivoltine population, times for five of the males flown to the 3% *E* and six of the males flown to the 0% *E* were not recorded. The time required to take flight generally increased as the percent *E* in the pheromone blend decreased in both the univoltine and bivoltine populations. This relationship also was evident in those individ-

TABLE 2. TIME IN SECONDS TO INITIATE FLIGHT IN 2-DAY-OLD MALE Z STRAIN *Ostrinia nubilalis* EXPOSED TO 3%, 1%, 0.5%, OR 0% *E*11-14:OAc IN Z11-14:OAc[a]

		3% *E*	1% *E*	0.5% *E*	0% *E*
Bivoltine biotype					
Responding insects	n	25	29	28	32
	$\bar{x}$	2.1a	4.7a	11.1b	14.9b
	S.D.	4.7	6.2	14.2	15.3
Insects completing flight to source	n	25	26	24	6
	$\bar{x}$	2.1a	4.8a	10.0b	4.8a
(TS + DIS)	S.D.	4.7	6.3	13.3	7.2
Univoltine biotype	n	29	28	24	22
Responding	$\bar{x}$	3.4a	9.6ab	10.4ab	17.5b
insects	S.D.	8.6	15.1	14.9	18.3
Insects completing	n	27	22	5	2
flight to source	$\bar{x}$	2.0a	8.2b	6.5	0.5
(TS + DIS)[b]	S.D.	3.7	13.3	2.6	0.7

[a] Means in the same row followed by the same letter are not significantly different at the 5% level as determined by Duncan's multiple-range test for unequal sample sizes.
[b] Means at 3% *E* and 1% *E* are significantly different at the 5% level using a *t* test for samples with unequal variances.

uals who completed the behavioral sequence except in those treatments with very few responding insects.

The results of flights of insects flown first to 0% *E* then to 3% *E* sources within 30 min are summarized in Table 3. With the bivoltine population, only 20% of the sample completed the behavioral sequence to 0% *E*, but then 97% of this sample completed the sequence to 3% *E*. These percentages are significantly different at the 0.001 level using a *t* test for proportions. All insects that displayed at 0% also displayed or touched the source at 3% *E*, with the mean time to take flight at 0% *E* of 12.3 ± 13.9 sec and at 3% *E* of 3.5 ± 6.0 sec. The univoltine population again exhibited lower sensitivity to the pheromone sources with only 7% completing the behavior sequence at 0% *E*, and then 83% of the same sample displaying or touching the source at 3% *E*. The mean time to take flight at 0% *E* was 20.4 ± 21.2 sec and at 3% *E* was 11.8 ± 19.3 sec, both of which are significantly longer than the bivoltine times as determined via *t* tests.

E *Strain.* Table 4 shows the response of *E* strain males to blends containing 0, 0.5, 1, and 3% of *Z* isomer. Statistical analysis of the data as described above shows that the 0% *Z* treatment has significantly ($P < 0.01$) fewer insects completing the behavioral sequence than the other three treatments. Fifty percent of the males presented the 0% *Z* treatment failed to orient to the pheromone

TABLE 3. FINAL BEHAVIORAL RESPONSE OF 2-DAY-OLD ADULT MALE *Ostrinia nubilalis* FLOWN TO PURE Z11-14:OAc THEN TO 3% *E*11-14:OAc IN Z11-14:OAc[a]

	No.	0% *E*	Then	3% *E*
Bivoltine biotype	2	NR		DIS
	1	ACT		DIS
	20	TF		DIS
	5	DIS		DIS
	1	DIS		TS
	1	TF		TF
Percent of sample touching source and/or displaying		20a		97b
Univoltine population	5	NR		DIS
	15	TF		DIS
	2	OR		DIS
	2	DIS		DIS
	1	TF		TS
	1	NR		NR
	1	TF		NR
	1	TF		ACT
	1	TF		TF
	1	ACT		TF
Percent of sample touching source and/or displaying		7a		83b

[a] Using a t test for proportions, percentage responding to 0% *E* is significantly less than responding to 3% *E* in both populations ($P < 0.01$).

TABLE 4. FINAL BEHAVIORAL RESPONSE OF 2-DAY-OLD-ADULT MALE *E* STRAIN *Ostrinia nubilalis* TO 3%, 1%, 0.5%, OR 0% Z11-14:OAc IN *E*11-14:OAc[a]

	Z in pheromone blend (%)			
	3	1	0.5	0
NR	2	0	2	8
ACT	1	0	0	3
TF	2	0	2	4
OR	0	0	0	4
30 cm	0	0	1	6
TS	4	6	7	4
DIS	21	24	18	1
Percent of sample touching source and/or displaying	83a	100a	83a	17b

[a] Percentages in the same row followed by the same letter are not significantly different at the 5% level using the post hoc multiple comparisons procedure of simultaneous $\sqrt{\chi^2}$ to generate confidence intervals for simple contrasts.

plume and only 17% were able to complete the flight to the pheromone source. High percentages of males did orient in the plume and complete the sequence to the other three treatments, similar to the bivoltine *Z* strain. With the *E* strain, however, the 1% treatment produced a higher percentage of insects completing the behavioral sequence than did the 3% treatment. This difference was not statistically significant under the analyses applied.

Analyses of Natural Sex Pheromone Blends. Female sex pheromone glands (pools of 10 glands, three replicates) of each ECB race were analyzed by capillary GLC for the amount of the minor isomer in the populations used for this study. The bivoltine *E* strain had 1.0% *Z* isomer in *E*11–14:OAc, whereas the bivoltine Z strain had 2.2% *E* isomer in Z11–14:OAc, and the univoltine *Z* strain had 2.0% *E*. Quantitation of the pheromone showed that females of the *E* strain stored substantially larger quantities of pheromone than either *Z* strain population: bivoltine $E = 13.8 \pm 10.6$ ng ($N = 89$); bivoltine $Z = 4.2 \pm 3.6$ ng ($N = 15$); univoltine $Z = 2.5 \pm 1.5$ ng ($N = 27$).

DISCUSSION

These experiments demonstrate that in the majority of the insects the natural blend is important for orientation, upwind flight, and source location. The two Z populations have different sensitivities for responding to the ratio of the minor component, with the majority of the bivoltine Z borers completing the behavioral sequence to a blend containing 0.5% *E*, whereas the univoltine borers require at least 1% *E* for a similar response. The 0% *E* pheromone source elicits activity and/or random flight in the majority of the moths tested [40/42 bivoltine Z (BZ) and 23/32 univoltine Z (UZ)]. This general activity has been reported previously by Webster and Cardé (1984) using glass tube olfactometers, but their study was unable to determine any behavioral differences between the 0% *E* and 3% *E* treatments. We found that very few of the moths were able to orient to and find the pure Z11–14:OAc component. Since 95% of the moths presented the optimal blend of 97:3 *Z/E*11–14:OAc were able to complete the behavioral sequence, we have shown that the pheromone blend significantly improves both populations' ability to locate pheromone sources in flight tunnel situations.

Studies with the bivoltine *E* strain also show that a blend of the two pheromone components is necessary for orientation and subsequent behavioral responses and that it is similar to the bivoltine *Z* strain in its high sensitivity to the 99.5:0.5 blend of isomers. However, with the bivoltine *E* strain, the 99:1 blend elicited the highest percentage of response. This follows female production since the *E* strain was found to produce a 99:1 *E/Z* ratio and the bivoltine *Z* strain was found to produce a 97.8:2.2 *Z/E* ratio. While the studies reported

here reveal the importance of both pheromone components in the ECB pheromone blend, they are part of a larger study presently underway designed to characterize the genetic basis for response differences in these three ECB populations.

Acknowledgments—We thank Dr. C.J. Eckenrode, Jr., P.S. Robbins, and M.L. Hessney for generously supplying ECB adults from the three distinct populations, and Dr. C.E. Linn for assistance in the behavioral analysis. This research was supported in part by NSF grant BSR-8500234.

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ODOR SIMILARITY BETWEEN STRESS-INDUCING ODORANTS IN WISTAR RATS

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Abstract—Odor similarity to the odor of 4-mercapto-4-methyl-2-pentanone (I) was measured on eight rats by generalization of a conditioned avoidance response to *cis*- and *trans*-8-mercapto-*p*-menthan-3-one (II and III), 3-mercapto-3-methyl-2-pentanone (V), and *t*-amyl mercaptan (VI). Previously, these odorants had been found to induce stress in rats in an open-field situation. In the present experiment, rats generalized the avoidance response learned with I, for V, VI, and to a lesser extent *trans*-isomer III, implying odor similarities; *cis* isomer II was discriminated. Odor similarity between mercapto ketone I and mercaptan VI is surprising since VI lacks the keto group.

Key Words—Avoidance behavior, generalized avoidance, mercapto ketones, mercaptans, odor similarity, rat, stress, structure activity relationship.

INTRODUCTION

In a previous experiment, Vernet-Maury et al. (1984) found that several mercapto compounds induced stress response in Wistar rats placed in an open-field situation. Stress was analyzed by quantifying several stress behavior responses and by measuring plasma corticosterone concentrations. The mercapto compounds were either synthetic equivalents or were structurally related to volatiles identified in fox droppings.

Stress response of the laboratory rat is apparently inherited and is probably related to particular components of the complex odorous signals released by the fox. Among the odorants inducing a stress response were several mercaptoketones (I, II, III, V) and one mercaptan (VI) (Figure 1). These have similar odor quality for humans and are reminiscent of male cat urine[1] (Polak et al., 1987).

[1] It has not yet been reported whether these odorants actually occur in cat urine.
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I
4-MERCAPTO-4-METHYL-2-PENTANONE

II
cis-8-MERCAPTO-p-MENTHAN-3-ONE

III
trans-8-MERCAPTO-p-MENTHAN-3-ONE

V
3-MERCAPTO-3-METHYL-2-PENTANONE

VI
t-AMYLMERCAPTAN

AA
ISOAMYLACETATE

DE
DECANAL

PE
PHENYLETHANOL

AN
ANETHOLE

MA
METHYLANTHRANILATE

FIG. 1. Structural formulae of odorants used.

The purpose of the present experiment was to evaluate whether analogous odor similarities and structure odor relationships would be found in the rat by a generalization procedure.

An extensive literature in vision and audition has shown that an animal trained by conditioning to respond to one stimulus will subsequently give responses that may vary with related stimuli. Generally, such experiments produce a gradient of response generalization (e.g., Guttman and Kalish, 1956), representing the stimulus variation along a known physical dimension. With olfactory stimuli, such generalization experiments are more difficult to design, because the quality of the stimulus cannot be modified along one physically measurable dimension equivalent to, e.g., wavelength. Mason and Stevens (1981) showed that salamanders trained to avoid one odor stimulus subsequently avoided two homologs. Rats trained to discriminate between two sexual odors (Slotnick and Born, 1979), or pure odorants without bilogoical relevance (Braun and Marcus, 1969), generalized to two related odors. However, for mice, the introduction of four new odorants during the generalization phase caused general response inhibition (Kaneko, 1979). Aversive conditioning of rats with lithium chloride, as used with taste (Halpern and Tapper, 1971; Domjan, 1975; Dugas du Villard et al., 1981), has been tried by us for several years (Polak, 1978). Unfortunately, reliable generalization (nondiscrimination) responses

could not be obtained due to the extinction of aversive learning during the first test session.

In the present experiment, we have used a method which in principle is the same as that used by Slotnick and Born (1979) and Braun and Marcus (1969). Thirsty rats were trained to discriminate between 4-mercapto-4-methyl-2-pentanone (I) and isoamyl acetate and tested for generalization of the response to odorants structurally related to I, namely *cis*-8-mercapto-*p*-menthan-3-one (II), *trans*-8-mercapto-*p*-menthan-3-one (III), 3-mercapto-3-methyl-2-pentanone (V), and *t*-amyl mercaptan (VI) (Figure 1), as well as the unrelated odorants decanal (fatty odor), 2-phenylethanol (rose odor), anethole (anise odor), and methyl anthranilate (tangerine odor).

Our goal was to test as many odorants as possible per rat, in order to detect individual characteristics of the response patterns. Consequently, we have tested all four pairs on each of a small number of rats ($N = 8$) rather than each pair separately on four sets of rats.

METHODS AND MATERIALS

Stimuli

Odorants. The structural formulas of odorants used in this study are shown in Figure 1. In Table 1 are given the chemical name, code in Roman numeral, the supplier, the purity, the method of analysis (GLC or TLC), the purification

TABLE 1.

Code	Name	Source	Estimated purity
I	4-mercapto-4-methyl-2-pentanone (19872-62-7)	Givaudan Research, Dubendorf, Switzerland	TLC: one spot
II	*cis*-8-mercapto-p-menthan-3-one (34349-25-2)	[a]	TLC: ±99%, one impurity
III	*trans*-8-mercapto-*p*-menthan-3-one	[a]	TLC: one spot
V	3-mercapto-3-methyl-2-pentanone	PFW B.V., Amersfoort, Holland	TLC: one spot GLC: 99%
VI	*t*-amyl mercaptan (2-methyl-2-butanethiol) (1679-09-0)	Jansen Chimica, Beerse, Belgium[b]	GLC: 99.5%

[a] The mixture of *cis*(+) and *trans*(−) isomers (ratio 40:60) was furnished by Givaudan. The isomers were separated and purified by M. Colin, I.C.S.N.-CNRS, Gif-sur-Yvette, France, according to an LLC procedure furnished by K.B. de Roos, Research Laboratory, PFW B.V., Amersfoort, Holland, using deactivated silica gel.

[b] The technical grade (GLC 98.7%) was purchased from Jansen, Beerse, Belgium, and purified by PFW B.V. by preparative LLC and GLC to 99.5%.

method where applicable, and the Chemical Abstracts registry number in parentheses. Chemical Abstracts nomenclature has been added in parentheses wherever it differs from the common usage names used in the text.

Isoamnyl acetate (AA), decanal (DE), phenylethanol (PE), anethole (AN), and methyl anthranilate (MA) were of best commercial quality available. These were not analyzed further.

Solvents. An odorless grade of diethylene glycol (DEG) was used as solvent to prepare odorant dilutions. An odorless grade of commercial mineral water was used, pretreated with 6 mg/liter of odorless chelating agent, ethylenediamine tetraacetic acid tetrasodium salt (EDTA) (J.T. Baker Chemicals B.V., Deventer, Holland). This treatment is essential to complex traces of heavy metal ions capable of neutralizing the mercapto (thiol) odor. Dissolved EDTA did not change the odor background for humans.

Stimuli Concentrations. These are listed in Table 2. Odorants were prediluted in DEG (0.1–0.0001%) and added to EDTA-treated water immediately prior to use each day. All concentrations were volume/volume in DEG or water. Headspace concentrations were not determined.

Stability of Stimuli. Because mercaptoketones and mercaptans are unstable and prone to oxidation to less intense odors in the presence of air, light, and trace metals, they were stored at low temperature ($<0°C$) as such or in DEG solutions.

Animals

Eight adult male Wistar rats (6–8 weeks old, 250 g) were housed in individual cages in a temperature-controlled room (23°C) provided with food pellets and water. Water deprivation began 24 hr after arrival in order to motivate

TABLE 2. CONCENTRATIONS USED

Odorants	Concentration (10^{-3} μl/liter water, equivalent to ppb)
I	0.1, 0.25, 0.5,[a] 1
II	10
III	50
V	15, 100
VI	1
Isoamyl acetate	1000, 2500, 5000,[a] 10,000
Decanal	500
Phenylethanol	10,000
Anethole	5000
Methyl anthranilate	50

[a] Concentration used during training

drinking during the experimental sessions. Water intake was limited to 16–20 ml/24 hr, and the weight of each rat was thus kept at 80% of ad libitum weight. Rats were weighed daily, just before the experimental session. Eight rats was the maximum that could be handled in eight successive 1-hr daily individual sessions.

Apparatus

The apparatus consisted of a 24.5 × 20 × 24-cm steel box from Cambden Instrument (USA). The front wall was a door made from clear plexiglas through which the rat's behavior was observed. The floor was a 1-cm spaced steel bar grill (Figures 2A, B).

Odor stimulus was delivered by unassisted evaporation from a film formed on the ball-bearing closing the nickel water-delivery spout. The evaporating film was estimated to have a surface of 20 mm^2. The water spout was attached to a 2-ml Plexiglas syringe containing an aqueous solution of the odorant. This removable syringe was clipped into a Plexiglas holder attached to the box. It was introduced for 10 sec through a 2-cm-diameter hole in the wall at 10 cm above the floor. In front of the spout was placed a vertical brass barrier (2.5 × 2 cm), with a 0.5-cm-diameter hole through which the rat could smell and lick. The tongue alone could touch the spout. The rat made approximately 60 licks per 10 sec, drinking 0.2 ml of aromatized water.

In the presence of a negatively reinforced stimulus (odorant I, see below), each lick induced an electric shock to the tongue with a constant intensity of 100 μA; the rat closed the circuit by simultaneously touching the spout and the brass barrier with its tongue.

Odor Stimulus

Odorant dilutions were prepared fresh every day, in 100 ml water pretreated with 6 ppm EDTA. DEG was adjusted so that the final concentration in all trials was the same (100 μl/100 ml water). During the session, the syringe was filled just before the trial with an aqueous solution. The concentrations used in water were adjusted so as to be just perceptible by the experimenter at the water spout exit. The concentrations used are listed in Table 2. It should be noted that, prior to the experiment, response thresholds of the nonmercapto odors were evaluated with naive rats. Behavioral threshold concentrations were a minimum of 10× lower than those actually used in the present experiment.

Procedure

The experiment began one week after arrival of the rats. Each rat had a 1-hr daily session at 24-hr intervals during six days per week for about six weeks.

Pretraining. On each of five days prior to conditioning, each rat spent 20

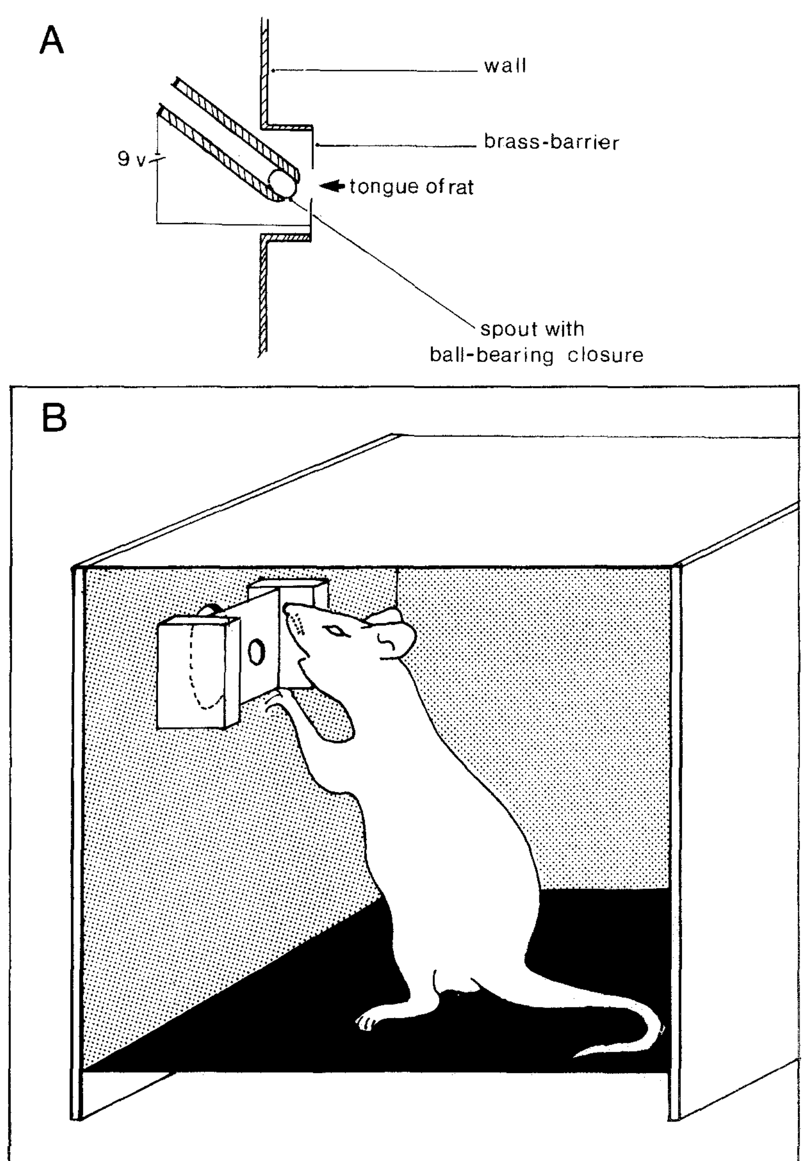

FIG. 2. Experimental box, general view (B) and detail of stimulus delivery (A). For description see text.

min in the experiment box in order to learn to drink water from the spout without shock. The duration of each trial was reduced from one day to the next, attaining 10 sec on the fifth day.

Training. Rats were trained to discriminate between isoamyl acetate (5 ppm) and mercapto odorant I (0.5 ppb) during 1-h sessions. In a training session, each of these two odorants was presented for 20 trials according to a pseudorandom sequence that allowed no more than three consecutive presentations of the same odorant. For all animals isoamyl acetate (AA) was the positive stimulus (S^+) and the mercapto odorant I was the negative stimulus (S^-). An approach response was defined as the first lick made on the spout. During the S^+ trials rats were positively reinforced by being allowed to drink from the spout for 10 sec. During the S^- trials, rats were negatively reinforced by an electrical shock on the tongue with each lick. An avoidance response was defined as the absence of licking for 10 sec.

The trial began with the introduction of a syringe holding aromatized water solution and ended with withdrawal of it. In the case of an approach response with S^-, the syringe was removed as soon as the rat withdrew from the spout after a few licks with shock.

To minimize pheromonal odor markings (Stevens and Koster, 1972; Valenta and Rigby, 1968), syringes and spouts were changed for each trial. After one session, spouts were washed with alcohol and water, attached to other syringes, and the inside of the box was wiped with water, before introducing the next rat.

Rats received a water ration in the home cage, 1 hr after the end of their session.

The criterion of learning was set at 15/20 correct approach responses to S^+ or avoidance responses to S^-.

Extension to Several Intensity Levels. During training, AA and I were presented at one concentration level. When the learning criterion was reached, these odorants were presented at different concentrations five times at each session, in order to minimize responses based on intensity rather than quality (Table 2).

Generalization to Other Odorants. In this phase of the experiment, the same eight rats were tested for responses to four mercapto odorants and four nonmercapto odorants. A total of 50 trials per rat were given per daily session in which one new mercapto odorant and one nonmercapto odorant were tested for generalization. Each new odorant pair was tested during three successive sessions, so that each rat was exposed to four new odorant pairs in 12 sessions.

The session began with 10 training trials. Five presentations of S^+ (AA) with positive reinforcement were given interspersed with five presentations of S^- (I) with negative reinforcement. Subsequently, 28 generalization trials were given; six presentations of each new product (mercapto or nonmercapto) were interspersed with eight presentations of S^+ and eight presentations of S^-. In

trials with a new product, no shock was given, and the rats were given an opportunity of positive reinforcement by allowing them to drink for 10 seconds in the case of a nonavoidance response. This modification of classical methods of generalization, where negative or positive reinforcement is not given, was necessitated by the mode of presentation of the odorant stimulus. The session ended with 12 retraining trials, with six presentations of S^+ and six presentations of S^-.

Because only eight animals were used in this experiment, the sequence of odor pairs presented was the same for all rats (VII with decanal, V with phenylethanol, III with anethole, II with methyl anthranilate).

RESULTS

Training

The performance of eight rats during training is shown in Figure 3. AA and stimulus I were effective stimuli for conditioning. Because approach responses with S^+ rapidly reached a maximum of 20/20 and were not affected by any experimental modifications, we have considered the responses with S^-.

The mean number of trials necessary to obtain the learning criterion (15/20) avoidance responses for S^- was 209 $\pm$ 39 and was reached within nine days.

In two subsequent training sessions with four concentrations of the stimuli, avoidance responses with the lowest level of S^- were less than 75%, but never lower than 58%. They were unchanged at the other concentrations.

Generalization

Student's test for paired samples showed that the mean total of avoidance responses with S^- (I) decreased only in the first generalization session in comparison to the last training session (t = 3.41; $P < 0.05$) (Figure 4). This perturbation of performance was certainly due to modifications in the experimental situation (increase in number of trials, introduction of new odorants such as decanal, etc.), rather than to a process of extinction. In the following sessions, the performance improved again.

Group results for the tested odorants are shown in Figure 5. The percentage of avoidance responses concerns the 20 generalization trials given per session (six trials with each new odorant and eight trials with S^-).

The mean percentage avoidance responses were transformed into an arcsin score for each group of products and subjected to two-way within-subjects ANOVA. Tukey's test was then performed to determine whether response differences occurred.

Group VI, DE, I. Analysis showed differences between odorants [$F(2, 14)$

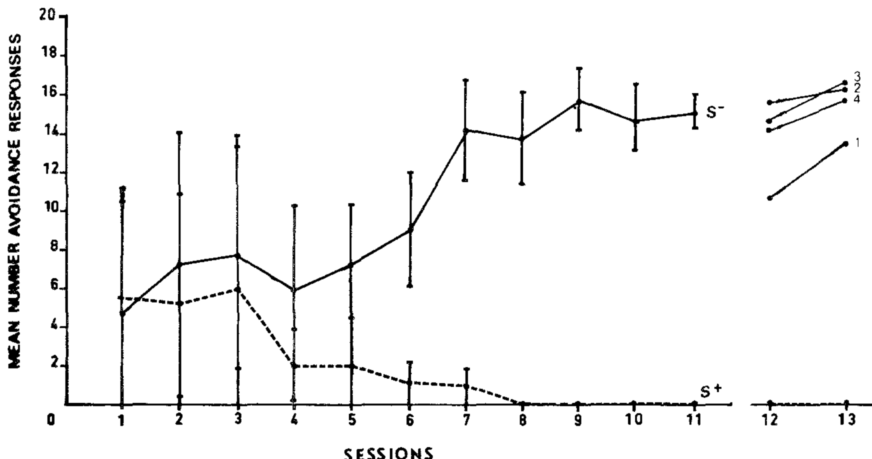

FIG. 3. Mean of avoidance responses for eight rats during training (13 sessions). Solid line: S^- is 4-mercapto-4-methyl-2-pentanone (I); dotted line: S^+ is isoamyl acetate (AA). After criterion was reached (sessions 12, 13), S^- and S^+ were presented at four concentration levels (v/v) for S^-: 1 = 0.1 ppb, 2 = 0.25 ppb, 3 = 0.50 ppb, and 4 = 1 ppb; for S^+: 1 = 1000 ppb, 2 = 2500 ppb, 3 = 5000 ppb, and 4 = 10,000 ppb.

= 32.56, $P < 0.001$] and between sessions [$F(2,14) = 12.77$, $P < 0.001$]. Tukey's test ($P = 0.05$) on the odorant variable showed that the responses in all three sessions differentiate decanal (DE) from VI and I and that VI was not differentiated from I, despite the fact that avoidance to VI decreased significantly across sessions.

Group V, PE, I. Analysis showed a difference between odorants [$F(2, 14) = 65.02$, $P < 0.001$] and between sessions [$F(2, 14) = 13.81$, $P < 0.001$]. Tukey's test showed that V differed in response to both I and phenylethanol (PE), while in the second and third sessions the response of V did not differ any longer from that of PE. The latter differed from I in all three sessions.

Group III, AN, I. Analysis showed a difference between odorants [$F(2, 14) = 20.44$, $P < 0.001$] and between rats [$F(7, 28) = 5.54$, $P < 0.01$], but not between sessions. Because of this response variability, differences could not be analyzed group-wise. A chi-square test done on individual rat frequency avoidance responses showed that two rats avoided III as often as I ($\chi^2 = 0.13$, and $\chi^2 = 1.95$), one rat avoided III less than I ($\chi^2 = 7.08$, $P < 0.01$) but more than anethole (AN) ($\chi^2 = 7.09$, $P < 0.01$), and five rats did not avoid III significantly compared to AN.

Group II, MA, I. Analysis showed only a difference between odorants [$F(2, 14) = 218.4$, $P < 0.01$]. Tukey's test showed that II and MAN, both with few avoidance responses, did not differ from each other, but did differ from I.

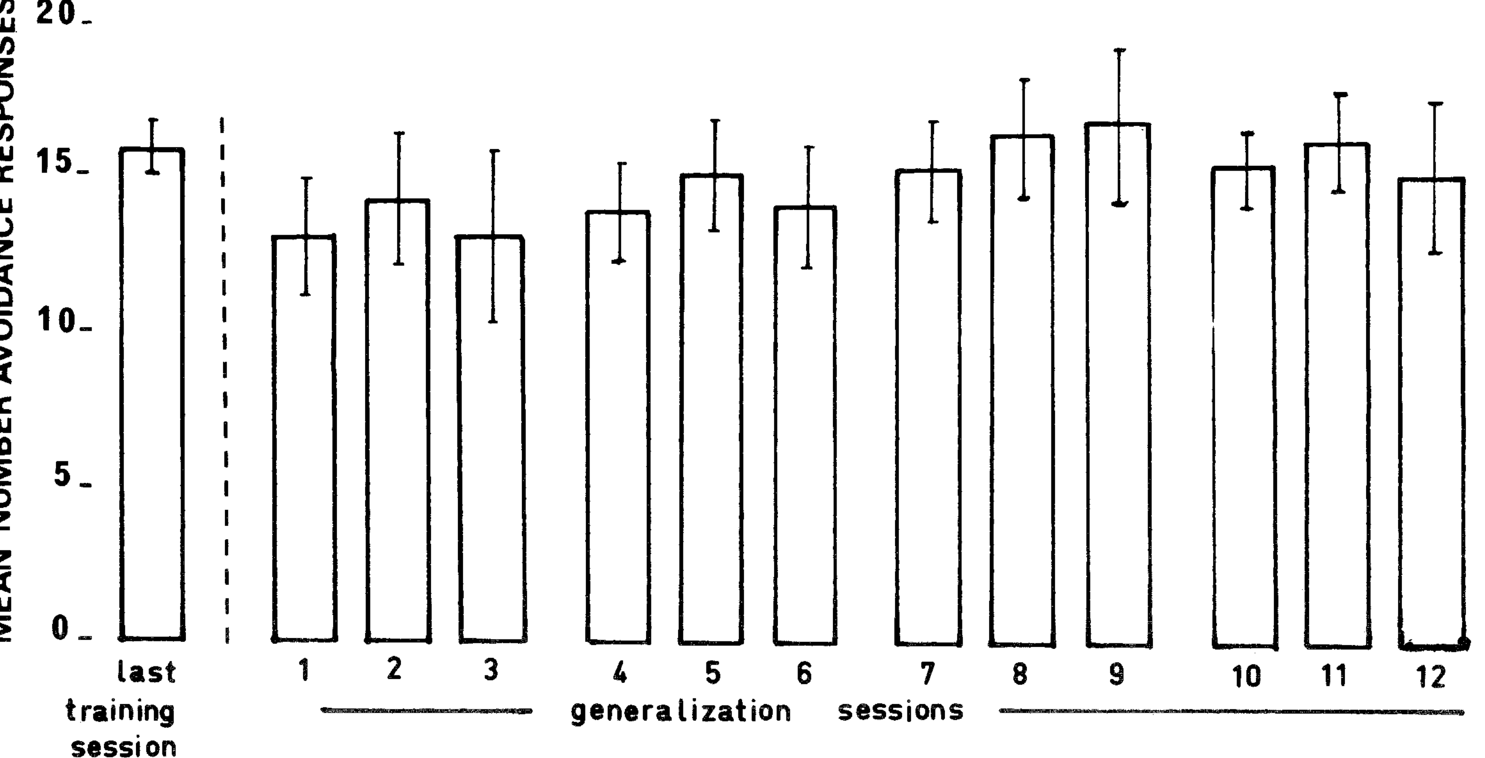

FIG. 4. Mean of avoidance responses with training odorant 4-mercapto-4-methyl-2-pentanone (I) during the last training session, and during generalization sessions (20 trials with I per session). $N = 8$ rats. *t*-Amylmercaptan (VI) and decanal (DE) were tested in sessions 1, 2, and 3; 3-mercapto-3-methyl-2-pentanone (V) and phenylethanol (PE) in sessions 4, 5, and 6; *trans*-8-mercapto-*p*-menthan-3-one (III) and anethole (AN) in sessions 7, 8, and 9; and *cis*-8-mercapto-*p*-menthan-3-one (II) and methyl anthranilate (MA) in sessions 10, 11, and 12.

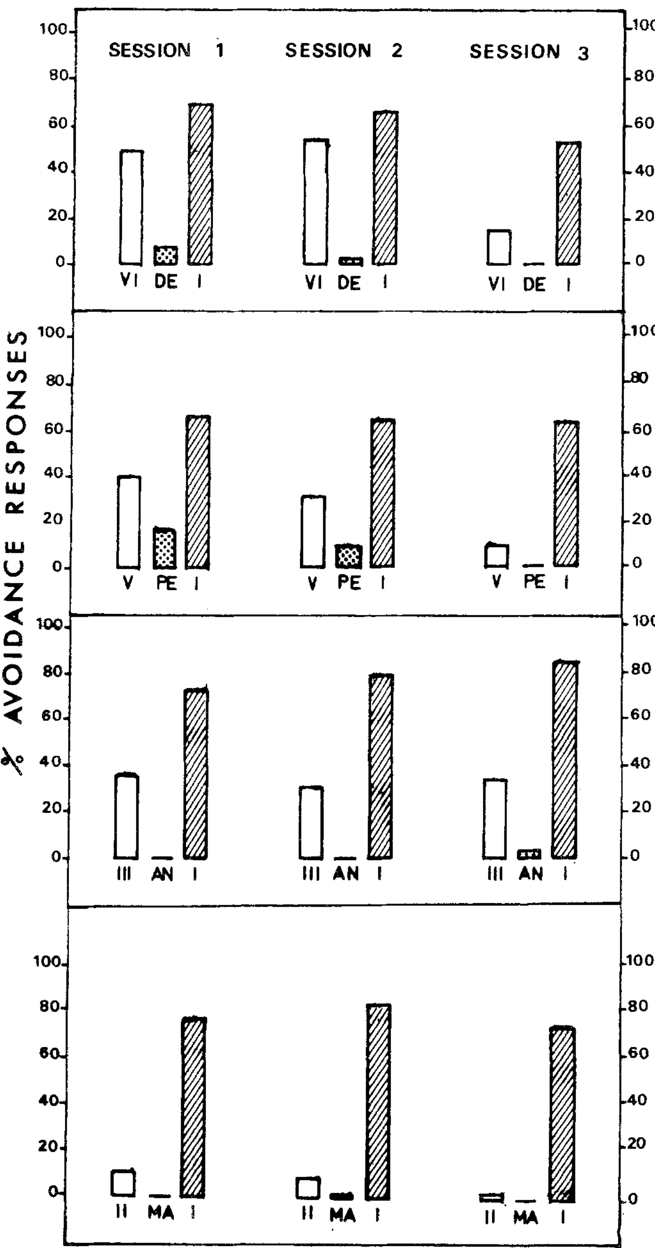

FIG. 5. Total percentage of avoidance responses for eight rats with mercapto odorants VI, V, III, II (open histograms); nonmercapto odorants DE, PE, AN, MA (stippled histograms) and training odorant I (striped histograms) during the three generalization sessions. For statistical analysis, see Results in text.

DISCUSSION

In the experiment of Vernet-Maury et al. (1984), the behavioral stress response was assessed by observing naive unconditioned rats upon entry into a standard ''open field'' box containing odorized air. In addition, physiological stress was determined by measuring the plasma corticosterone concentration.

While we did not quantify the behavior, signs of stress behavior, such as freezing (Cattarelli et al., 1974a, b; Riess, 1945) were never observed at any of the odorant concentrations tested under our experimental conditions. When training began, the rats approached the water spout spontaneously and drank water flavored with either isoamyl acetate or with mercaptoketone I in the same manner and with similar response latency (median 0.62 sec with AA and 0.65 sec with I). Odorant I was thus an effective stimulus for eliciting a conditioned response. Learning criterion was not reached faster with it than with a neutral odorant such as octanol-2 as S^- versus isoamyl acetate (Polak, 1983). In the generalization phase, avoidance responses observed with the mercapto odorants show that the rats were able to generalize the response learned to stimuli other than the training stimulus. The low percentage of avoidance responses with nonmercapto odorants proves that the rats did not systematically avoid any new odorant, it also suggests that avoidance response with new mercapto odorants is due to common odor properties with the training odorant I.

However, the generalization is only partial and decreases with time. This could be due to our experimental procedure. Thus, odor concentrations were not adjusted to equal intensity per rat. Only training odors I and isoamyl acetate were presented at four different concentrations with a 20-fold spread. We do not know whether all rats perceived the test odorants within this intensity range. The interaction between quality and intensity for the test odors could be different from I and consequently reduce the strength of conditioned association (Panhuber, 1982).

Also, the rats had the option of drinking water flavored with all odorants except S^- (I). This generalization procedure differs from usual methods (e.g., Braun and Marcus, 1969) that suppress any reinforcement during generalization sequence. Our procedure may lead rats to seek small differences between training stimulus and test stimuli and thus reduce the generalization responses.

In addition, results show that the first odorant (VI) was more avoided than the last one (II). New odorants were presented in a fixed order and not with a random series, and this order effect could have contributed to an extinction of generalization. But, if this order effect dominated, a continuous decrease of avoidance responses would be expected from the end of testing one mercapto odorant to the start of the next one. Figure 5 shows that any order effect, if present, did not mask qualitative generalization.

In spite of these reservations, the results suggest that odorants V and VI share similar odor aspects with the training stimulus (I), while for III this applies

to only three of eight rats. For human subjects, these mercapto odorants (I, II, III, V, VI) were quite similar, as shown by poor discrimination (Polak et al., 1987).

In terms of odor structure relationships, the similar odorants also share certain structural features as already discussed elsewhere (Polak, 1983; Vernet-Maury et al., 1984; Polak et al., 1987). In this experiment too we made the surprising observation that the keto group is not essential to odor similarity, since mercaptoketone I is confused with mercaptan VI. Dreiding models of the alkyl mercapto odorants (I, V, VI) can take up spatial conformations that overlap equally well with either the *cis* isomer (II) or the *trans* isomer (III) of the larger cyclical molecule 8-mercapto-*p*-menthan-3-one. Since *cis* (II) was better descriminated from I than *trans* (III) was, it suggests that the odor-active conformers of I, V, and VI correspond closer to the configuration of III than that of II.

CONCLUSION

When several odorants cause a stress response, it does not necessarily follow that they all have similar odor quality. It could signify recognition of a common origin, such as a predator.

2,4,5-Trimethyl-Δ^3-thiazoline, an active principle of fox droppings (Vernet-Maury, 1980) was found to be equally or more stressful to rats than the mercaptoketones (Vernet-Maury et al., 1984), which to humans at least are of an entirely different odor type.

Of the five mercapto compounds common to the present experiment and the stress experiment, three [4-mercapto-4-methyl-2-pentanone (I), 3-mercapto-3-methyl-2-pentanone (V), *t*-amyl mercaptan (2-methyl-2-butanethiol) (VI)] were all stressful (Vernet et al., 1984) and frequently confused by most rats. In contrast, *cis*-8-mercapto-*p*-menthan-3-one (II) was as stressful as I, but was discriminated from I by all rats in the present experiment. The stressful stimulus *trans*-8-mercapto-*p*-menthan-3-one (III) was intermediate and not uniformly generalized. Finally, this experiment suggests that the odor similarity to I can be principal element of the stress response for V and VI, but not for II.

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RESIDUAL ACTIVITY OF OVIPOSITION-DETERRING PHEROMONE IN *Rhagoletis pomonella* (Diptera: Tephritidae) AND FEMALE RESPONSE TO INFESTED FRUIT

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Abstract—Under dry conditions, oviposition-deterring pheromone (ODP) of the apple maggot fly (*Rhagoletis pomonella* Walsh) on host fruit proved deterrent to ovipositing females for at least three weeks, with a half-life of ca. 11 days. There was no difference in decline of residual activity under lab vs. field conditions or between fly-deposited ODP vs. an application of a water extract of ODP. A decline in pheromone activity resulted from exposure to both natural and simulated rainfall. For natural rainfall, greatest losses (50–61%) in activity resulted from high-intensity rains, with substantially less activity lost (13–35%) following light or moderate intensity rains. *R. pomonella* females discriminated against fruit without ODP but with conspecific larvae. Discrimination against infested fruit was manifested within fewer days following infestation of small fruit (9 mm diam.) as compared to larger fruit (15 mm diam.).

Key Words—*Rhagoletis pomonella*, apple maggot fly, pheromone, oviposition deterrent, Diptera, Tephritidae.

INTRODUCTION

A resource partitioning system that relies on a chemical stimulus such as a pheromone to mediate against overcrowding may be influenced by a diversity of factors such as the production, release, reception, and residual properties of the stimulus involved. In regard to the latter, persistence of a pheromone may

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vary over time according to the species of insect and the nature of the message conveyed. For example, a repellent pheromone deposited by *Xylocopa* bees following extraction of nectar from passion flowers persists for only about 10 min, the time required for at least partial nectar replenishment (Frankie and Vinson, 1977), whereas *Pieris brassicae* butterflies deposit an oviposition-deterring pheromone (ODP) during egg-laying that is deterrent for more than 14 days, the maximum time required for egg incubation (Schoonhoven et al., 1981).

Among other phytophagous insects that utilize oviposition-deterring pheromones to signal recognition of previously infested plants or plant parts, deterrent components from occupied resources may be emitted until completion of larval development, such as pheromonal release by larvae of *Ephestia*, *Plodia*, and *Heliothis* (Prokopy et al., 1984). On the other hand, as far as is known, the ODPs produced by over a dozen different species of tephritid fruit flies are characterized by moderate residual activity and water solubility (Averill and Prokopy, 1987). As a result, several researchers have questioned the effectiveness of these pheromones as mediators of uniform egg dispersion and larval competition: pheromonal activity may break down prior to completion of larval development and, in climates with moderate to high precipitation, activity conceivably may be lost rather quickly (Katsoyannos, 1975; Girolami et al., 1981; Prokopy et al., 1984). Here, one might suspect selection would favor female detection of larvae, or their effects.

Although studies aimed at understanding the ecological significance of these chemical stimuli are of interest to many researchers, most data concerning residual activity of ODPs have been generated by applied entomologists: if ODPs could be isolated, identified, and synthesized, spraying host crops might become an important new approach to pest management, especially if used in conjunction with appropriate traps to capture deterred females (Prokopy, 1972, 1975; Boller, 1981).

In the laboratory and field, we investigated the residual activity of *R. pomonella* ODP over time under dry conditions as well as following exposure to varying intensities and durations of natural and simulated rainfall. Further, because it appeared that host discrimination mediated by pheromone broke down before completion of larval development, we determined whether females could discriminate against larva-infested fruit.

METHODS AND MATERIALS

All flies bioassayed in lab tests and utilized for fruit infestations or for pheromone collections emerged from puparia formed by larvae that infested *Crataegus* hawthorns. (Hawthorn is the native host fruit of *R. pomonella*). Adults were maintained at 25°C, 60% relative humidity, and 16hr:8hr light–dark photoperiod in 30 × 30 × 30-cm Plexiglass-screen cages and provided a diet of sucrose, enzymatic yeast hydrolyzate, and water.

Unless indicated, for all bioassays of female response to various fruit treatments, Downy hawthorns (*Crataegus mollis*) were used. A total of 5–9 treated and control assay fruit was hung 6–8 cm apart from the ceiling of a Plexiglass-screen observation cage (30 × 30 × 30 cm). Unless otherwise stated, four cages were observed simultaneously and at least 20 different flies were bioassayed. A single mature *R. pomonella* female, which had just begun oviposition in a clean fruit attached to the end of a dissecting probe, was introduced into the assay cage by placing the probe near the cage floor. The female was allowed to fly to an assay fruit overhead and subsequently allowed to visit assay fruit for up to 2 hr. Females were excluded from tests if they rejected several (ca. 5) successive clean fruit. Acceptance (attempting oviposition before leaving) or rejection (leaving without attempting oviposition) was recorded for each visit to a fruit. When a female did accept a fruit, she was, immediately following egg deposition, gently transferred to a nonassay fruit, where she commenced and completed ovipositior dragging. In this way, contamination of assay fruit with pheromone was minimized.

Residual Activity of ODP over Time under Dry Conditions. Fresh-picked, 15-mm-diam. sour cherries were placed in a high humidity plastic box, and either 14, 10, 7, 3, or 0 days prior to behavioral bioassays of pheromone activity, each of several fruit was pheromone marked by five *R. pomonella* females. This level of pheromone deposition is known to be highly deterrent to arriving females (Averill and Prokopy, unpublished data). We used cherries in place of hawthorns because they were available and less likely to rot as rapidly. To obtain pheromone-marked fruit free of egg infestation, females that had just oviposited in nonassay fruit were transferred to assay fruit, where they commenced and completed ovipositor dragging. Unmarked control fruit were held in an identical manner as treated fruit. All treatments were bioassayed simultaneously. Bioassays were run with five treated fruit (marked 14, 10, 7, 3, or 0 days prior to bioassay) plus two clean control fruit. The experiment was replicated twice.

A second series of sour cherries was maintained as above, but at 14, 10, 7, 3, or 0 days prior to bioassay, several fruit were swabbed with a water extract of ODP. Pheromone extract was prepared as follows: pheromone was collected by rinsing hawthorns used for oviposition with a known volume of distilled water. The amount of pheromone collected was estimated by counting the number of oviposition punctures in each washed fruit: 1 puncture = 1 dragging bout equivalent (DE). In this experiment, we applied a concentration of 30 DE/fruit, an amount known to elicit a high level (ca. 89%) of fruit rejection by egg-laying females (Averill and Prokopy, unpublished data). Bioassays were set up as in the above experiment, and the experiment was replicated twice.

Residual activity of pheromone in the field was evaluated using 18- or 19-mm-diam. unpicked *C. mollis* fruit. Five *R. pomonella* females were allowed to deposit pheromone on a single fruit either 21, 16, 12, 8, 4, or 0 days prior

to bioassay (= treated fruit). As with laboratory-held fruit, marking females were not allowed to oviposit in the assay fruit. All pheromone-marked and clean control fruit were protected from rainfall by plastic hoods with mesh sides and bottom. Bioassays were run with six treated fruit plus two clean, control fruit.

Effect of Rainfall on Activity of ODP. C. mollis fruit, 15–16 mm diam, which had been picked the previous season and refrigerated for up to nine months, were pheromone-marked in the lab. Females were allowed to deposit an amount of pheromone equivalent to 6 dragging circles/fruit (1 dragging circle = ovipositor extended over a distance equivalent to the circumference of the fruit). Fruit were pheromone-marked 10 min to 2 hr prior to rainfall initiation, and along with clean control fruit, where hung by wires among natural growing clusters of *C. mollis* in the field. Several additional pheromone-marked fruit were similarly hung, but were rain-protected by plastic hoods as described above. Individual bioassays were run with two each of: rain-exposed, clean control fruit; rain-exposed, pheromone-marked fruit; and non-rain-exposed, pheromone-marked fruit. Following each rain event, fruit were bioassayed using a minimum of 12 flies.

Effect of Simulated Rain on Activity of ODP. Fruit were prepared as for field tests, but were hung on wires and exposed to simulated rainfall. Artificial rain was produced using an adjustable sprinkler attached to a garden hose. Fruit, 16 mm diam., marked with six dragging circles were bioassayed following two different intensities of artificial rain: light–moderate (4.5 mm/hr) and extremely heavy (32 mm/hr). Several of these fruit were collected following each of 0, $\frac{1}{2}$, 1, or 2 hr of rain exposure (= treated fruit). Unmarked control fruit were exposed to the rainfall for 2 hr. Bioassays consisted of four treated and two control fruit. Each simulated rain type was replicated twice, on different days.

In both the natural and simulated rainfall tests, percentage loss in pheromone activity following a rain treatment was established by: (1) calculating the difference between percentage acceptance of clean control fruit and pheromone-marked, non-rain-exposed fruit, (2) calculating the difference between percentage acceptance of pheromone-marked, rain-exposed fruit and pheromone-marked, non-rain-exposed fruit, and (3) determining percentage loss by calculating what percentage the second value was of the first.

Effect of Presence of Developing Larva on Fruit Acceptance by Ovipositing Females. We ran the following tests using picked sour cherries and *C. oxyacantha* hawthorns because a bacterial rot destroyed the unpicked *C. mollis* fruit we initially infested.

Fresh-picked, 15-mm-diam. sour cherries were placed in a high-humidity plastic box (= day 0). On days 0, 5, and 9, several fruit were infested by allowing a female to oviposit a single egg. Females were not allowed to deposit pheromone. Control fruit were held in an identical manner as infested fruit. Bioassay of infested fruit was run on day 14, so that fruit possibly containing a single first, second, or third instar larva could be bioassayed simultaneously.

The response of a total of nine individual flies was observed in each of three bioassay cages containing six presumably infested fruit plus two control fruit. Upon completion of bioassays, dissection of fruit revealed that a total of five bioassay fruit contained no larvae, five contained a first instar, four a second instar, and five a third instar.

For *C. oxyacantha* tests, naturally infested, 9-mm-diam. fruit were picked on the day of bioassay. Fruit were inspected for oviposition punctures, thoroughly washed to remove ODP, and then six singly punctured fruit and two unpunctured fruit were included in a bioassay. Fifteen flies were observed in each of four bioassay cages. Dissection of the presumably infested fruit revealed that two contained no larvae, four contained a first instar, six a second instar, and 12 a third instar.

RESULTS

Under dry conditions, a relatively linear decline in activity of *R. pomonella* ODP over time was observed both under lab and field conditions (Figure 1). Analysis of covariance (Dunn and Clark, 1974) to test for differences among the separate least-squares lines of the three test conditions ($F = 0.29, p > 0.10$,

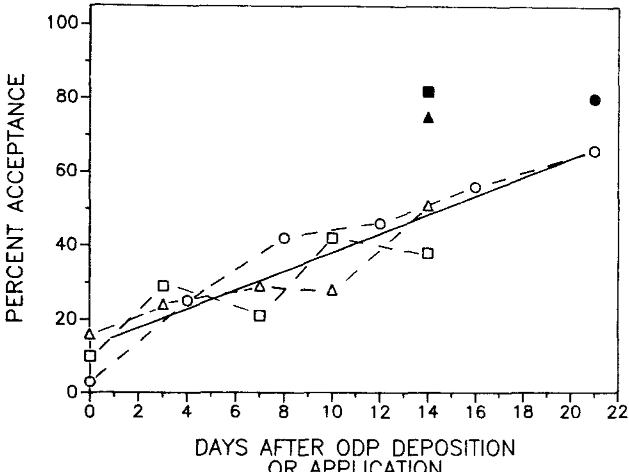

FIG. 1. The residual activity over time of *R. pomonella* ODP under dry conditions in the lab or field. Treatments were: fly-marked fruit that were held in the lab (□) and the corresponding clean control fruit (■); field-exposed, fly-marked fruit (○) and control fruit (●); ODP-extract-marked fruit that were held in the lab (△) and control fruit (◀). "Fly-marked" fruit were pheromone-marked by five *R. pomonella* females. All treatments within a test were bioassayed simultaneously; thus, values for control fruit are represented by a single point. The least-squares regression line for all data points (solid line) is shown ($y = 12.6 + 2.5x$; $r^2 = 0.86$, $N = 16$).

NS) indicated that the combined data could be well described by a single regression line. Thus, there were no significant differences in rate of decline of activity under lab vs. field conditions or between female-deposited vs. extract applied ODP. Further, the pheromone proved moderately stable, even on growing fruit under natural conditions, with some activity persisting after three weeks. For the combined data, the half-life of ODP was 10.7 days. This was calculated from the regression equation of days after ODP deposition or application against the log of percent rejection of fruit treatments: $y = 4.29 - 0.0645x$. Because overall rejection of unmarked control fruit was approximately 20%, the regression line was shifted by subtracting 20% from each percent rejection value to account for this "background."

A distinct decline in pheromone activity resulted from exposure to both natural and simulated rainfall (Figures 2 and 3). In nature, the most severe impact on pheromone persistence followed a torrential 20 min downpour (9 mm) and a heavy $4\frac{1}{2}$-hr rain (25 mm) where 61% and 50%, respectively, of activity was lost. Substantially less activity (ca. 13%) was lost following ex-

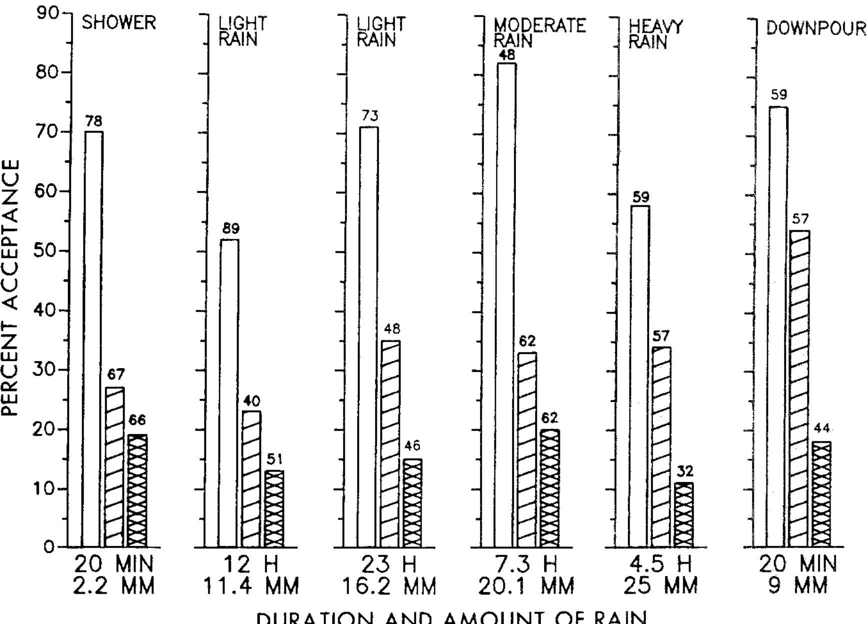

FIG. 2. The residual activity of *R. pomonella* ODP exposed to various durations and intensities of natural rainfall. Treatments were: clean control, rain-exposed fruit ([open bar]); ODP-marked, non-rain-exposed fruit ([cross-hatched bar]); ODP-marked, rain-exposed fruit ([hatched bar]). Values above bars represent the number of female arrivals on each fruit treatment.

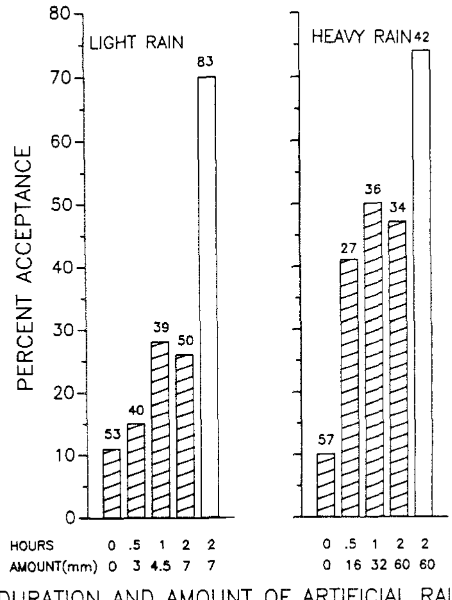

FIG. 3 The residual activity of *R. pomonella* ODP exposed to various durations of a light or heavy simulated rainfall. Treatments were ODP-marked, rain-exposed fruit (▨) and clean control, rain-exposed fruit (▭). Values above bars represent the number of female arrivals on each fruit treatment.

posure to a 20-min shower (2.2 mm), whereas an intermediate loss (21–35%) in activity resulted from two longer-term (12 and 23 hr) light rains (11.4 and 16.2 mm) and a 7.3-hr moderate rain (20.1 mm) (Figure 2). Tests of simulated rainfall (Figure 3) produced similar losses in ODP activity: following $\frac{1}{2}$-, 1-, or 2-hr exposures to a light–moderate rain (3, 4.5, or 7 mm), approximately 8, 34, and 30% total activity, respectively, was lost. Following $\frac{1}{2}$-, 1- or 2-hr exposures to a very heavy simulated rainfall (16, 32, or 60 mm), approximately 62, 76, and 70% total activity, respectively, was lost.

Females were able to discriminate against fruit containing second or third instar conspecific larvae (Table 1). Fewer ($P < 0.05$, G test) females attempted oviposition in cherries (test A) that contained a third-instar larva (31% acceptance) than in controls (70%) or fruit containing a first-instar larva (63%). Discrimination against infested fruit was stronger in the smaller *C. oxyacantha* hawthorns (test B). Acceptance of fruit containing a second-instar larva (33%) was less ($P < 0.05$, G test) than that of controls (56%) or fruit containing a first-instar larva (53%), and only 2% of arriving females accepted fruit containing a third-instar larva.

TABLE 1. FEMALE *R. pomonella* ACCEPTANCE OF 15-mm-diam. SOUR CHERRIES (TEST A) OR 9-mm-diam. *Crataegus oxyacantha* HAWTHORNS (TEST B) INFESTED WITH 1 CONSPECIFIC LARVA

Treatment	Number of female arrivals on fruit	boring attempts (%)[a]
Test A		
Clean control	33	70 a
1st instar	32	63 a
2nd instar	31	52 ab
3rd instar	32	31 b
Test B		
Clean control	50	56 a
1st instar	15	53 a
2nd instar	40	33 b
3rd instar	106	2 c

[a] Values in the same column followed by the same letter are not significantly different at the 5% level according to a pairwise *G* test.

DISCUSSION

Stability over time of the ODP of *R. pomonella*, as demonstrated here, confirms and expands on earlier work that showed high persistence of this pheromone for at least four days under dry laboratory conditions (Prokopy, 1972). Studies of several other tephritids have also shown moderate to substantial persistence in ODP activity under dry conditions (Averill and Prokopy, 1987). Perhaps the active components of these ODPs are similar in chemical identity.

Other phytophagous insects respond to oviposition deterrents (of either insect or plant origin) that persist for days or weeks, including those of the sorghum shootfly, *Atherigona soccata* (Raina, 1981), *Pieris brassicae* butterflies (Schoonhoven et al., 1981), and the European corn borer, *Ostrinia nubilalis* (Dittrick et al., 1983).

Data presented here and in other studies (Prokopy, 1972; Prokopy et al., 1982) demonstrate that *R. pomonella* ODP is highly soluble in water. Indeed, most known oviposition deterrents of phytophagous insects are water soluble, including the ODPs of all tephritids investigated to date (Averill and Prokopy, 1987), as well as of the alfalfa blotch leafminer (*Agromyza frontella*) (McNeil and Quiring, 1983), the sorghum shootfly (Raina, 1981), the European corn borer (Dittrick et al., 1983), and *P. brassicae* (Schoonhoven et al., 1981).

The water solubility of *R. pomonella* OPD may lessen its efficacy in field applications. Indeed, Katsoyannos and Boller (1980), found a reduced effect of

Rhagoletis cerasi ODP sprays on cherry trees following a heavy rainfall. In our simulated rain tests, some pheromone activity remained even following a 2-hr heavy washing. Perhaps some ODP compounds bind to fruit surface components, or, because ODP is released with gut contents during deposition of a pheromone trail (Prokopy et al., 1982), perhaps the presence of feces slows the removal or decay of ODP. This possibility, combined with partial protection of ODP-marked fruit afforded by foliage cover, may result in at least some retention of pheromone effectiveness even under substantial rainfall conditions.

Although *R. pomonella* ODP might seem a poor resource partitioning cue because of its water solubility and only moderate stability, its disadvantages may be balanced by such considerations as low physiological cost of producing ODP (Prokopy, 1981). Alternatively, ODP deposition and recognition may originally have served to deter a female from hawthorn fruit already containing one of her own eggs. In such a case, the pheromone may need be only short-lived, owing to the fact that a foraging female tends to lay a single egg per fruit until all clean fruit are exhausted in a cluster (Averill and Prokopy, unpublished data). She then usually moves to adjacent clusters until she has laid about 10 or so eggs per day. In the evening, the female often moves to and remains in tree top (Prokopy et al., 1972) and, because there usually are thousands of hawthorn fruit per host tree, it would be unlikely she would revisit the same clusters during the next few days.

Further, the pheromone may deter egg-laying initially, and, after time, other partioning factors may come into play. For example, *R. pomonella* larval infestation promotes premature abcission, and, as we have shown, females are able to detect developing larvae or their effects. With small fruit (ca. 9 mm), where larval competition is exceptionally intense (Averill, 1985), we found that females clearly were able to discriminate against fruit within 8–10 days following introduction of an egg. In larger fruit (15 mm diam.), significant discrimination occurred against fruit 12–14 days following infestation (when third-instar larvae were present), although reduced acceptibility occurred after 8–10 days.

The tephritids *R. fausta* and *A. suspensa*, which also lay a single egg per fruit, were not influenced by presence of first- or second-instar larvae in 15-mm-diam. host fruit (Prokopy et al., 1977), but response to presence of third-instar larvae or to larvae developing in smaller fruit was not tested. Among tephritids that lay a clutch of eggs per ovipositional bout, *R. completa*, *C. capitata*, *Dacus cucurbitae*, and *D. tyroni* all discriminate against fruit infested with early instar larvae (Cirio, 1972; Fitt, 1984; Prokopy and Koyama, 1982; Prokopy et al., 1978). As in *R. pomonella*, Fitt (1984) found that *D. tyroni* females more strongly discriminate against small fruit containing larvae. He suggested that in such fruit, larvae are relatively closer to the fruit surface than would be the case in a larger fruit. Thus, larval activity (movement) or effects (e.g., release of volatile deterrents of larval or fruit origin from lacerated tissue) would be more easily detected by ovipositing females.

We did not study how *R. pomonella* females discriminated against larval infested fruit. Discrimination occurred after landing, because similar numbers of females visited infested and uninfested fruit. Further, neural receptors on the ovipositor apparently were not involved because females were able to discriminate against infested fruit without probing. Females that did insert the ovipositor were just as likely to complete egg-laying in infested fruit as in uninfested fruit. These results suggest that females utilize short-range olfactory receptors or contact chemoreceptors or mechanoreceptors to discriminate against fruit containing larvae. In studies of other tephritids, Girolami et al. (1981) reported that volatile deterrents released form olive tissues attacked by *Dacus oleae* elicit female deterrence, and Fitt (1984) showed that oviposition was inhibited by decomposed host tissue from which larvae had been removed. Fitt (1984) suggested that chemical changes in the host may be due to proliferation of bacteria that release an inhibitory chemical. Bacteria are thought to be important or essential for larval development of several tephritid species, including *R. pomonella* (Allen and Riker, 1932; Allen et al., 1934; Prokopy, 1977; but see Howard et al., 1985). Further, the possibility that female *R. pomonella* are able to detect larval movements within the fruit cannot be ruled out. Two parasitic wasps, *Biosteres longicaudatus* and *Opius oelleus*, utilize host vibration cues to locate their larval hosts (Lawrence, 1981; Glas and Vet, 1983).

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STEREOSPECIFIC SYNTHESIS OF (Z,Z)-3,5-TETRADECADIENOIC ACID, A COMPONENT OF *Attagenus elongatulus* (CASEY) PHEROMONE

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Abstract—A simple stereospecific synthesis of (*Z*,*Z*)-3,5-tetradecadienoic acid is described based upon coupling of 1-decyne and 3-butyn-1-ol to give 3,5-tetradecadiyn-1-ol. Subsequent reduction of the diyne to (*Z*,*Z*)-3,5-tetradecadien-1-ol and oxidation of the dienol gave the desired acid. *Attagenus elongatulus* were strongly attracted to the pure acid.

Key Words—Coleoptera, Dermestidae, *Attagenus elongatulus*, (*Z*,*Z*)-3,5-tetradecadienoic acid, Pheromone.

INTRODUCTION

A major component of the sex pheromone of the dermestid beetle *Attagenus elongatulus* (Casey) was identified as (Z,Z)-3,5-tetradecadienoic acid (Fukui et al., 1977). The synthesis of this acid has been reported (Silverstein et al., 1967; Rodin et al., 1970) by methods which depended upon gas chromatographic separation of geometrical isomers. Here we report a simplified, stereospecific synthesis of (*Z*,*Z*)-3,5-tetradecadienoic acid.

METHODS AND MATERIALS

1-Decyne and 3-butyn-1-ol were purchased (Farchan Div., Albany International).

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1-Bromo-1-decyne was prepared by a reported method for a homolog (Brandsma, 1971) in 88% yield, bp 55–57°/0.5 torr, pure by GC (EGSS-X). [^{13}C]NMR (delta values–assigned carbon): 14.03–C10, 19.75–C3, 22.08–C9, 31.80–C8, 37.43–C1, 80.52–C2.

3,5-Tetradecadiyn-1-ol was prepared as described by Brandsma (1971) for a homolog in 88% yield following low-temperature crystallization from pentane–hexane, 98% pure by GC (EGSS-X). [^{13}C]NMR: 14.03–C14, 19.17–C7, 22.68–C13, 23.72–C2, 31.84–C12, 60.96–C1, 65.05, 67.32, 73.69, 78.44–C3–6.

(Z,Z)-3,5-Tetradecadien-1-ol was prepared by reduction of the above diynol with dicyclohexylborane as described for reduction of a similar diynol (Svirskaya et al., 1980; see also Zweifel and Polston, 1970) in 82% yield, bp 112–114°/0.1 torr, pure by GC (EGSS-X and 007-CPS-2 50 m capillary, Quadrex Corp.). [^{13}C]NMR: 14.10–C14, 22.74–C13, 27.62–C7, 31.19–C2, 31.97–C12, 62.39–C1, 123.35. 126.66, 126.86, 133.48–C3–6.

(Z,Z)-3,5-Tetradecadienoic acid was prepared by oxidation of the alcohol with Jones reagent (Djerassi, 1956), i.e., 2.67 M CrO_3 in H_2SO_4. A number of experiments was carried out to determine suitable conditions. A typical experiment was as follows: To a solution of 2.0 g (9.52 mmol) of (Z,Z)-3,5-tetradecadien-1-ol in 200 ml acetone was added dropwise at 10–16°C 14.2 ml Jones reagent while stirring vigorously under N_2. After 10 min, the mixture was poured into 500 ml ice and saline (saturated NaCl solution). The product was extracted with ether and washed twice with saline. The ether solution was dried and, after filtration and evaporation, gave 1.76 g of yellow oil.

The crude product was applied in approximately four equal portions to a column (4.7 × 45 cm) of 30 μm silica gel and eluted with 1% methanol in hexane. The eluate was monitored with a UV detector at 256 nm. The last eluted peaks from each run were combined to give 0.8 g of oil that was crystallized from hexane to give 0.11 g colorless crystals. GC of a methylated sample (diazomethane) on the 007-CPS-2 capillary showed only one peak. The filtrate was evaporated to yield 0.54 g of oil that was purified on a reversed-phase C-18 column to separate 0.14 g (Z,Z)-3,5-tetradecadienoic acid. Nonanoic acid was identified as the main acid impurity by MW (MS) and comparison with the GC retention of an authentic sample of the methyl ester. GC of the methyl esters of the crude acid fraction also had trace amounts of two tetradecadienoate isomers. These isomers were separated on both the 007-CPS-2 and on a 30 m DB-1 (J & W Scientific) capillary that was coupled to a Finnegan mass spectrometer equipped for CI (chemical ionization) with isobutane. The structure of the (Z,Z)-3,5-tetradecadienoic acid was confirmed by [^{13}C]NMR: 177.7–C1; 134.8, 127.0, 122.6, 120.9–C3–6; 32.8–C2; 31.9–C12; 27.7–C7; 22.7–C13; 14.06–C14; and by MS-CI which gave MW 238.

RESULTS AND DISCUSSION

The syntheses scheme is outlined in Figure 1. The conversion of 1-decyne to 1-bromo-1-decyne was effected by Brandsma's (1971) procedure. The bromodecyne was coupled by Chodkiewitz reaction (Brandsma, 1971) to yield 3,5-tetradecadiyn-1-ol. The alternative coupling of 1-decyne and 4-bromo-3-butyn-1-ol gave only ca. 38% conversion in our hands.

Our attempts to reduce 3,5-tetradecadiyn-1-ol by hydrogenation over Lindlar's catalyst (Lindlar and Dubuis, 1966) were unsatisfactory, giving a complex mixture of products. Chemical reduction using dicyclohexylborane gave smooth conversion of 3,5-tetradecadiyn-1-ol to (*Z,Z*)-3,5-tetradecadien-1-ol (Svirskaya et al., 1980).

Oxidation of (*Z,Z*)-3,5-tetradecadien-1-ol to the corresponding acid was carried out in moderate yield with the Jones reagent (Djerassi, 1956). Oxidation of an alcohol to the corresponding acid with chromic acid has been plagued by oxidation of the in situ-formed hemiacetal to ester (Craig and Horning, 1960) so that yields in the 20–50% range are obtained (Holland and Gilman, 1974). Holland and Gilman reported an improved procedure involving reversed addition, i.e., slow addition of the alcohol to the chromic acid solution, and were able to reduce the yield of ester substantially. In our hands this method gave good results with a model compound but poorer results than we obtained with the normal addition procedure with tetradecadienol as substrate, probably due

$$CH_3(CH_2)_7C{\equiv}CH \xrightarrow[\text{2) } Br_2]{\text{1) } n\text{-}C_4H_9Li} CH_3(CH_2)_7C{\equiv}CBr \quad \text{yield 88\%}$$

$$\xrightarrow[\text{Chodkiewitz}]{HC{\equiv}C(CH_2)_2OH} CH_3(CH_2)_7C{\equiv}C\text{-}C{\equiv}C(CH_2)_2OH \quad \text{yield 88\%}$$

$$\xrightarrow{(C_6H_{11})_2BH} CH_3(CH_2)_7\underset{H}{C}{=}\underset{H}{C}\text{-}\underset{H}{C}{=}\underset{H}{C}(CH_2)_2OH \quad \text{yield 82\%}$$

$$\xrightarrow{CrO_3} CH_3(CH_2)_7\underset{H}{C}{=}\underset{H}{C}\underset{H}{C}{=}\underset{H}{C}CH_2CO_2H \quad \text{yield 35\%}$$

FIG. 1. Scheme for synthesis of (*Z,Z*)-3,5-tetradecadienoic acid.

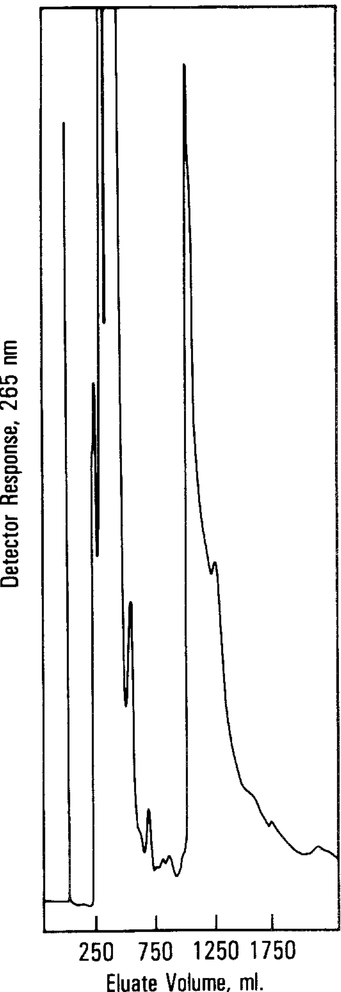

FIG. 2. Separation of acid fraction on 45 × 4.7-cm silica column. Eluent: 12 ml/min of 1% methanol in hexane. Detector: UV, 265 nm, and 2 AUFS. Sample: 305 mg of oxidized (*Z*,*Z*)-3,5-tetradecadien-1-ol. The acid fraction comprised eluate volume from 750 to 1500 ml.

to over oxidation. The Holland and Gilman method subjects the oxidation products to prolonged contact with excess oxidant.

Rodin et al. (1970) reported oxidation of (*Z*)-5-tetradecen-3-yn-1-ol with chromic acid of unspecified strength [reference is made to Fieser and Fieser (1967), but several different chromic acid compositions are mentioned on the cited page including that which we used]. A yield of 67% was reported, but no

purity was specified. A subsequent purification was effected by preparative GC to furnish a purified methyl ester in unspecified yield.

Pyridinium dichromate (Corey and Schmidt, 1979) oxidation of the tetradecadienol gave substantial amounts of α,β-unsaturated acids. Similar results were obtained when a stepwise approach was tried by oxidation of the dienol to aldehyde with dipyridine chromic anhydride complex (Valicenti and Holman, 1976). The aldehyde contained much α,β-unsaturation.

Phase transfer oxidation of alcohols to aldehydes and ketones has been described (Pletcher and Tait, 1978; Landini et al., 1979; Brown et al., 1971). The method of Brown does not require a phase transfer agent, but was applied only to secondary alcohols. We investigated a similar procedure to Brown's for the oxidation of 1-octanol in ether. The product mixture contained 1-octanol, octanal, octyl octanoate, octanoic acid and probably octanal trimer, but all of these were resolvable on a 10-ft Carbowax MTPA glass GC column so that oxidation could conveniently be followed by GC. A simple procedure of shaking 5 min while cooling with tap water an ether solution (25 or 250 ml) of 10 mmol 1-octanol with 0.5, 1, or 2 equivalents of Jones reagent (an equivalent = oxidation to acid) was followed. This procedure with two equivalents of oxidant at the higher dilution gave an 80% yield of octanoic acid and only 12% of octyl octanoate or about the same yields as reported by Gilman and Holland. When this procedure was applied to oxidation of the dienol, we obtained a 61% yield of crude acids, of which 24% was at the desired acid retention (GC of methyl esters) and 36% had retention equivalent to methyl nonanoate.

The best result in the oxidation of the dienol to (Z,Z)-3,5-tetradecadienoic acid was obtained using a 200% excess of the Jones reagent, whereby 71% of the product weight was estimated to be in the acid fraction. The acid fraction consisted principally of the desired dienoic acid (ca. 53%) and nonanoic acid (ca. 35%) (Fig. 2). Trace amounts of isomeric dienoic acids were also present as established by capillary GC-MS-CI. The (Z,Z)-3,5-dienoic acid was separated by reversed-phase chromatography or by crystallization. Absence of isomers in the purified acid was established by capillary GC (007-CPS-2).

Test showed (Z,Z)-3,5-tetradecadienoic acid attractive to *Attagenus elongatulus* (Burkholder, personal communication).

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ALLELOCHEMICALS FROM PALMER AMARANTH, *Amaranthus palmeri* S. WATS.[1]

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Abstract—The presence of Palmer amaranth (AMAPA) residues in the soil reduced fresh weight accumulation in onions and carrots and markedly decreased seedling field establishment of carrots. Solid-phase separation techniques were used to isolate fractions containing water-soluble organic compounds from AMAPA residues and soil amended with such residues. At concentrations of 20–100 mg/liter most of the organic solids thus extracted were inactive in seed germination assays using onion, carrot, AMAPA, and tomato seeds. Extracts from the roots of AMAPA increased 72-hr germination percentages in carrot, AMAPA, and tomato. A time-study of AMAPA residue decomposition in soil showed an increase in extractable inhibitors of onion germination after 62 days but no other significant changes in the activity. The most active allelochemicals from AMAPA proved to be volatile compounds. Volatiles emitted by soil containing AMAPA residues and by the dried and partially rehydrated leaf and flower residues themselves reduced carrot and tomato seed germination to less than 7%. Freshly harvested aerial AMAPA inhibited only carrot seed. Germination of AMAPA and carrot seeds was retarded by exposure to volatiles from dried AMAPA residues. Residues from AMAPA grown in Texas and Louisiana exhibited comparable inhibitory activity after air-drying two weeks. Onion seeds were also inhibited by volatiles from AMAPA residues.

Key Words—Allelopathy, seed germination, *Amaranthus palmeri*, *Allium cepa*, *Daucus carota*, *Lycopersicon esculentum*, onion, carrot, tomato, volatiles, aqueous extracts, weed residues.

[1] Names of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

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INTRODUCTION

Inhibitory allelochemical effects have been attributed to both crop residues (Barnes and Putnam, 1983; Guenzi and McCalla, 1962; Guenzi et al., 1967; Kimber, 1967, 1973; McCalla and Duley, 1949; McCalla and Haskins, 1964; Patrick et al., 1963) and weed residues (Bhowmik and Doll, 1982, 1983, 1984). Water extracts of various weeds have been shown to inhibit plant growth (Bhowmik and Doll, 1984; Gressel and Holm, 1964; Guenzi and McCalla, 1962; Guenzi et al., 1967; Kimber 1967, 1973; Patrick et al., 1963) and seed germination (Bieber and Hoveland, 1968; Bradow, 1985; McCalla and Duley, 1949), and there is evidence that volatile substances released by plants and plant residues also inhibit plant growth (Del Moral and Muller, 1970; Heisey and Delwiche, 1983; 1984; Katsin et al., 1977; Vokou and Margaris, 1982) and seed germination (Evenari, 1949; French and Leather, 1979; Holm, 1972; Katsin et al., 1977; Vokou and Margaris, 1982).

Field studies (Menges, 1985) and laboratory seed germination studies of aqueous and organic solvent extracts (Bradow, 1985; Fischer and Quijano, 1985) indicated the presence of both inhibitory and promotive allelochemicals in *Amaranthus palmeri* S. Wats. (Palmer amaranth, AMAPA). Because fresh weight accumulation and time of maturity can be significantly influenced by the amount of time seedlings require to emerge (Currah, 1978), and because field emergence is related to the germination properties of onion and carrot seeds (Bedford and Mackay, 1973), seed germination assays were used for further screening. Although tomato was not part of the field studies, tomato seeds were quite sensitive in the preliminary studies and were thus included in these bioassays (Bradow, 1985). Volatiles and water-soluble organic compounds isolated from AMAPA residues and soil containing AMAPA residues were screened for biological activity. The paper describes laboratory and greenhouse experiments undertaken to locate and identify those volatile and water-soluble organic compound released by AMAPA residues which may inhibit seed germination in carrot, onion, and AMAPA itself.

METHODS AND MATERIALS

Aqueous Extracts from AMAPA and Soil. Aqueous extracts were prepared from the following soil samples: (1) soil (Hidalgo sandy clay loam from Weslaco, Texas); (2) soil + AMAPA, the same soil amended with 1.2 g/kg AMAPA residues (Menges, 1985); and (3) soil + AMAPA incubated in the greenhouse for 0, 20, 41, or 62 days. In this time study, 2000-g samples of soil + AMAPA were placed in 3.8-liter plastic nursery pots, wet to field capacity, and incubated (mean daily ambient temperature = 18–29°C). Uniform moisture content was maintained by bottom irrigation with deionized water.

For each extraction, 2000 g of specific soil sample was added to 1.5 liters deionized water, pH 6.8. The slurry was stirred briefly, covered with aluminum foil, and allowed to stand at room temperature (21°C, average) for 2 days with occasional stirring. The slurry was filtered through a layer of Celite Analytical Filter Aid (Fisher No. 0211) above a sheet of Whatman No. 2 filter paper in a 32-cm porcelain Buchner funnel, and the soil cake was washed with 200 ml deionized water. The filtrate, pH 7.5, was additionally filtered through a Durapore® HVLP 0.15-μm filter (Millipore) fitted with a prefilter. This aqueous extract was subjected to the solid-phase treatment described below. An additional 200-g sample of soil + AMAPA was separately extracted with 150 ml deionized water, and the filtered solution (pH 7.4) was used directly in the in vitro seed germination bioassays described below.

Similarly, water extracts were made from the aerial and root portions of AMAPA plants field-grown at the Southern Regional Research Center (SRRC) from seeds collected in Weslaco, Texas. The plants were harvested at a height of 1.2–1.7 m after flower anthesis but before seed dispersal. The day after harvesting, aerial and root AMAPA were chopped into segments less than 3 cm in length. A 300-g sample of chopped aerial AMAPA was extracted with 3 liters deionized water (pH 5.5 after filtration) and subjected to the solid-phase separation sequence below. A 100 g-sample of chopped root AMAPA was extracted with 1 liter deionized water (pH 5.9 postfiltration) and similarly treated.

Aqueous-Extract Solid-Phase Separations. The specific aqueous extracts were separately subjected to the following series of solid-phase separations. An aqueous extraction filtrate was drawn by vacuum through two SepPak® C_{18} cartridges (Waters Assoc., No. 51910) connected in series. Adsorbed organic compounds were eluted from this pair of cartridges with 10 ml acetonitrile, followed by 10 ml methanol. The combined eluant solutions (containing a precipitate) were placed on a rotary evaporator and taken to dryness under vacuum (neutral residue).

Eluant passing through the first SepPak® pair was acidified to pH 3 with concentrated HCl and passed through a fresh SepPak® pair. Elution of adsorbed organic compounds from the cartridges and collection of the acidified residue were identical with that used to obtain the neutral residue.

The acidified eluant from the second solid-phase separation was extracted with 3 × 50 ml dichloromethane. The combined extracts were dried over anhydrous sodium sulfate, and the volatiles removed on a rotary evaporator with a room temperature water bath (dichloromethane residue). When the aqueous extracts from soil, soil + AMAPA, aerial AMAPA, or root AMAPA were fractionated using the solid-phase separation scheme described, no starting substrate yielded sufficient dichloromethane residue for bioassay, indicating the effectiveness of the C_{18} packing in removing organic compounds from the aqueous solution. The aqueous layer was reduced to dryness, using an 80°C water bath (final residue).

The water-extracted soil + AMAPA/Celite cake was air-dried, broken into small pieces, and added to 1 liter 0.3 M ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) solution which had been adjusted to pH 7.5 with NaOH (Kaminsky and Muller, 1977). After standing overnight, the mixture was filtered as described in the previous section for the primary aqueous extraction of soil samples (EDTA-treated residue). The EDTA treatment was omitted in the case of the soil-less AMAPA samples.

In Vitro Seed Germination Bioassay. Dried soil and plant extract residues obtained from the solid-phase separations were dissolved in deionized water, pH 6.8 at 20, 50, or 100 mg/liter. Germination of seeds of onion, *Allium cepa* L., cv. Texas Early Grand 502 (Baxter's, Weslaco, Texas); carrot, *Daucus carota* L., cv. Danvers Half-long (Burpee's, Warminster, Pennsylvania); tomato, *Lycopersicon esculentum* Mill., cv. Homestead (Burpee's) and AMAPA collected in Weslaco, Texas, were used to compare the bioactivities of these solutions and parallel deionized water controls. Assay seeds were incubated in the dark as previously described (Bradow, 1985), except that a temperature cycle of 10 hr at 31°C and 14 hr at 21°C was substituted for isothermal incubation at 25°C. Osmolarities of the test solutions were determined with an Osmette A (Precision).

Seed Germination Bioassays of Volatiles from Soil and AMAPA Residues. Using separate 160-mm (id) glass desiccators, seeds of onion, carrot, AMAPA, or tomato, as described in the in vitro aqueous extract assays, were germinated on porcelain desiccator plates covered with double sheets of deionized water-saturated Whatman No. 1 filter paper. Circles (22 mm diameter) had been removed from the center of the paper to allow diffusion of volatiles from samples in the desiccator wells. The filter paper sheets were divided into eight equal segments containing the same number of seeds (25 AMAPA, or 20 of the other three species), and each segment was treated as a replicate for purposes of statistical analysis.

Test samples consisted of one of the following: 170 g soil, 170 g soil + AMAPA from a field amended with 8 or 1.5 kg AMAPA/m^2 (Menges, 1985), 20 g fresh weight or 5 g dry weight of root or aerial AMAPA. Aerial and root AMAPA were chopped into segments smaller than 3 cm before drying. Portions of SRRC-grown aerial AMAPA were air-dried in the laboratory for up to 22 weeks before assay for bioactive volatiles. Weslaco AMAPA was shipped in Dry Ice from Texas, and all refrigerated or frozen AMAPA was allowed to dry in the open at room temperature for 24 hr before use. Weslaco AMAPA was divided into roots, mature seedheads, green stems, red pigmented woody mature stems, and leaves and flowers. Portions of the Weslaco aerial AMAPA were air-dried 15 days, and part of the Weslaco root AMAPA was dried 1 week in the laboratory before comparative assays. Controls consisted of 50 g fine sand.

A test sample or control in a 5 × 10-cm glass crystallizing dish was placed

in each desiccator well and 10 ml deionized water were added. Each desiccator was sealed with minimal stopcock grease, wrapped light-tight in aluminum foil, and placed on a greenhouse bench or in a biological incubator (Percival I-32LLVL) operated on a 10 hr/31°C day/21°C night cycle. During the periods of the greenhouse experiments, the mean daily ambient air temperature varied from 18 to 29°C with a maximum of 34°C and a minimum of 15.5°C. Seed germination, using radicle emergence as the criterion, was evaluated after 72 hr incubation. In most experiments the crystallizing dish and contents were removed and an additional 10 ml deionized water were added to each desiccator well. The seeds were then further incubated in the resealed desiccators for 96 hr before a second evaluation of germination and relative seedling growth. In several experiments the test samples were allowed to remain throughout the 7-day germination period. Control assays were repeated at bimonthly intervals as checks on assay seed vitality.

Statistical Analyses. Data from the completely random experimental designs for each seed species were analyzed separately. Germination count data were normalized by the transformation $(X + 0.5)^{1/2}$ and subjected to appropriate analyses of variance (Sokal and Rohlf, 1981). A significance level of $P = 0.05$ was used for the aqueous extract data and of $P = 0.01$ for the volatiles data. The time-study data were also subjected to regression analysis (significance level, $P = 0.05$).

RESULTS AND DISCUSSION

Solid-Phase Separation of Aqueous Extracts of Soil and Soil + AMAPA Mixtures. Like the aqueous extracts from aerial AMAPA residues (Bradow, 1985), the aqueous extract from soil + AMAPA residues had sufficient osmotic activity (50 mosm) to inhibit seed germination in carrot, AMAPA, and tomato (59%, 24%, and 94% inhibition, respectively). Onion seeds were unaffected. Dilution of the extract 1:1 with deionized water produced a solution of 24 mosm which inhibited tomato 79% and had no significant effect on the other assay seeds. The bioassay seeds incubated in 50 mosm solutions of polyethylene glycol 8000 (Michel, 1983) were similarly inhibited (data not shown).

The fractionation of the extracts from soil and soil + AMAPA resulted in milligram quantities of the neutral and acidified residues which were tested at a maximum level of 20 mg/liter. The osmolarities of the resuspended residues never exceeded 10 mosm, and the initial solution pH levels were circumneutral.

When applied as solutions with osmolarities below inhibitory levels, none of the redissolved extracts from soil or soil + AMAPA had any significant effect on AMAPA germination, and the extracts from soil + AMAPA had no effect on either carrot or onion germination (Table 1). Secondary treatment with EDTA produced an acid residue from soil which increased onion germination

TABLE 1. EFFECTS ON SEED GERMINATION OF REDISSOLVED RESIDUES FROM SOLID-PHASE SEPARATIONS OF AQUEOUS EXTRACTS FROM WESLACO, SOIL AND SOIL + AMAPA RESIDUES

	Germination (%)[a]							
	ONION		CARROT		AMAPA		TOMATO	
RESIDUE (mg/liter)	Soil	Soil + AMAPA	Soil	Soil + AMAPA	Soil	Soil + AMAPA	Soil	Soil + AMAPA
Neutral (20)	65.6	73.1	39.4	33.7	28.0	20.5	48.1	32.5*
Acid (20)	51.9*	66.3	31.3*	42.5	25.5	24.5	50.6	66.3
Final (20)	61.3	68.1	40.0	46.9	25.5	20.5	56.3	55.6
(100)	62.5	61.9	25.6*	36.9	32.0	21.0	28.7*	59.4
EDTA/neutral (20)	71.9	68.1	41.9	38.7	22.0	25.0	45.0	74.4*
EDTA/acid (20)	76.3*	63.1	31.9*	45.0	24.4	23.5	17.5*	34.4*
Control ($\bar{X} \pm$ SE)	65.3 ± 1.3		37.9 ± 1.4		24.7 ± 0.7		55.2 ± 1.2	

[a] Percentages are means of eight replications. Asterisk (*) indicates values significantly different from parallel deionized water control ($P = 0.05$) according to linear contrast testing. Control values are experiment means, shown for comparison.

while significantly inhibiting that of tomato and carrot. The EDTA/acid residue from soil + AMAPA also inhibited tomato seeds to a lesser degree. The EDTA/ neutral residue from soil + AMAPA increased tomato seed germination, while the initial neutral residue from that source was highly inhibitory of the same seed species. Where sufficient extract residues were obtained to increase the bioassay level to 100 mg/liter, all soil + AMAPA residues were inactive. Carrot and tomato seeds were inhibited by 100 mg/liter final residue from soil, but not soil + AMAPA. Any further increase in residue concentration raised the test solution osmolarity above 20 mosm.

Time Study of Changes in Activity of Solid-Phase Separation Extracts from Soil + AMAPA. Incubating the soil + AMAPA mixture under greenhouse conditions produced no significant changes in the activity of the various extract residues with respect to carrot or AMAPA germination (data not shown).

Onion germination was unaffected by the initial neutral residue (Table 2), but the EDTA/neutral residue from 41-day soil + AMAPA promoted onion germination at 100 mg/liter, and the 62-day EDTA/neutral residue very significantly inhibited onion seeds. At 100 mg/liter, the acid residue also inhibited onion germination, and the final residue showed a linear increase in inhibitors of onion seed germination over the 62-day incubation. Concentration was a significant factor only in the activity of the acid residue at 62 days. Incubation time was the significant factor in the effects of the residues on tomato germination as well. The initial neutral residue markedly inhibited, but after 20 days the activity of this residue became promotive. There was also a linear increase with time in tomato seed germination promotion by the final residue. Germination in the parallel water controls remained constant during the nine weeks of the experiment (analysis of variance insignificant at $P = 0.01$). Again, concentration was a significant factor only in assays of the acid residue effects. The 100 mg/liter solutions of the comparable 20-day acid residue inhibited tomato seeds and the comparable 41-day residue had no effect at 100 mg/liter and promoted at 20 mg/liter.

With time, water-extractable inhibitors of onion and promoters of tomato seed germination accumulated in soil amended with AMAPA. However, within the constraint of osmolarity below the inhibitory threshold, nothing in the results from the solid-phase separations indicates any significant or consistent suppression of seed germination by water-soluble substances from AMAPA or soil-containing AMAPA residues.

Solid-Phase Separation of Aqueous Extracts from Root and Aerial AMAPA. To avoid osmotically active salts found in extracts from soil-containing samples, root and aerial AMAPA samples were separately subjected to solid-phase separation. Significant differences existed in the responses of all four test seeds to root versus aerial extract residues (Table 3). Neutral, acid, and final root residues significantly promoted onion, carrot, and tomato seed germination at 20 mg/liter. Increasing the concentration to 100 mg/liter had no effect on the

TABLE 2. CHANGES WITH TIME (DAYS) IN ACTIVITY OF SOLID-PHASE SEPARATION RESIDUES FROM WESLACO SOIL + AMAPA

Residue	Days	Germination (%)[a] Onion 20 mg/liter	Onion 100 mg/liter	Tomato 20 mg/liter	Tomato 100 mg/liter
Aerial AMAPA					
Neutral	0	73.1	—	32.5 c	—
	20	71.3	71.9	76.3 a	67.5 ab
	41[b]	67.5	63.1	76.3 a	68.7 ab
	62[c]	72.5		73.7 a	
EDTA/neutral	0	68.1 bc	—	66.3 ab	—
	20	69.4 bc	65.6 c	59.4 bc	81.3 a
	41	75.6 ab	78.7 a	87.5 a	81.3 a
	62	36.3 d	31.3 d	57.5 c	58.1 c
Acid	0	66.3 bc	—	66.3 ab	—
	20	73.7 ab	72.5 ab	67.5 ab	33.1 c
	41	74.4 ab	71.3 a	78.7 a	56.9 b
	62	61.9 c	33.1 d	78.1 a	83.7 a
Final	0	68.1 b	68.1 b	55.6 c	58.1 c
	20	81.3 a	74.4 ab	61.9 bc	78.7 a
	41	71.3 ab	57.5 c	78.7 ab	86.9 a
Control ($\bar{X} \pm$ SE)		65.3 ± 1.0		55.2 ± 1.5	

[a] Percentages are means of eight replicates. Values associated with a given seed species and followed by the same letter are not significantly different ($P = 0.01$) according to linear contrast testing.
[b] Tested at 20 and 50 mg/liter.
[c] Tested at 50 mg/liter.

TABLE 3. EFFECTS ON SEED GERMINATION OF SOLID-PHASE SEPARATION RESIDUES FROM SRRC-GROWN AERIAL AND ROOT AMAPA

	Germination (%)[a]							
	Onion		Carrot		AMAPA		Tomato	
Extract	20 mg/liter	100 mg/liter	20 mg/liter	100 mg/liter	20 mg/liter	100 mg/liter	20 mg/liter	100 mg/liter
Aerial AMAPA								
Neutral	71.3 bc	71.9 bc	38.1 cde	32.5 de	20.5 cd	15.0 de	73.7 bc	73.1 bc
Acid	69.4 bc	70.0 bc	44.4 cd	27.5 e	15.0 de	14.0 e	64.0 cd	55.6 d
Final	76.9 ab	70.0 bc	33.1 de	34.4 de	14.5 de	19.0 de	60.0 d	73.1 bc
Root AMAPA								
Neutral	77.5 ab	81.3 a	54.4 ab	56.9 ab	27.0 bc	30.5 ab	93.1 a	96.3 a
Acid[b]	76.9 ab	75.6 ab	53.7 ab	57.5 ab	35.5 ab	34.5 ab	91.9 a	93.7 a
Final	74.4 ab	65.0 c	61.9 a	43.1 cd	40.5 a	15.0 de	91.9 a	85.0 ab
Control	65.2 ± 1.1 c		37.9 ± 1.4 cde		24.7 ± 0.7 bc		55.2 ± 1.5 d	

[a] Percentages are means of eight replicates. Values associated with a given seed species and followed by the same letter are not significantly different ($P = 0.01$) according to linear contrast testing.
[b] Assayed at 20 and 50 mg/liter.

activities of the neutral and acid root residue solutions, but the 100 mg/liter final root residue had no effect on onion or carrot seeds. Only the 20 mg/liter final root residue promoted AMAPA germination. In contrast, aerial residues had no effect on carrot germination. At 20 mg/liter, the final aerial residue increased onion germination, and all aerial residues but the 20 mg/liter neutral inhibited AMAPA seeds, in comparison to the parallel controls. The neutral aerial and 100 mg/liter final aerial residues promoted tomato seed germination.

The solid-phase separation technique was ineffective in separating the aqueous root extract into neutral, acid, and final residues of differing bioassay activities. The C_{18} cartridge pairs may have become saturated, allowing the extract components to pass through into the next separation stage; or different compounds of the extract may account for the activity found in the neutral and acid residues. Again, the results from the solid-phase separation of aqueous extracts from aerial root portions of AMAPA do not indicate the presence of water-soluble germination inhibitors.

Volatiles from Soil Containing AMAPA Residues. Under greenhouse (GH) conditions (Table 4), onion, carrot, and tomato seed germination was markedly inhibited by volatiles emitted from soil amended with AMAPA residues. The unamended soil had no effect, compared to the GH control No. 1 which was incubated under the same GH temperature conditions (mean day temperature, 26°C; mean night temperature, 20°C). When the GH controls were repeated during a 7-day period while the mean day temperature was 29°C and the mean night temperature was 20°C, germination of onion, carrot, and tomato seeds was significantly reduced, while that of AMAPA seeds increased (GH control No. 1 vs. GH control No. 2). This lack of reproducibility under GH conditions led to the use of an environmental chamber (EC) operated at temperatures (31°C day, 21°C night) which approximated the temperature conditions in Weslaco during the summer field tests.

After three days of exposure to volatiles from soil + AMAPA, carrot seed germination was markedly reduced, in comparison to both seeds exposed to volatiles from soil and the controls. Soil + AMAPA volatiles also inhibited tomato seeds to a lesser, but still significant, degree. AMAPA seeds were not affected.

Under EC conditions, both the controls and the unamended soil assays were reproducible for all four test seeds, but the inhibition of onion seeds by AMAPA residues in the soil was considerably less than under the first set of GH conditions. The degree of homogeneity in the soil + AMAPA samples also significantly affected the test results. The second soil + AMAPA replicate in the EC tomato seed bioassay contained less apparent AMAPA residue and proved to be significantly less inhibitory than the earlier replicate using a more representative soil + AMAPA sample. Volatiles from soil amended with 1.5 kg AMAPA/m^2 did not inhibit any of the assay seeds (data not shown). This level of residues was not active in field studies (Menges, 1985).

TABLE 4. GERMINATION INHIBITION BY VOLATILES FROM SOIL + AMAPA UNDER GREENHOUSE AND ENVIRONMENTAL CHAMBER CONDITIONS

	Germination (%)[a]							
	Onion		Carrot		AMAPA		Tomato	
Volatiles source	3 days	7 days	3 days	7 days	3 days	7 days	3 days	7 days
Greenhouse								
Soil (GH)	76.3 a	85.0 a	29.4 b	68.1 a	18.0 cd	31.0 c	89.4 a	97.5 a
Soil ± AMAPA	6.9 c	71.3 bc	0.6 c	51.3 b	15.5 d	57.5 ab	0.0 d	94.4 a
Control								
Rep. 1	75.6 a	83.7 ab	43.1 a	61.9 a	16.5 d	44.0 b	83.7 a	93.1 ab
Rep. 2	53.7 b	74.4 abc	25.0 b	71.3 a	31.0 ab	58.0 ab	52.5 b	87.5 b
Environmental chamber								
Soil								
Rep. 1	63.1 ab	80.0 abc	32.5 ab	66.9 a	37.5 a	63.5 a	73.7 a	96.3 a
Rep. 2[b]	66.9 a	83.7 ab	37.5 a	73.7 a	33.0 ab	59.0 a	81.9 a	96.3 a
Soil + AMAPA								
Rep. 1	47.5 b	71.9 bc	6.9 c	70.0 a	29.0 ab	66.0 a	14.4 c	90.0 ab
Rep. 2[b]	53.7 ab	67.5 c	1.9 c	32.5 c	26.0 bc	60.5 a	53.7 b	90.0 ab
Control								
Rep. 1	53.7 ab	74.4 abc	30.6 ab	71.3 a	24.5 b	58.0 ab	73.3 a	87.5 b
Rep. 2	58.1 ab	71.9 bc	32.5 b	71.9 a	23.0 bc	53.5 ab	71.9 a	87.5 b

[a] Percentages are the means of eight replications within a single desiccator. Values in columns which are followed by the same letter are not significantly different ($P = 0.01$) according to linear contrast testing.
[b] Seeds exposed to volatiles throughout 7-day assay period.

After 3-day incubations under GH or EC conditions, the soil samples were removed from the desiccators and the seeds allowed to incubate an additional 4 days. After the 7 days, both the GH and EC onion and tomato bioassays showed no significant residual effects from exposure to soil or soil + AMAPA. When the AMAPA-amended soil remained in the desiccators throughout the entire 7-day EC incubation, only carrot was significantly inhibited. The decreases in tomato and carrot germination after a 3-day exposure to AMAPA volatiles were retardations of germination, rather than reductions in the capacity to germinate. With time, tomato seeds overcame this retardation in the presence of the AMAPA volatiles, but continued exposure markedly reduced carrot seed germination.

Volatiles from Freshly Harvested and Air-Dried AMAPA Residues. Compared to the appropriate controls, a 3-day exposure to volatiles from freshly harvested aerial AMAPA significantly inhibited only carrot germination (Table 5). Aerial AMAPA lost an average of 75% fresh weight after one week of air-drying; the dry weight then stabilized. The sample weights of dried aerial AMAPA were decreased to reflect this water loss. Aerial AMAPA, dried one week and partially rehydrated by the addition of 10 ml H_2O during the assays, released volatiles which significantly inhibited all four seed species at 3 days. The dried aerial AMAPA remained inhibitory of all but onion seeds throughout 22 weeks of air-drying. Onion seeds were not significantly inhibited by volatiles from the dried AMAPA after the second week of drying until, at 22 weeks, volatiles inhibitory of onion seed germination were again present.

When the freshly harvested root and aerial AMAPA samples were removed from the desiccators, all four test seeds reached germination percentages which were not significantly different from those of the controls. The 1-week dried aerial AMAPA residually inhibited onion, carrot, and AMAPA seed germination. This significant residual inhibition of carrot and AMAPA by the dried AMAPA persisted throughout the 22-week period. The 2-week and 6-week dried aerial AMAPA also had a residual inhibitory effect on tomato germination recovery. The 9-week dried data was lost in a power and equipment failure.

Comparison of Activities of Volatiles from Weslaco and AMAPA SRRC Residues. Aerial and root AMAPA, shipped in Dry Ice from Weslaco, also emitted volatiles inhibitory of seed germination (Table 6). The Weslaco aerial AMAPA and mature seedheads were highly inhibitory of all the assay seeds. This is in marked contrast to the inactivity of freshly harvested SRRC aerial AMAPA which had no effect except in the carrot bioassay. The green stem tissue from Weslaco was significantly inhibitory of onion and tomato germination, but less so than the leaves and seed heads. The mature woody stems did not inhibit carrot, AMAPA, or tomato germination significantly. Only the Weslaco seedhead AMAPA had significant residual effect on the germination of all four seeds. Exposure to volatiles from nonwoody Weslaco aerial AMAPA also

TABLE 5. EFFECTS OF 3-DAY EXPOSURE TO VOLATILES FROM FRESHLY HARVESTED AND AIR-DRIED SRRC-GROWN AERIAL AMAPA RESIDUES UPON SEED GERMINATION AFTER 3 AND 7 DAYS

	Germination (%)[a]							
	Onion		Carrot		AMAPA		Tomato	
Weeks dried	3 days	7 days	3 days	7 days	3 days	7 days	3 days	7 days
0	59.4 a	78.1 a	4.4 b	61.3 a	31.0 a	61.5 a	53.7 a	93.1 a
1	31.3 de	58.7 b	0.0 c	29.4 b	7.0 b	10.5 b	2.5 bc	87.5 a
2	33.1 cd	83.1 a	2.5 b	8.7 c	0.5 d	4.0 d	3.1 bc	66.3 b
6	58.7 a	73.7 a	6.9 b	8.7 c	6.0 bc	12.5 b	3.1 bc	66.9 b
9	52.5 ab	—	5.0 b	—	6.5 b	—	3.7 bc	—
12	43.7 bc	73.7 a	0.0 c	11.3 c	2.5 bcd	4.5 d	6.9 b	91.3 a
22	37.5 c	56.9 b	5.0 b	28.1 b	1.5 cd	5.5 c	1.3 b	95.0 a
Controls (±SE)	58.1 (±1.5) a	75.6 (±1.3) a	32.5 (±1.3) a	65.6 (±1.7) a	23.0 (±1.4) a	52.0 (±1.5) a	66.3 (±1.4) a	91.9 (±1.6) a

[a]Percentages are the means of eight replications within a single desiccator. Values in columns which are followed by the same letter are not significantly different ($P = 0.01$) according to linear contrast testing. Nine week, 7-day data lost in equipment failure.

TABLE 6. COMPARISON OF EFFECTS OF 3-DAY EXPOSURE TO VOLATILES FROM UNDRIED WESLACO- AND SRRC-GROWN AMAPA RESIDUES UPON SEED GERMINATION AFTER 3 AND 7 DAYS

	Germination (%)[a]							
	Onion		Carrot		AMAPA		Tomato	
Volatiles source	3 days	7 days	3 days	7 days	3 days	7 days	3 days	7 days
Weslaco								
Leaves/flowers	5.0 c	43.1 bc	0.6 c	71.9 a	5.0 c	16.0 c	0.0 c	96.9 a
Seed heads	3.7 c	26.3 c	0.0 c	54.4 b	0.5 c	3.0 c	1.3 c	69.4 b
Green stems	27.5 b	46.3 bc	21.1 ab	72.5 a	16.5 ab	51.0 a	21.3 b	92.5 a
Mature stems	31.3 b	53.1 ab	17.5 ab	70.0 a	23.5 ab	44.0 a	55.6 a	95.0 a
Roots	31.3 b	60.0 ab	6.9 bc	55.0 b	14.0 b	36.5 ab	64.3 a	96.3 a
SRRC								
Aerial	59.4 a	78.1 ac	4.4 bc	61.3 ab	31.0 a	63.0 a	53.7 a	93.1 a
Roots	50.0 ab	73.7 a	13.7 b	73.1 a	24.5 ab	65.0 a	56.9 a	93.7 a
Control (±SE)	58.1 (±1.5) a	75.5 (±1.3) a	32.5 (±1.3) a	72.1 (±1.3) a	23.1 (±1.4) ab	52.3 (±1.5) ab	66.3 (±1.4) a	92.0 (±1.3) a

[a] Percentages are the means of eight replications within a single desiccator. Values in columns which are followed by the same letter are not significantly different ($P = 0.01$) according to linear contrast testing.

had a residual inhibitory effect on onion seeds. AMAPA seed germination was residually retarded by Weslaco seedheads and leaves and flowers.

Volatiles from freshly harvested SRRC-grown AMAPA significantly inhibited only carrot germination and had no residual effect on any of the assay seed species. Weslaco AMAPA roots emitted volatiles which reduced onion, carrot, and AMAPA germination after 3 days of exposure, the carrot germination reduction persisting after the volatiles source was removed.

After drying for 2 weeks and partial rehydration, Weslaco leaf/flowers and seedhead AMAPA were still highly inhibitory of onion, carrot, and tomato (Table 7). Dried SRRC Aerial AMAPA inhibited carrot, AMAPA, and tomato.

Volatiles from 1-week dried roots of SRRC AMAPA inhibited tomato and particularly carrot seeds. Weslaco root volatiles were less active.

Residual inhibition in carrot, AMAPA, and tomato seeds exposed to volatiles from dried Weslaco AMAPA was generally less profound than that observed in seeds exposed to dried SRRC aerial AMAPA. After 7 days, carrot seed germination was reduced by less than 20% by the 3-day exposure to Weslaco seedhead AMAPA, while similar exposure to 2-week dried SRRC aerial AMAPA nearly eliminated carrot germination. Volatiles from the dried Weslaco seedhead AMAPA residually inhibited onion and AMAPA germination, and AMAPA seeds showed continuing effects from exposure to Weslaco leaves/flowers AMAPA.

Before air-drying, the woodier stem and root AMAPA samples were less inhibitory of seed germination than the Weslaco leaves/flowers and seedhead AMAPA. Except in the carrot assay, freshly harvested SRRC aerial and root AMAPA were inactive. Air-drying for a week or more increased the inhibitory activity of freshly harvested AMAPA, but decreased that of the Weslaco aerial and seedhead AMAPA. This suggests that biochemical decomposition and tissue disruption beyond the chopping process common to all samples increased the production or release of the inhibitory volatiles.

One possible source for such inhibitors would be the microflora present on all the AMAPA samples. After the 3-day desiccator incubation, all AMAPA samples showed visible fungal populations, and preliminary electron micrographs have shown similar extensive mixed fungal (and bacterial) growth on the fresh SRRC aerial AMAPA, the freshly thawed Weslaco AMAPA, and on both the air-dried Weslaco and SRRC AMAPA samples. With similar fungal networks on both noninhibitory and inhibitory AMAPA samples, equivalent O_2/CO_2 ratios should exist in all assays, but the possibility that germination was reduced by increased CO_2 concentrations due to the presence of microflora will be examined in studies designed to investigate the effects of specific volatile inhibitors upon seed respiration. The possibility that the fungi are the source of the inhibitory volatiles is also being investigated, although preliminary studies indicate that fungi collected from the AMAPA samples and cultured on potato dextrose agar (PDA) emit no volatiles which inhibit onion, carrot, or AMAPA

TABLE 7. COMPARISON OF EFFECTS OF 3-DAY EXPOSURE TO VOLATILES FROM REHYDRATED AIR-DRIED WESLACO- AND SRRC-GROWN AMAPA RESIDUES UPON SEED GERMINATION AFTER 3 AND 7 DAYS

	Germination (%)[a]							
	Onion		Carrot		AMAPA		Tomato	
Volatiles source	3 days	7 days	3 days	7 days	3 days	7 days	3 days	7 days
Weslaco								
Leaves/flowers	20.6 b	52.5 bc	10.6 cd	78.7 a	12.5 abc	24.5 b	25.6 c	95.0 a
Seed heads	12.5 c	32.5 c	7.2 cd	55.6 ab	5.5 bc	8.5 c	35.0 bc	90.0 a
Green stems	38.1 abc	60.0 ab	25.6 bc	80.0 a	18.0 ab	45.5 a	70.0 a	94.4 a
Mature stem	39.4 ab	55.0 abc	40.0 a	78.7 a	18.5 ab	43.0 ab	61.3 ab	95.6 a
Roots	40.6 ab	58.1 abc	22.5 bc	72.5 a	16.5 ab	50.0 a	58.1 ab	96.3 a
SRRC								
Aerial	33.1 abc	83.1 a	2.5 d	8.7 c	0.5 c	4.0 c	1.9 c	66.3 b
Roots[b]	48.1 a	56.9 abc	11.9 cd	55.6 ab	26.0 a	51.0 a	35.6 bc	88.7 ab
Control (±SE)	58.1 (±1.5) a	75.5 (±1.3) ab	32.5 (±1.3) ab	72.1 (±1.3) a	23.0 (±1.4) a	52.3 (±1.5) a	66.3 (±1.4) a	92.0 (±1.3) a

[a]Percentages are the means of eight replications within a single desiccator. Values in column which are followed by the same letter are not significantly different ($P = 0.01$) according to linear contrast testing.
[b]Roots dried one week.

germination. Germination of tomato seeds incubated 3 days above fungi on PDA was reduced to 62% of that of a control incubated above sand.

The dramatic reductions and residual inhibitions of seed germination in four different seed species by volatiles from AMAPA residues and soil containing those residues indicate the presence of a very important factor in allelopathic interactions which has been mostly overlooked. Classic natural products and allelochemical isolations generally involve freeze-drying and/or multiple solvent removal steps, all of which remove any volatile compounds of the plant residues and soil under study. The potent volatile inhibitors emitted from AMAPA residues could well explain the decreased stands of carrots in fields amended with such residues, and the residual effects of exposure to the volatiles could be important factors in fresh weight reduction and delayed maturity in crops. Delay in seed germination and seedling emergence reduce yields in carrots and onions, particularly under less than ideal growing conditions (Bedford and Mackay, 1973). Volatiles from AMAPA residues are clearly an important factor in the system under study, and current efforts are directed toward identification and bioassay of the inhibitory components of the volatiles.

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OZONE-INDUCED CHANGES IN HOST-PLANT SUITABILITY: INTERACTIONS of *Keiferia lycopersicella* AND *Lycopersicon esculentum*

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Abstract—Tomato pinworms, *Keiferia lycopersicella* (Walsingham), survived better and developed faster on tomato plants, *Lycopersicon esculentum* Mill., damaged by ozone than on plants not subjected to ozone fumigation. Other measures of fitness, including survival during pupation, sex ratio of adults, female longevity, and fecundity, were not affected. Analyses of ozonated foliage at zero, two, and seven days following fumigation demonstrated a transient but significant increase (18–24%) in soluble protein concentration. Although the concentration of the total free amino acids in ozonated foliage did not increase significantly, significant changes were observed in at least 10 specific amino acids, some of which are critical for either insect development or the production of plant defensive chemicals. A reduction in total nitrogen in ozonated foliage at seven days postfumigation indicated that nitrogen was being translocated to other portions of the plant. The implications of increases in assimilable forms of nitrogen in ozonated foliage, which lead to improved host-plant suitability for insect herbivores, are discussed both in relation to some current ecological theories and in regard to pest-management strategies.

Key Words—Ozone, nutrition, insect–plant interactions, nitrogen, secondary plant compounds, *Keiferia lycopersicella* Lepidoptera, Gellechiidae, tomato, *Lycopersicon esculentum.*

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INTRODUCTION

Ozone, a colorless, highly reactive gas formed from the interaction of the products of fossil fuel combustion and oxygen, causes more plant damage in the United States than any other air pollutant (EPA, 1978). Losses in southern California are reportedly in excess of 275 million dollars annually (Leung et al., 1982). Upon entering leaves through the stomata (Dugger et al., 1962), ozone reacts with plant membranes and cellular structures such as chloroplasts, resulting in structural damage and, in some cases, the onset of premature senescence (Mudd et al., 1969; Chang, 1971a; Heath, 1980). Such physiological changes reportedly decrease photosynthesis in several species (Ormrod et al., 1981; Reich and Amundson, 1985); both photosynthesis (Todd and Probst, 1963) and partitioning of photosynthates are affected in tomatoes (McCool and Menge, 1983).

An important action of ozone on foliar tissues is the apparent release of large amounts of soluble nitrogenous compounds from plant structural forms. Nitrogen in soluble form (soluble proteins and free amino acids) is apparently more easily digested by insects than many protein complexes in plants (Slansky and Feeny, 1977). Ozone has been shown to preferentially oxidize certain amino acids, specifically cysteine, methionine, tyrosine, histidine, and cystine (Chang, 1971b; Mudd and Freeman, 1977). This action breaks structural proteins into their component parts, causing membrane dissociation and producing significant increases in free amino acid concentrations (Menzel, 1971; Ting and Mukerji, 1971; Craker and Starbuck, 1972), and in some reports, soluble protein levels (Beckerson and Hofstra, 1979). However, these results cannot be generalized across all plant species, as some exceptions have been reported (Ting and Mukerji, 1971; Tingey et al., 1973).

The implications of increased concentrations of readily available forms of nitrogen for insect herbivore development are considerable (Mattson, 1980; White, 1984). Researchers have clearly demonstrated that nitrogen availability affects such basic life processes as growth rates, survival, and reproductive capacity (Onuf, 1978; Prestidge, 1982). White (1984) speculated that improved host quality resulting from an increase in free amino acid concentration following an episode of plant stress would greatly enhance survival of newly enclosed larvae. Although a variety of physiological changes have been reported for plants (primarily evergreens) stressed by SO_2 (Alstad et al., 1982), increased concentrations of free amino acids in foliage due to SO_2 injury have been suggested as the causal factors for improved survival in both aphids (Dohmen et al., 1984) and beetles (Jaeger et al., 1972). Since air-pollution incidents typically occur over broad areas of crop production (Perkins, 1974), the cumulative effects on herbivores at the population level may be quite significant. Thus, potential changes in plant physiology due to stress or direct injury resulting from air

pollutants may well have more serious consequences than surveys of direct economic losses have suggested.

Little research is available concerning the impact of ozone on insect–plant interactions. In the most detailed study to date, Endress and Post (1985) demonstrated that the Mexican bean beetle preferred to feed on excised soybean foliage damaged by ozone, but the authors only speculated as to the physiological mechanisms underlying the changes in foliage suitability. In a related study, Jeffords and Endress (1984) reported a preference by gypsy moth larvae for ozonated foliage excised from white oak. Hughes et al. (1981) showed a similar preference by the Mexican bean beetle for foliage damaged by SO_2.

Earlier research on the direct impact of ozone on insects suggested there were no significant effects on two species of cockroaches or on fire ants (Levy et al., 1974). Prolonged exposure to high ozone concentrations on housefly populations caused insignificant variations in growth and survival, but apparently stimulated oviposition (Beard, 1965). However, insects in these two latter studies were not fed living plant material, so any potential ozone-induced changes in nutritional quality of their food source would not occur.

The first objective of the study reported here was to evaluate the influence of ozone on interactions between *K. lycopersicella*, a specialist herbivore of economic significance in the United States, and its host plant, *L. esculentum*. Secondly, we wished to document some of the chemical and physiological changes in ozonated tomato plants which might alter the nutritional or defensive status of the foliage.

METHODS AND MATERIALS

Tomato pinworm (TPW) larvae, *K. lycopersicella*, used in all tests were reared in the laboratory from a colony initiated in 1982 with insects collected from commercial tomatoes in Orange County, California. The culture was maintained at 26 ± 1°C in a photoperiod of 14L : 10D on tomato plants (variety VFN 7718). All tomatoes used in these studies (VFN 7718) were grown from the same seedlot in a glasshouse and were planted in one-gallon pots containing UC soil mixture (Matkin and Chandler, 1957). Fertilization was standardized within each test. All plants were eight weeks old (six true leaf stage) at the initiation of each experiment.

Ozone fumigation chambers utilized in these tests were either large (2.5 m diameter) outdoor chambers (Musselman et al., 1986) or smaller (1.37 m diameter) chambers located in a glasshouse (1.1 air exchanges/min, similar to Heck et al., 1978). Temperatures and relative humidities during fumigation ranged from 20 to 25.5°C and 35 to 42%, respectively. The outdoor chambers operated continuously, and plants received all ambient ozone present. Glass-

house fumigation chambers, which are suitable for precise metering of ozone, were utilized for developmental tests since temperature could be maintained near 26°C, the optimum for TPW (Lin and Trumble, 1985). Regardless of size, both multiple chamber facilities were equipped with electrical arc ozone generators and activated charcoal filters for filtered air (low ozone) fumigation. Ozone concentrations within individual chambers were recorded using Dasibi UV ozone monitors linked to data loggers.

Insect Development, Survival, and Fitness: TPW developmental rates and survival on ozonated plants, and the potential effects of direct exposure to ozone, were evaluated in glasshouse chambers using three treatments: (1) control, no ozone; (2) two ozone fumigations prior to TPW infestation of plants; and (3) two ozone fumigations prior to, and two fumigations after, TPW infestation. All fumigations lasted three hours and were two to three days apart; treated plants received 0.28 ppm ozone and control plants received filtered air. Such concentrations of ozone are not unrealistic for agroecosystems in the Los Angeles–Riverside area, as ozone levels usually exceed 0.20 ppm (first-stage alert) on at least 30 days per year, and levels of 0.35 ppm (second-stage alert) occur annually.

Five plants with 10 eggs per plant (i.e., 50 eggs/treatment) were exposed in each treatment. Age of the eggs was standardized by using eggs oviposited during a 2-hr period. Eggs were transferred to test plants prior to randomization for the three ozone treatments. Following the last fumigation, the tomato plants were moved to the laboratory (maintained at 26 $\pm$ 2°C) and placed on trays covered with sand. The pots were covered at this time to prevent larvae exiting the foliage from pupating in the soil.

The time of host abandonment, signaling the cessation of feeding and the onset of the prepupal stage, was used to determine developmental rate and survival of larvae. The sand, serving as a pupation medium for larvae exiting the leaves, was sifted at 8-hr intervals until all surviving larvae had exited the leaves. The numbers of larvae successfully pupating and emerging as adults were subsequently recorded.

A second experiment, designed to evaluate general fitness parameters of TPW, was conducted in the outdoor fumigation chambers. Three treatments were investigated: high ozone = 35.8 ppm-hours (ambient levels plus four, 4-hr fumigations with 0.28 ppm ozone over the course of 10 days); ambient ozone concentrations = 31.6 ppm-hours; and a filtered air control = 7.7 ppm-hours. Total dose is reported because the daily ozone dosage varies with ambient in field studies. The filtered air treatments contained background levels of ozone due to the inability of the activated carbon filters to completely remove ozone. Forty-five plants per treatment were arranged in a randomized complete block design within nine fumigation chambers which had been previously selected at random for treatments. Each plant was infested with five eggs, for a total of

225 eggs/treatment, prior to randomization. Following the fumigations, the plants were returned to the laboratory and prepared as in the previous test. The pupation medium was sifted at 24-hr intervals, and all pupae were segregated by treatment and held to adult emergence. The first time this experiment was conducted, only sex ratio data were collected. In a second trial, with the identical exposure and infestation schedule (high ozone = 38.8 ppm-hours; ambient = 34.2 ppm-hours; control = 12.4 ppm-hours), males and females emerging from within each treatment were paired and allowed to oviposit on an artificial substrate (Schuster and Burton, 1982). Total egg production by each female was recorded at 48-hr intervals. Longevity of the females was assessed by recording survival at 24 hr intervals. All data from these experiments were analyzed using analysis of variance followed by Duncan's (1955) multiple-range test (DMRT) as appropriate.

Nitrogen, Free Amino Acid, and Chlorogenic Acid Analyses: Eighteen tomato plants were exposed to four, 2-hr, 0.18 ppm ozone fumigations at three-day intervals in glasshouse chambers. Eighteen control plants received identical treatment but were fumigated with ozone-free air. Immediately following the last fumigation, individual leaves, consisting of seven leaflets each, from six randomly chosen plants per treatment were divided into roughly equal aliquots for analysis of total nitrogen, soluble proteins, free amino acids, and chlorogenic acid. Samples were weighed immediately after collection and frozen for later analysis. Enough foliage was available on a single plant to provide four pairs of samples for each of the variables analyzed. Leaves were chosen on the basis of position on the main stem, starting with the third fully expanded leaf from the top; above this level not all leaves had been exposed to all four fumigations. At 7 and 14 days postfumigation, foliage from six ozone-fumigated plants and six control plants were evaluated in an identical fashion, except that only soluble protein and chlorogenic acid concentrations were assessed at 14 days postfumigation.

In a related test, five tomato plants were exposed to two, 3-hr fumigations at 0.28 ppm of ozone. Fumigations were three days apart. As in the previous trial, five additional plants were fumigated for an equivalent time with ozone-free air. Two foliage samples per plant from control and treated plants were collected at two days postfumigation as described previously and analyzed for soluble protein content.

Total nitrogen was analyzed using the micro-Kjeldahl technique (McKenzie and Wallace, 1954). The technique was modified by replacing the mercuric oxide catalyst with copper sulfate and by utilizing bromocresol green in place of methylene blue as an indicator.

Soluble protein was quantified using methods modified from Hare (1983, Jones, Hare, and Compton, unpublished). Frozen samples (1–2 g fresh weight) were ground and extracted with 10 ml 0.1 M NaOH for 30 min at room tem-

perature. Leaf tissue was removed by centrifugation (11,500*g* for 10 min) and the supernatant was brought to 10 ml. Protein concentration of the NaOH solution was measured with the Bradford (1976) reagent using ribulose 1,5-biphosphate carboxylase (RuBPCase) (Sigma Chemical Co.) as the standard. Samples were diluted when necessary to avoid deviations from linearity due to NaOH at high protein concentrations. Duplicate readings were made on each extraction. Values are reported as milligrams RuBPCase equivalent protein per gram (fresh weight) of foliage.

Free amino acids were qualitatively and quantitatively evaluated by extraction from additional foliage samples (1–2 g, fresh weight) with 10 ml 0.2 M trichloroacetic acid (TCA) for 30 min at room temperature. Samples were gravity-filtered through Whatman No. 1 filter paper to remove leaf tissue and then centrifuged at 11,500*g* for 10 min to remove small particles and precipitated protein. The filtrate was subsequently brought to a volume (in ml) 10 times the original weight of the leaf tissue, diluted 1:4 with additional TCA, and analyzed by automated ion-exchange chromatography at the Biotechnological Instrumentation Facility at the University of California, Riverside. Specific amino acid concentrations are reported as micrograms of amino acid per gram (fresh weight) of foliage. Total amino acid concentration was calculated as the sum of the individual amino acids per sample.

Chlorogenic acid content of foliage was quantified using high-pressure liquid chromatography (after Elliger et al., 1981).

Chemical data were analyzed by nested analysis of variance (ANOVA). The variation between treatments was tested over the variation among plants within treatments, and the variation among plants was tested over the variation between samples within plants.

Pearson product-moment correlation coefficients were calculated between plant means of percent nitrogen, soluble protein concentration, and total free amino acid concentration in order to determine if these three parameters varied independently.

RESULTS AND DISCUSSION

Insect Survival, Development, and Fitness. Survival of larvae developing on ozonated foliage was significantly greater than larvae fed control plants (Table 1). Survival was more than doubled for TPW fed plants exposed to two fumigations. A significant decrease in survival was observed for TPW developing on foliage exposed to four fumigations, as opposed to two fumigations. Whether such an effect was due to contact toxicity, irritation, or some other cause, warrants additional investigation. Upon entering the tentiform leaf-mining stage (instars 3 and 4), adverse effects of ozone were apparently lessened, as counts of mines on the foliage equaled numbers of larvae exiting from leaves.

TABLE 1. DEVELOPMENTAL RESPONSE OF *K. lycopersicella* TO OZONATED AND CONTROL *L. esculentum*[a]

Treatment	% Completing larval development	% Successfully pupating[b]	% Adult emergence[b]
Control	28 a	93 a	100 a
Two O_3 fumigations	62 c	94 a	95 a
Four O_3 fumigations	46 b	100 a	94 a

[a] Five replicates of 10 larvae per treatment; numbers in column followed by the same letter are not significantly different ($P \leq 0.05$), arcsine transformation, DNMRT.
[b] Percentage of those individuals completing the previous stage.

No significant differences were detected in either percent pupation or successful adult emergence from pupal cases (Table 1). Although TPW larvae feeding on ozonated plants survived better, no corresponding improvement in other fitness parameters was observed; significant differences were not detected for ratios of males to females, female longevity, or fecundity (Table 2).

Developmental rates for TPW larvae feeding on ozonated tomato plants were faster by more than 32 hr than larvae feeding on control plants (Figure 1). Mean times for emergence from the plants (setting the first emergence as time zero) were 75.9 hr for larvae fed control foliage, 55.5 hr for TPW fed on plants exposed to two ozone fumigations, and 49.4 hr for larvae fed tomatoes fumigated four times. These values represent increases in overall larval development

TABLE 2. INFLUENCE OF OZONATED AND CONTROL PLANTS ON SELECTED BIOLOGICAL PARAMETERS OF *K. lycopersicella*

Treatment	Test[a]	Sex ratio (male–female)	Mean longevity per female (days)[b]	Mean No. eggs per female (N)[c]
High ozone[d]	1	0.92	—	—
	2	0.81	6.1	15.9 (18)
Ambient	1	0.85	—	—
	2	0.67	5.5	19.4 (14)
Filtered air	1	0.76	—	—
	2	1.12	5.6	16.8 (25)

[a] Test 1 = 3 replicates of 75 TPW per treatment; test 2 = 3 replicates of 50 larvae per treatment.
[b] Based on 3 replicates of 11–36 females/treatment monitored daily.
[c] N = paired males + females; oviposition evaluated on artificial substrate, see text for details.
[d] See text for fumigation, ambient and filtered air dosages of ozone.

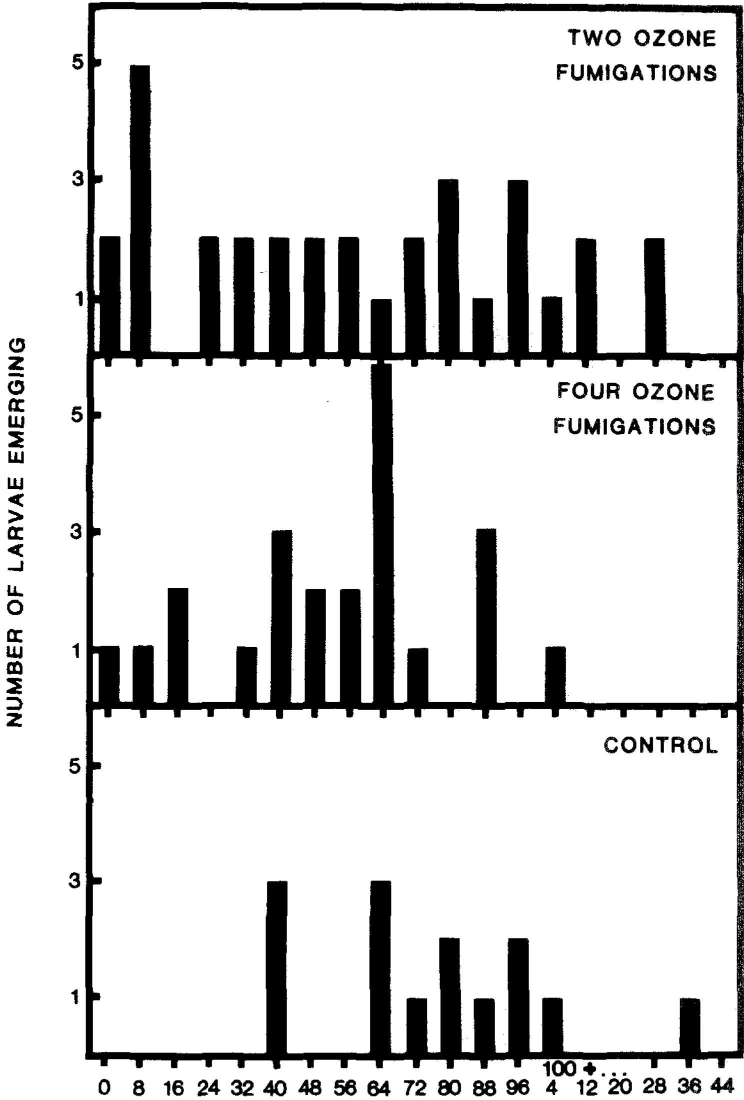

FIG. 1. Effects of ozone fumigation on time of emergence for larvae exiting ozonated and control foliage. Ozone fumigations in glasshouse chambers; dosage for each fumigation was 0.28 ppm.

rates of 1.3%, 7.1%, and 10.6%, respectively, as compared to expected larval developmental time predicted at 26°C by Lin and Trumble (1985). Although the observed increase in developmental rate may have occurred in response to ozone-induced stomatal closure in tomato foliage (which would elevate temperature through a decrease in evaporative cooling), Dugger et al. (1962) reported that ozone fumigation resulted in either no change or an increase in stomatal opening in beans.

If the observed effect of increased survival and developmental rate on ozonated plants proves applicable to other agricultural systems, two interesting implications for pest management programs become evident. First, programs dependent on developmental models driven by temperature will underestimate both stage of development and population size. To our knowledge, this effect has not yet been reported, but this manuscript reports the first results suggesting that the exposure of host plants to ozone is an important developmental factor for phytophagous insects. Also, those programs dependent on monitoring fruit and/or foliage for determination of threshold levels may require more frequent sampling (examples for the TPW include Wellik et al., 1979; and Wolfenbarger et al., 1975). Second, increased developmental rates may affect the success of biological control agents. For example, even though increased density of TPW may allow increases in the most common parasite species (Oatman et al., 1979), the time period for most successful parasitization would be shortened to less than the reported 48-hr period (2- to 3-day-old TPW at 26°C; Cardona and Oatman, 1971), thus decreasing the opportunity for parasites to utilize the most effective "window" for oviposition.

Nitrogen, Free Amino Acid, and Chlorogenic Acid Analyses. Since the biological effects of feeding on ozonated foliage were more evident for larvae than adults, we speculated that either the nutritional suitability of the foliage was improved for the neonate larvae as suggested for stressed plants by White (1984), or the plant's "defensive" system was being adversely affected. Analyses of total nitrogen content suggested that ozone-damaged foliage should be less nutritious than untreated leaves by one week following exposure to ozone fumigations (Table 3). However, total nitrogen analysis with the Kjeldahl method also includes nitrogen from structural proteins, nonprotein amino acids, and nitrogen incorporated in defensive compounds (Hare, 1983). Significant enhancement of soluble protein concentrations for up to two days postfumigation provided an increase in readily assimilable nitrogen available for the larvae, which suggests a plausible explanation for the observed improvement in larval survival. Similar increases in soluble protein concentrations have been documented for beans exposed to ozone (Beckerson and Hofstra, 1979), but reports in the literature have not been consistent (Ting and Mukerji, 1971).

Ten of the 20 amino acids quantified from ozonated foliage varied significantly from concentrations in control leaves (Table 4). Reductions in methionine and tyrosine immediately following fumigation were anticipated from ear-

TABLE 3. ANALYSES OF SOLUBLE PROTEINS AND TOTAL NITROGEN IN OZONATED AND CONTROL PLANTS AT SELECTED INTERVALS POSTFUMIGATION

Days post fumigation	No. of fumigations	Fumigation interval (days)	Ozone (ppm) concentration	Soluble protein		% Total nitrogen	
				Ozone	Control	Ozone	Control
0	4	3	0.18	14.699[a]	12.025	5.483[b]	5.190
2	2	3	0.28	6.638[c]	6.592	—	—
7	4	3	0.18	7.766[b]	7.352	5.481[d]	5.957
14	4	3	0.18	4.701[b]	4.835	—	—

[a] Significantly different from control plants at $P \leq 0.08$, $F_{(1, 10)} = 3.44$, based on two samples per plant and six plants per treatment.
[b] Concentrations were not significantly different from control plants.
[c] Significantly different from control plants at $P \leq 0.03$, $F_{(1, 8)} = 6.65$, based on two samples per plant and five plants per treatment; these data from a separate test, see text for details.
[d] Significantly different from control plants at $P \leq 0.03$, $F_{(1, 10)} = 6.66$, based on two samples per plant and six plants per treatment.

TABLE 4. COMPARISONS OF FREE AMINO ACID CONCENTRATIONS AT ZERO AND SEVEN DAYS POSTFUMIGATION IN OZONATED AND CONTROL PLANTS

	0 days postfumigation		7 days postfumigation	
Amino acid[a]	Ozone	Control	Ozone	Control
Alanine	103.78 **	80.78	157.51 **	133.23
Aspartic acid	272.35 NS	221.79	344.53 *	305.95
Glutamine	39.58 **	92.79	383.84 NS	353.24
Glycine	16.77 **	13.28	35.66 *	27.80
Histidine	7.99 NS	7.29	85.55 *	68.72
Methionine	5.89 **	9.46	4.52 NS	7.49
Proline	67.01 **	107.20	96.11 **	178.18
Serine	141.61 **	113.12	919.23 *	442.88
Tyrosine	9.98 **	16.63	39.91 **	77.74
Valine	68.89 **	54.83	246.30 NS	238.83

[a] Includes only those amino acids with substantial changes in concentration, amino acid concentration in $\mu g/g$. Horizontal comparisons within 0 or 7 days postfumigation are not significant (NS), significant at the $P \leq 0.1$ level (*), or significant at the $P \leq 0.05$ level (**) as noted.

lier reports (Chang, 1971b; Mudd and Freeman, 1977), but significant reductions in glutamine (57%) and proline (37%) had not been previously noted. Of the other amino acids which reportedly react with ozone, cysteine and cystine were only nominally present in tomato foliage, and histidine was not significantly affected. Unlike previous studies (Menzel, 1971; Craker and Starbuck, 1972), significant increases in the total pool of free amino acids were not detected. However, a trend toward higher concentrations of total free amino acids in ozone-treated plants was evident (ozonated plants = 1020.1 and 3310.5 $\mu g/g$; control plants = 968.6 and 2601.3 $\mu g/g$ at 0 and 7 days postfumigation, respectively).

Comparisons between amino acid concentrations immediately following fumigation and concentrations at seven days postfumigation suggest an explanation for the variable reports of amino acid fluctuations in the literature. While proportions of some amino acids remained approximately the same on both sampling dates, the relative amounts of others, specifically serine, glutamine, histidine, and valine, increased considerably. Whether the tomato plant is stimulated to compensate for ozone-induced reductions in certain amino acids such as glutamine, or if the leaves are entering a premature senescence causing nitrogenous compounds to be converted to soluble forms available for reallocation, should be the focus for additional research. The general increase in free amino acid concentration in both ozonated and control plants over the course of the experiment probably reflects a general mobilization of energy reserves from the leaves to flowers or immature fruit (Ting, 1982). Regardless of the

cause of amino acid concentration differences between ozonated and control plants, time elapsed between ozone fumigation and leaf sampling can affect the quantities of amino acids, soluble protein, and total nitrogen measured.

The implications of ozone-induced changes in amino acid concentrations are potentially quite significant for insect populations. Of the 10 rat-essential amino acids generally considered necessary for insect growth (Dadd, 1973), only methionine and valine differed significantly in ozonated foliage. Since growth of TPW was not inhibited by the transient reduction in methionine concentration, and valine concentrations increased, changes in these amino acids were not limiting. Vanderzant (1966) reported that even relatively small changes in concentrations of free amino acids can have significant and negative effects on insect development if the amino acids compete for sites on the absorption system. House and Barlow (1964) documented the negative effects of increasing the amounts of amino acids in an artificial diet, and thereby altering the osmotic balance of the diet. However, since our data indicated: (1) the total pool of free amino acids was not significantly increased at either 0 or 7 days following ozone fumigation, (2) the increase in total free amino acids between day 0 and day 7 was greater than the increase between ozonated and control foliage, and (3) the concentrations of free amino acids in ozonated and control plants were similar to those used in successful artificial diets for other phytophagous lepidopterans (Vanderzant, 1957; Arai and Ito, 1964), neither competition for absorption sites nor changes in osmotic balance of the host would be likely to exert a negative effect in our system.

Potentially critical changes in free amino acid concentrations occurred in the key supplementary amino acids (terminology after Dadd, 1973), all of which increased significantly in ozonated foliage. Alanine, glycine, aspartic acid, and serine have been reported as important growth factors for the silkworm (Ito and Arai, 1966, 1967), while proline was documented as "semi-essential" for the silkworm (Ito and Arai, 1965) and critical to the development of many dipterans (Friend, 1968; Gingrich, 1964). Thus, since changes in concentrations of these amino acids were relatively major (up to 207% increase for serine), increased availability of supplementary amino acids probably accounts for at least some of the improved survival demonstrated for TPW fed on ozonated tomato foliage. None of the correlation coefficients between percent nitrogen, soluble protein, or total free amino acid concentration were significantly different from zero in either data set, suggesting that exposure to ozone affected these three aspects of plant nitrogen metabolism independently.

Since insect survival could also have been affected by the concentration of plant defensive chemicals, we considered which compounds would be most likely to be affected by ozone. Of the secondary plant compounds documented in commercial tomatoes which have proven antibiotic properties against lepidopterans, only chlorogenic acid has an "exposed" double bond susceptible to rapid oxidation by ozone (J. Kumamoto, personal communication). Ozone-in-

duced reductions in tyrosine would not effectively inhibit productivity of chlorogenic acid in tomatoes, since this amino acid is only an important precursor in monocotyledonous plants (Rhodes and Wooltorton, 1978). Since chlorogenic acid production can be an induced response, occurring after stress from either disease (Carrasco et al., 1978) or insects (Elliger et al., 1981), oxidation should prevent chlorogenic acid levels from increasing as long as contact with ozone is frequent. We found that chlorogenic acid levels did not differ significantly from controls either immediately following the last fumigation or at seven days postfumigation. However, by 14 days after the last fumigation, significant increases in chlorogenic acid concentration were observed in ozonated plants (ozone-treated plants = 31.1 μg/g fresh weight, control plants = 16.3 μg/g, ANOVA, $P \leq 0.05$). Therefore, although stress generated by ozone may induce chlorogenic acid production in tomato plants, benefits relating to insect control may be minimized by repetitive exposures and subsequent oxidation. Nonetheless, the increase in developmental rates and survival of TPW on ozonated foliage cannot be explained by suppression of chlorogenic acid production in our experiments.

CONCLUSIONS

The observed increase in survival and developmental rates of TPW larvae feeding on ozonated tomato foliage was due to a complex of factors, the most important of which were nutritional. Concentrations of readily assimilable nitrogenous compounds such as soluble protein and important supplementary amino acids proved to be considerably better indicators of host suitability than total nitrogen analysis. Although chlorogenic acid production in tomatoes may be induced by exposure to ozone, the plant response occurred too late to effect the enhanced growth and survival of TPW and could therefore be eliminated as a primary cause for the observed effects.

Although our data show an increase in soluble nitrogenous compounds as predicted by White's (1984) hypothesis, this apparent agreement may be an artifact. White proposed that the breakdown of insoluble proteins to smaller, more soluble compounds was a general response to plant stress and adaptive to the extent that the increase in free amino acid concentration stimulated seed production during stressful periods. While this may be true for some stresses, (e.g., drought), we do not believe that our results necessarily reflect an adaptive response of plants to ozone damage. Rather, the increases in concentration of soluble nitrogen following ozone exposure are more likely to be simply the direct consequence of the chemical reactivity of ozone with plant proteins and amino acids (Craker and Starbuck, 1972; Mudd and Freeman, 1977).

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OVIPOSITION DETERRENT FROM EGGS OF *Callosobruchus maculatus*: Spacing Mechanism or Artifact?

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Abstract—Females of *Callosobruchus maculatus* (F.) avoid ovipositing on host seeds already bearing conspecific eggs, and thus distribute eggs evenly among seeds. This behavior was presumed to be mediated by an ether-soluble "oviposition marker" that is deposited with the egg and can be extracted from egg-laden artificial hosts (glass beads). Ablation experiments revealed that the true factors promoting an even dispersion of eggs were perceived by the maxillary and labial palpi. In contrast, receptors on the antennae were largely responsible for avoidance of seeds treated with "oviposition marker." Taken together, these results suggest that a careful distinction should be drawn between factors that promote spacing of eggs under natural conditions and general oviposition deterrents that may be isolated from both sexes.

Key Words—Ablation, egg dispersion, oviposition deterrent, foretarsi, palpi, antennae, Coleoptera, Bruchidae, *Callosobruchus maculatus*.

INTRODUCTION

Female insects use a variety of cues to locate suitable oviposition sites. In some phytophagous species, females tend to reject host plants that already bear conspecific eggs (Prokopy et al., 1984). This behavior produces a statistically uniform (nonrandom) dispersion of eggs among hosts. Not surprisingly, detection and avoidance of egg-laden hosts seems especially prevalent in species where the potential for competition among larvae is relatively high (Rausher, 1979). "Egg-load assessment" (Rothschild and Schoonhoven, 1977) may be elicited by visual, olfactory, or tactile stimuli, but in most cases studied so far, it is

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mediated by contact chemoreception of pheromones deposited during or following egg-laying (Prokopy et al., 1984; Städler, 1984).

Seed beetles (Bruchidae) of the genus *Callosobruchus* are well known to distribute eggs evenly among seeds (e.g., Avidov et al., 1965). The legless hatching larva must develop within the seed upon which the egg is laid, and a clear fitness advantage is conferred on females that seek unused hosts (Mitchell, 1975). Gravid females of *C. maculatus* (F.) not only distinguish between egg-laden and pristine seeds, but also assess egg loads quantitatively, i.e., when all seeds bear eggs, females prefer to oviposit on seeds with below-average egg densities (Messina and Renwick, 1985a). This tendency appears to be adaptive, since larval survivorship decreases linearly with small increases in larval density within seeds (Messina and Renwick, 1985b). A chemical basis of egg recognition is likely because females lay more eggs on clean seeds than on seeds from which an egg has been removed (Giga and Smith, 1985; Wasserman, 1985; Messina and Renwick, 1985c). Physical (tactile) cues may also be involved; attachment of egg models to seeds deters oviposition to some extent (Messina and Renwick, 1985c).

Oshima et al. (1973) and Messina and Renwick (1985c) extracted chemical oviposition deterrents from glass beads laden with eggs of *C. chinensis* (L.) and *C. maculatus*, respectively. In both studies, however, deterrents were similarly obtained from egg-free beads that had been exposed to males. Such lack of specificity casts doubt on the role of these chemicals as "oviposition markers." The ability of females to perceive differences in egg density implies that the relevant cues are deposited only during each previous oviposition. Females were not deterred from ovipositing on egg-free seeds that had been directly exposed to males or virgin females (Messina and Renwick, 1985c).

In order to help identify the stimulus used in egg recognition, we determine in this study the sense organs involved. We compare how ablation of female sense organs influences both the ability to disperse eggs evenly and the response to oviposition marker. In a previous study on the sensory basis of bruchid egg recognition, Avidov et al. (1965) found that *C. chinensis* females distributed eggs uniformly among seeds despite antennectomy.

METHODS AND MATERIALS

A laboratory colony of *C. maculatus* was established in 1982 from infested cowpea seeds [*Vigna unguiculata* (L.) Walp.] in northern Cameroon. We maintained the colony and conducted all experiments in a dark incubator at 30°C and 50–60% relative humidity. Black bean seeds (*Phaseolus vulgaris* L.) were used as the experimental host because their dark coats permit high visibility of eggs.

Ablations were performed by anesthetizing newly emerged females with CO_2 for ca. 1 min and placing them under a dissecting microscope. Fine forceps were used to snip off each organ at its base. Antennae (A), foretarsi (F), and palpi (P, maxillary and labial) were removed alone or in combination. To test the ability of females to disperse eggs evenly, we placed each female plus one male into a Petri dish containing 15 seeds. Control females in this experiment were similarly anesthetized, and were subjected to removal of an elytron. Each female was allowed to lay her lifetime complement of eggs (usually 40–80 eggs) on her allotment of 15 seeds. After the female died, we calculated the mean egg density and the variance in egg density. The variance–mean ratio was used as a standard, unbiased index of dispersion (Myers, 1978), where $s^2 < \overline{X}$ indicates an even distribution, and $s^2 > \overline{X}$ indicates a clumped distribution. We conducted 2×2 contingency tests (with Yates' correction) to compare control and treated females in the frequency at which they distributed eggs evenly or unevenly. Fifty to seventy-five females were used per ablation treatment. We discarded a small number of dishes in which the female laid <20 eggs, as these individuals were probably injured during ablation.

We used the method of Oshima et al. (1973) and Messina and Renwick (1985c) for extracting chemical deterrents. Five hundred mated females were placed into a jar containing 400 g of glass beads ($\simeq 1500$ beads of 3–5 mm diameter). Beetles were sieved off after four days. We sampled 20 beads to estimate the mean number of eggs/bead. Beads were washed vigorously for ca. 1 min with 200–300 ml of diethyl ether, and each wash was divided in half for use in two separate trials. The half-strength wash was then concentrated to 70 ml. For each trial, 200 seeds were dipped into the 70 ml of ether wash (treated) or into plain ether (control). Seeds were apportioned into 20 divided Petri dishes, with 10 control and 10 treated seeds/dish (5 seeds/quadrant). Each dish received three females whose sense organs had been ablated unilaterally (10 dishes) or bilaterally (10 dishes). Unilateral ablations served to check the activity of the extract and to control for any traumatic effect of the procedure. After 16 hr, we recorded the number of eggs laid on control or treated seeds in each set of dishes. Data were analyzed with paired t tests.

RESULTS

Lifetime fecundity was similar among females in the control and ablation treatments, even though ablated females lost up to three sense organs (Table 1; means were not compared statistically because females were not always derived from a single batch). Since most females deposited 60–70 eggs on the 15 available seeds, mean egg density in a dish was typically 4–5 eggs/seed. As expected, 80% of the control group tended to distribute eggs evenly, i.e., the

TABLE 1. NUMBER OF *C. maculatus* FEMALES TENDING TO DISTRIBUTE EGGS EVENLY ($s^2 < \bar{X}$) OR UNEVENLY ($s^2 > \bar{X}$) AMONG SEEDS FOLLOWING VARIOUS ABLATIONS.

Ablation	Eggs/ female ($\bar{X} \pm$ SE)	Females distributing eggs		Median $s^2/\bar{X}$ ratio	P[a]
		Evenly	Unevenly		
Control	62.7 ± 2.7	45	11	0.57	—
Antennae (A)	63.7 ± 3.1	46	7	0.55	>0.10
Forestarsi (F)	69.2 ± 2.8	47	4	0.42	>0.10
Palpi (P)[b]	77.2 ± 3.1	11	43	1.54	<0.001
A + F	63.7 ± 2.8	39	16	0.64	>0.10
A + P	67.1 ± 3.2	8	44	1.86	<0.001
F + P	64.3 ± 3.2	12	46	1.71	<0.001
A + F + P	66.8 ± 2.8	2	52	2.50	<0.001

[a]2 × 2 contingency test (with Yates' correction) comparing ablated and control females in the frequency of even and uneven distributions.
[b]Maxillary plus labial palpi.

variance in egg density was lower than the mean (Table 1). The median s^2/X ratio for the 56 control females was well below unity. The tendency toward uniform distribution of eggs persisted after removal of antennae or foretarsi. Thus any impairment of locomotion caused by removal of foretarsi was too slight to affect egg dispersions. However, removal of the palpi, alone or in combination with other organs, caused a reversal in oviposition behavior; most females tended to clump their eggs ($s^2 > \bar{X}$), and the median $s^2/\bar{X}$ ratio rose accordingly (Table 1). In no treatment was there an approximately equal number of females distributing eggs evenly and unevenly, which would be expected if a particular ablation led to random oviposition.

Two results suggest that the antennae and foretarsi could play a minor or supplementary role in egg recognition: (1) a slightly higher proportion of females distributed eggs unevenly in the A + F treatment than in the control treatment (and the median $s^2/\bar{X}$ ratio rose slightly), and (2) virtually all females clumped their eggs in the A + F + P treatment (Table 1). Alternatively, females in the A + F + P treatment may have been so drastically deprived of sensory input that they continued to oviposit but stopped moving between seeds.

Mean egg densities ranged between 8 and 12 eggs/glass bead after 500 females were allowed to oviposit on 1500 beads for four days. Thus between 12,000 and 18,000 eggs contributed to each ether wash, which was then divided in half. Since ca. 4 ml of the 70 ml, half-strength wash was taken up in coating the 200 dipped seeds, each treated seed used in the choice-test bioassay received roughly 1.5–3.0 egg-equivalents of ether-soluble material.

TABLE 2. OVIPOSITION PREFERENCES OF *C. maculatus* FEMALES SUBJECTED TO UNILATERAL OR BILATERAL ABLATIONS AND OFFERED SEEDS DIPPED IN ETHER (CONTROL) OR ETHER WASHES OF EGG-LADEN GLASS BEADS (TREATED)

	Unilateral, eggs added to			Bilateral, eggs added to		
Ablation	Control	Treated	P^a	Control	Treated	P^a
Antennae	158	72	<0.01	85	85	>0.10
Foretarsi	71	24	<0.05	80	31	<0.05
Palpi	199	110	<0.01	175	93	<0.01
A + F	190	123	<0.05	108	110	>0.10
A + P	98	65	<0.05	32	55	>0.10
F + P	127	51	<0.05	63	41	>0.10

[a] Paired *t* test, 10 replicate dishes/trial.

Females subjected to unilateral ablations consistently laid more eggs on control seeds than on seeds dipped in washes of glass beads (Table 2). This confirmed the deterrence of the extract and ruled out any negative effect of the ablation procedure. Avoidance of treated seeds continued after bilateral ablation of foretarsi or palpi, but disappeared after bilateral ablation of antennae, whether antennae were removed alone or in combination with other organs (Table 2). An unexpected result was that removal of the foretarsi and palpi also caused a lack of significant discrimination (F + P, Table 2), although females still tended to lay more eggs on control seeds.

DISCUSSION

The results in Tables 1 and 2 present a paradox. Females deprived of maxillary and labial palpi, but bearing intact antennae, failed to distribute eggs evenly, and in fact usually aggregated their eggs. Yet the putative oviposition marker was mainly perceived by the antennae, as antennectomized females bearing intact palpi no longer discriminated between control and extract-treated seeds. We must conclude that a different factor was influencing behavior in each bioassay. The ether-soluble "oviposition marker" probably has no relevance to the natural spacing of eggs, otherwise females missing their palpi but retaining their antennae would have distributed eggs evenly (Table 1). Conversely, the true factor(s) promoting an even dispersion of eggs must not have been present in detectable amounts in the ether-extract bioassay, otherwise females missing their antennae but retaining their palpi would have avoided the treated seeds (Table 2). We cannot reject the hypothesis that the ether-soluble material plays an auxiliary role in egg dispersion, but we have no evidence that it does.

A distinction should therefore be drawn between general oviposition deterrents, which may be identified from choice-tests (as in this study), and factors that promote an even spacing of eggs. Uniform dispersion of eggs by *C. maculatus* may well be mediated by an ether-insoluble chemical or by the physical shape of the egg. This may explain why females continue to avoid ovipositing on egg-laden seeds despite repeated washing of seeds with ether (Messina and Renwick, 1895c). Giga and Smith (1985) claimed that ether-soaked, egg-laden seeds inhibited oviposition less than control, egg-laden seeds, but their data showed little difference in oviposition rate on the two seed types (3.31 vs. 3.19) and no statistical comparison was given.

Other studies may also be interpreted as showing that isolation of an oviposition-deterring chemical is not tantamount to explaining an observed even spacing of eggs. Using a behavioral assay, Prokopy et al. (1982) isolated oviposition deterrents from several body parts of the apple maggot fly, *Rhagoletis pomonella* (Walsh), but in an electrophysiological assay, only a few of the extracts stimulated tarsal hairs known to be receptive to the true oviposition-deterring pheromone. The behaviorally deterrent extracts that failed to stimulate the tarsal hairs may contain compounds that are of little importance in explaining the fly's avoidance of occupied hosts. Szentesi (1981) obtained strong oviposition deterrents from seeds exposed to either sex of the dry bean weevil, *Acanthoscelides obtectus* Say, yet this insect tends to distribute eggs in a random or clumped manner (Umeya and Kato, 1970; Pouzat, 1983; Jarry and Chacon, 1983). Thus, extraction of an oviposition deterrent need not imply an even distribution of eggs in nature. For an insect such as *C. maculatus* that does disperse its eggs evenly, the likelihood that an isolated oviposition deterrent is indeed the natural spacing mechanism will be increased if ablation of the sense organ responding to the chemical also leads to random or aggregated oviposition.

The maxillary and labial palpi were the primary organs used for egg recognition by *C. maculatus.* Removal of palpi did not simply lead to random oviposition; females deprived of these organs tended to clump their eggs. This reversal in behavior was not completely unexpected. Once egg loads on seeds no longer influence oviposition choices, a female may prefer particular seeds because her remaining sense organs respond to subtle variation in seed size or texture (cf. Mitchell, 1975; Pouzat, 1983). In various Bruchidae, the palpi are already known to play a dominant role in legume host recognition, in plant-mediated stimulation of oogenesis (Szentesi, 1976; Pouzat, 1978), and in discrimination between host species (Messina, Renwick and Barmore, unpublished). The sensory basis of avoidance of egg-laden hosts is known for only a few phytophagous insects (e.g., Crjnar and Prokopy, 1982; Städler, 1984), but in at least one species, the large cabbage white butterfly (*Pieris brassicae* L.),

the same sense organ mediates chemoreception of both host plants and conspecific eggs (Behan and Schoonhoven, 1978; Klijnstra, 1985).

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CHEMISTRY OF MALE MANDIBULAR GLAND SECRETIONS OF *Philanthus basilaris* CRESSON AND *Philanthus bicinctus* (MICKEL) (HYMENOPTERA: SPHECIDAE)

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Abstract—Detailed chemical characterizations are provided for the species-specific mixtures of marking pheromones utilized by the male beewolves *Philanthus basilaris* and *P. bicinctus*. Successful analysis of these complex mixtures of 2-ketones, fatty acids, ethyl esters, and aldehydes was facilitated by the treatment of the crude extracts with 1,1-dimethylhydrazine and direct analysis of the resulting hydrazones, methyl esters, and starting ethyl esters by GC-MS.

Key Words—*Philanthus*, Hymenoptera, Sphecidae, pheromone, scent-marking, 2-alkanones, fatty acids, ethyl esters, alkenals, *N,N*-dimethylhydrazine, methyl esters, EI-MS, CI-MS, methoxymercuration, methylthiolation.

INTRODUCTION

Male sphecid wasps in the genus *Philanthus* are known to be territorial and to display a striking behavior that has been called "abdomen dragging." This

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behavior consists of alighting on plants within their territories and walking up and down the stem or leaf while pressing the hair brushes located at the base of their mandibles and/or the venter of their abdomens against the plant surface (Alcock, 1975; Borg-Karlson and Tengö, 1980; Evans, 1983; Gwynne, 1978; O'Neill, 1979, 1983). Since it is known that these males also possess large paired mandibular glands that open at the base of the mandibles adjacent to the hair brushes, a logical hypothesis is that these glands are the source of a pheromonal mixture that is being deposited while the wasps are "abdomen dragging" (Gwynne, 1978; O'Neill, 1979). Direct confirmation of this hypothesis was recently provided plus preliminary chemical evidence for the nature of the pheromonal blends (Schmidt et al., 1985). This paper provides a detailed chemical analysis of the pheromonal components deposited by *Philanthus basilaris* (Cresson) and *P. bicinctus* (Mickel) while "abdomen dragging" on the plant surfaces.

METHODS AND MATERIALS

Mandibular glands were obtained as previously described (Schmidt et al., 1985). Five to ten pairs of glands were pooled by species and suspended in ca. 200 μl of distilled-in-glass methylene chloride (Burdick and Jackson Co. Muskeegon, Mich.).[6] Aliquots of these stock solutions were used for subsequent analyses.

Derivatizations were conducted in 5 ml Reactivials (Pierce Chemical Company, Rockford, Illinois) with heating (when necessary) provided by a Reactitherm unit. *N,N*-Dimethylhydrazones were prepared as previously reported (McDaniel and Howard, 1985). Methyl esters were prepared either using diazomethane (Schlenk and Gellerman, 1960), or 1,1-dimethylhydrazine (McDaniel and Howard, 1985). Double-bond positions were determined either by gas chromatographic–mass spectrometric (GS-MS) analyses of methoxy ethers which were prepared by methoxymercuration–demercuration (Abley et al., 1970; Blomquist et al., 1980), sometimes with subsequent hydrogenation (Plattner et al., 1975), or by GC-MS analyses of methyl thioethers (Francis and Veland, 1981). Double-bond stereochemistries were determined by comparison of gas chromatograph (GC) retention times and coinjection with authentic standards using a 6-m × 2-mm ID stainless-steel column of 15% OV-275 on 100/120 Chromosorb P AW-DMCS operated isothermally at 220° in a Hewlett Packard 5710A gas chromatograph.

GC-MS analyses were conducted using a Hewlett Packard 5710A GC interfaced to a Hewlett Packard 5982 mass spectrometer equipped with a Hewlett Packard 5933 data system. The GC contained 1.8-m × 2-mm ID glass columns packed with either SP-2100 on 100/120 mesh Supelcoport or 0.65% OV-17 +

[6] Mention of a company or trade name is for identification purposes only and does not imply endorsement by the U.S. Department of Agriculture.

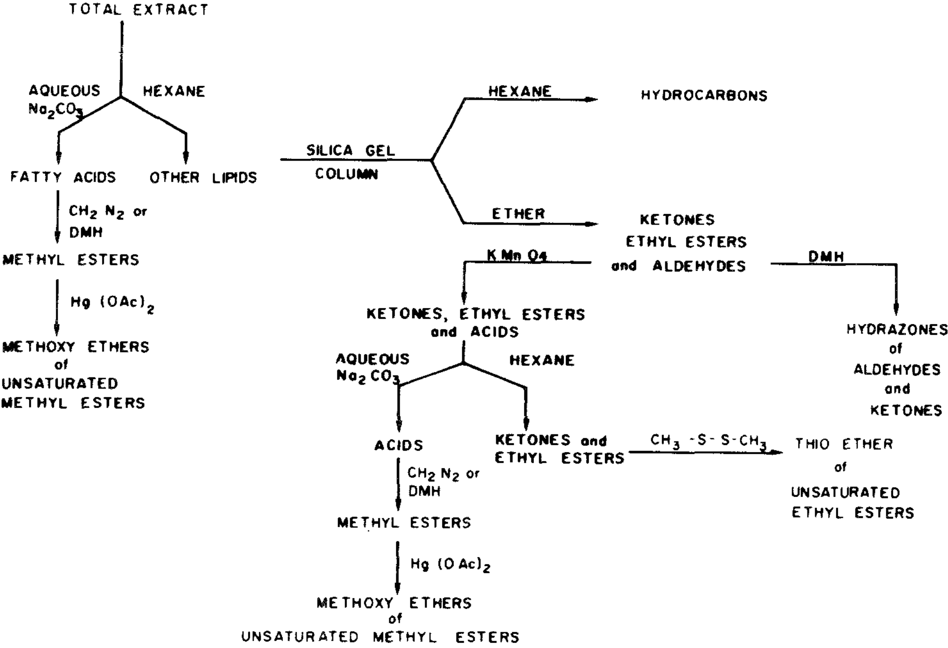

FIG. 1. Separation and analysis flowchart for the marking pheromone of *Philanthus basilaris* and *P. bicinctus*.

0.5% OV-210 on 100/120 mesh Ultrabond 20 M. Analyses involved temperature programming from 100 to 280°C at 8°/min. Electron-impact mass spectra (EI-MS) were generated at 70 eV; chemical-ionization mass spectra (CI-MS) were generated at 200 eV using ultrapure methane as both carrier and ionizing gas at a flow rate of 13 ml/min which generated an internal source pressure of 0.5 torr.

Figure 1 is a flow chart outlining the separation and derivatization procedures used in the course of the analyses.

RESULTS

The exocrine products of the sympatric species *P. basilaris* and *P. bicinctus* are essentially those of carbonyl compounds. Although quantitatively different, both species contain the same homologous series of 2-ketones and saturated and unsaturated carboxylic acids. They contain different unsaturated aldehydes, however, and *P. bicinctus* is also characterized by a homologous series of saturated and unsaturated ethyl esters that are not present in *P. basilaris*. A detailed analysis of each class of carbonyl compound is presented below and summarized in Table 1.

TABLE 1. DIAGNOSTIC MASS SPECTRAL DATA FROM MANDIBULAR GLAND COMPONENTS (OR THEIR DERIVATIVES) FROM *Philanthus basilaris* and *P. bicinctus*

Compound	Molecular formula	Molecular weight	Free compounds		EI-MS			
			EI	CI	Methyl esters	Methoxy ethers	Dithiomethyl ethers	*N,N*-Dimethyl hydrazones
2-Tridecanone[a,b]	$C_{13}H_{26}O$	198	58; 198	199				100; 240
2-Pentadecanone[a,b]	$C_{15}H_{30}O$	226	58; 226	227				100; 268
2-Heptadecanone[a,b]	$C_{17}H_{34}O$	254	58; 254	255				100; 296
Z-9-Hexadecenoic acid[a,b]	$C_{16}H_{30}O_2$	254		237; 255	74; 236; 268(M+)	143;201; 129; 215		
Hexadecanoic acid[a,b]	$C_{16}H_{32}O_2$	256		239; 257	74; 270(M+)			
Z-9-Octadecenoic acid[c]	$C_{18}H_{34}O_2$	282		265; 283	74; 264; 296(M+)	171; 229; 157; 243		
Z,Z-9,12-Octadecadienoic acid[d]	$C_{18}H_{32}O_2$	280		263; 281	74; 262; 294(M+)	171; 229; 157; 243; 129; 243; 115; 257		
Octadecanoic acid[a,b]	$C_{18}H_{36}O_2$	284		267; 285	74; 298(M+)			

Ethyl Z-9-hexa-decenoate[b]	$C_{18}H_{34}O_2$	282	88; 236; 282	237; 283		145; 231; 376(M+)	
Ethyl hexadecanoate[b]	$C_{18}H_{36}O_2$	284	88; 238	239; 285			
Ethyl Z-9-octade-cenoate[b]	$C_{20}H_{38}O_2$	310	88; 264; 310	265; 311		173; 231	
Ethyl octadecanoate[b]	$C_{20}H_{40}O_2$	312	88; 266	267; 313			
Z-9-Heptadecenal[a,e]	$C_{17}H_{32}O$	252	234	235; 253	157; 215 143; 229		86; 294 250; 295 (Cl-MS)
Z-9-Octadecenal[a,e]	$C_{18}H_{34}O$	266	248	249; 267	171; 215 157; 243		86; 308 224; 309 (Cl-MS)
Z-9-Nonadecenal[b,e]	$C_{19}H_{36}O$	280	262	263; 281	185; 215 171; 257		86; 322

[a]Contained in *P. basilaris*.
[b]Contained in *P. bicinctus*.
[c]Methoxy ethers of methyl esters.
[d]Reaction product hydrogenated before analysis.
[e]Methoxy ethers of methyl esters after oxidation and esterification of free aldehyde.

Ketones. These compounds are readily characterized by mass spectrometry. Their EI-MS contain characteristic ions at *m/z* 58 as the base peak and parent ions of approximately 3–5% intensity. CI-MS of these compounds contain the M+1 ion as the base peaks and small (1–2%) M-18 ions. Confirmation of these assignments were made by an analysis of the mass spectra of their *N,N*-dimethylhydrazones; EI-MS of these hydrazones contain ions at *m/z* = 100 as the base peak, with parent ions of ca. 20% abundance and significant M-44 ions which correspond to the loss of a dimethylamino radical from the parent species. CI-MS of the *N,N*-dimethylhydrazones contain the M+1 ion as the base peak and significant M-42 ions which presumably arise from the loss of methylimine from the protonated parent species.

Aldehydes. EI mass spectra of the unsaturated aldehydes contained no parent ions and only modest amounts (2–3%) of a M-18 ion. By contrast, the CI-MS of these compounds had the M+1 ion as their base peaks and significant (20–90%) M-17 ions, which presumably arise from a loss of water from the protonated parent species. These data, however, did not allow an unequivocal distinction between monounsaturated aldehydes and diunsaturated alcohols of the same carbon number.

Treatment of the mixture with 1,1-dimethylhydrazine (DMH), however, quantitatively converted all of the presumptive aldehydes to the corresponding hydrazones within 10 min. EI-MS of these hydrazones contained the ion at *m/z* 86 as their base peaks and parent ions of 10–20% abundance. Their CI-MS contained M+1 ions as the base peaks and prominent M-44 ions which probably correspond to the loss of dimethylamine from the protonated parent species.

An initial attempt was made to determine double-bond positions by methoxymercuration–demercuration of the crude glandular extracts (Abley et al., 1970; Blomquist et al., 1980). However, intractable product mixtures resulted; therefore, an aliquot of the crude extract was extracted with sodium carbonate to remove carboxylic acids and then loaded onto a silica gel minicolumn. Elution with hexane removed any possible cuticular hydrocarbon contaminants, and subsequent elution with ether yielded a mixture containing only the aldehydes, ketones, and ethyl esters. This mixture was oxidized using a neutral dilute permanganate solution which oxidized the aldehydes to carboxylic acids without alteration of the ketones or ethyl esters. These acids were partitioned into sodium carbonate solution, acidified, taken into ether, and converted to their methyl esters with diazomethane (Schlenk and Gellerman, 1960). Methoxymercuration–demercuration of these methyl esters proceeded smoothly and the double-bond positions were determined by EI-mass spectrometry. In all cases, the double bonds were Δ^9.

Double-bond stereochemistries of the aldehydes (as methyl esters) were determined via gas chromatography. In all cases only the *Z* stereoisomer was present.

Ethyl Esters. The presence of ethyl esters in *P. bicinctus* extracts was easily recognized by the characteristic ion at *m/z* 88 in their EI mass spectra. Although parent ion abundances were quite low (or sometimes undetectable when only small amounts of material were present), the ions at M-46 arising from a loss of ethanol were typically present in 3–5% abundance. CI-MS of these compounds had M+1 ions as their base peaks and M-45 ions of approximately 20% relative intensity.

As with the aldehydes, attempts to form methoxy derivatives using the crude glandular extracts were unsuccessful. Interestingly, thiomethyl ether formation was successful using the crude extracts (Francis and Veland, 1981). EI mass spectra of the resulting vicinal dithiomethyl ethers allowed the double-bond position of the ethyl esters to be assigned as Δ^9. Stereochemical assignment as the *Z* isomers was again based on GC coelution with known standards.

Carboxylic Acids. The presence of the carboxylic acids in the *Philanthus* extracts was first detected when the addition of 1,1-dimethylhydrazine to the total mixture produced not only the expected hydrazones of the aldehydes and ketones, but also a homologous series of methyl esters (see Discussion). The EI-MS of these methyl esters showed the expected ion at *m/z* 74, small parent ions, and M-32 ions in 3–5% abundance. CI-MS had the M+1 ion as base peak and significant M-31 ions, presumably resulting from loss of methanol from the protonated molecular ion.

To locate the positions and configurations of the double bonds of these acids, they were separated from the crude mixture by extraction with sodium carbonate and converted to their methyl esters using diazomethane. Methoxy ethers were prepared as above, but the resulting mixture of carbon number and positional isomers precluded unequivocal locations of the methoxyl groups. This problem was overcome by using the method of Plattner et al. (1975), whereby the mixture of methoxy ethers was injected onto a GC column in which the first few centimeters was packed with palladium chloride on Chromosorb W, with hydrogen used as the carrier gas. This reduced all remaining double bonds, and the eluting compounds were then all monomethoxyl methyl esters whose EI-MS were readily interpreted. As indicated in Table 1, the double-bond positions in the acids were all Δ^9 or $\Delta^{9,12}$. Assignment of the *Z* configuration to all double bonds was based on GC coelution with known standards.

DISCUSSION

The complex mixtures of carbonyl compounds present in the *Philanthus* mandibular gland secretions present an interesting challenge. The carboxylic acids tailed badly on our nonpolar GC columns and were difficult to detect in their free state. Although the aldehydes chromatographed well, their mass spec-

tra were not easily differentiated from those of diunsaturated alcohols of the same carbon number. Unlike our previous experience with unsaturated cuticular hydrocarbons, the total crude extract was not a suitable mixture for using the technique of methoxymercuration–demercuration to locate double bonds.

The use of DMH to convert the ketones and aldehydes to their hydrazones and the carboxylic acids to methyl esters (McDaniel and Howard, 1985) was the critical step in determining the chemistry of these carbonyl mixtures. DMH treatment of the crude glandular extract followed by direct GC-MS analysis allowed us to readily identify the basic structural features of all the classes of semiochemicals present. In the case of the ketones, the resulting hydrazone spectra were verification of what we had readily deduced from the underivatized parent compounds. In the case of the aldehydes, however, the resulting hydrazone spectra were clearly more diagnostic than those of the free aldehydes (Budzikiewicz et al., 1967; McDaniel and Howard, 1985). Conversion of the free acids to their methyl esters made possible the chromatographic separation and subsequent mass spectral identification of these compounds. All of these derivatizations and analyses were made on nanogram to microgram quantities and, for at least the DMH-induced reactions, all were essentially quantitative. Perhaps the greatest obstacle was the location of the double-bond positions in the aldehydes, acids, and ethyl esters.

Methoxymercuration has been successfully used by us and others to derivatize esters (Abley et al., 1970), alkenes (Blomquist et al., 1980), and dialkyl pyrrolidines (Jones et al., 1982). It is not clear in our case whether it is the free acids, ketones, or aldehydes that are hindering the reaction, either at the initial addition stage or the subsequent reduction step with sodium borohydride. The fact that methylthiolation (which has no reductive step) occurred smoothly with the ethyl esters, even in the crude extract, suggests that the problem with the methoxymercuration may have been in the addition step. Whatever the reason, isolation of the free acids and aldehydes (by oxidation to their respective acids) followed by conversion to their methyl esters allowed us to successfully locate the double bonds using methoxymercuration. In agreement with Plattner et al. (1975), we found that the dienoic compounds gave very complex mixtures of saturated and unsaturated methoxy esters. Their technique of hydrogenating the product mixture, however, eliminated the problem.

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Announcement

FOURTH ANNUAL MEETING OF THE INTERNATIONAL SOCIETY OF CHEMICAL ECOLOGY

The Fourth Annual Meeting of the International Society of Chemical Ecology will be held at the University of Hull, England from the evening of Monday 13th July through lunch on Friday 17th July 1987.

We are planning 4 symposia on the following topics:

1. The veracity of bioassays
2. Chemical ecology and plant protection
3. The applications of chemical ecology
4. Biochemical mechanisms of defence compounds

These topics are broad enough to include allelopathy, natural marine antifouling agents, pheromones, physiological and behavioral responses to allelochemicals, etc., and we hope that some/many of the contributed papers and poster sessions will reflect the main themes of the meeting. We plan to stimulate commercial and industrial interest in the Society by emphasizing the applications of our studies.

Members (and potential members) of ISCE are asked to send the title and abstract of a paper or poster that they wish to present at the meeting to Professor David A. Jones, ISCE 1987, Department of Plant Biology & Genetics, University of Hull, Hull HU6 7RX, England.

NOTE

The deadline for submitting details of oral presentations is February 28, 1987. There is no deadline for posters, but the sooner you submit your title and abstract the sooner you can receive an acknowledgement that may help you to obtain travel funds!

The full cost of accomodation, all meals, and excursion, the conference fee, and the conference dinner will be £140.00.

A reservation form will be issued to members with the Spring 1987 ISCE Newsletter. Early in 1987, forms will be sent to people who have addressed inquiries directly to David Jones in Hull.

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PRELIMINARY ISOLATION OF MALE-INHIBITORY PHEROMONE OF THE SPIDER *Schizocosa ocreata* (ARANEAE, LYCOSIDAE)

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Abstract—Compounds with pheromone activity were extracted from male *Schizocosa ocreata* silk using methanol as the solvent. Behavioral data suggest that these compounds mediate agonistic encounters among male spiders. Preliminary chemical analyses indicate that the pheromones are low-molecular-weight lipids, one being slightly polar, the other nonpolar.

Key Words—*Schizocosa ocreata,* Araneae, Lycosidae, spider pheromone, male pheromone, agonistic display, courtship inhibitor, pheromone bioassy.

INTRODUCTION

Chemical communication has been long recognized in spiders (Bristowe and Locket, 1926; Crane, 1949; Kaston, 1936). Most of this early research has concentrated on behavioral assays for female-produced sex pheromones, while some studies described chemical communication mediating behaviors associated with species recognition, maintenance of group cohesion (in social spiders), and location of mates. In all of these studies, silk has been used as a pheromone source. Recently, a few researchers have investigated the microscopic structure of spider chemoreceptors, or their electrophysiology. However, no one has reported a chemical analysis of any spider pheromones (reviewed in Tietjen and Rovner, 1982).

Extraction of female silk by common organic and inorganic solvents, such as alcohol and water, was shown to inactivate the pheromone-like activity in several spider species (Gwinner-Hanke, 1970; Hegdekar and Dondale, 1969; Kaston, 1936; Sarinana et al., 1971), a property that has hindered research into the chemical structure. In a few species, however, the successful transfer of an

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active pheromone from female silk to another substratum has been reported, but no details are given as to the chemical nature of the pheromone (Collatz in Weygoldt, 1977; Kaston, 1936; Suter and Renkes, 1982).

The present study describes the first chemical isolation of a spider pheromone. In this case, we extracted pheromones from the silk of male *Schizocosa ocreata.* Behavioral evidence suggests that these pheromones may be used during male–male interactions to affect the frequency of agonistic display and inhibit courtship behavior. In addition, we provide the first experimental evidence for male-produced pheromones used for a chemical-based mating interference strategy.

METHODS AND MATERIALS

General Methods. Spiders were collected in Saint Charles County, Missouri, as antepenultimate and penultimate instars and were individually raised to adults in 100 × 15-mm plastic Petri dishes. Water was provided ad libitum via a cotton-stoppered vial inserted through a hole in the lid. Adult house flies (*Musca domestica*) and *Tenebrio* sp. larvae were provided at weekly intervals. Upon molting to the adult instar, male spiders were visually isolated from one another (isolation of males makes them more likely to respond to chemical cues; Crane, 1949; Tietjen, 1977, 1979a).

Previous research (Tietjen, 1979a) indicated that pheromones were associated with the silk of various lycosid species, including *S. ocreata.* This being the case, we chose silk as a potential source for extraction of pheromone in these experiments. Silk deposited on 7.5-cm-diam filter paper cage cards was used directly in some experiments or the pheromone was extracted from the silk on the cards as described below.

Pheromone Extraction and Concentration. Spiders were not fed during silk deposition periods in order to decrease extraneous contamination on the cage cards. Preliminary bioassays with extracts of acetone, benzene, hexane, methanol, methylene chloride, and petroleum ether indicated that methanol was most effective in extracting behaviorally active pheromone from silk on male cage cards. Cage cards were soaked in 10 ml of methanol for 15 min. The dissolved pheromone was then deposited on filter paper (7.5 cm diam) and allowed to air-dry for 10 min. These papers provided the experimental stimuli, while papers soaked in methanol (with no dissolved pheromone) and allowed to air-dry provided the control. The dried extract was immediately bioassayed for pheromone activity except when additional chemical extractions of the pheromone was required. In these cases, the extract and stimulus filter papers were stored in a desiccator at −20°C until eventual use. These papers were brought to room temperature by first placing them in a refrigerator at 1.5°C for 20–30 min and then warming the papers to room temperature; this process decreased the amount

of condensation collecting on the stimulus cards and thus minimized any potential inactivation by water.

A relative measure of the amount of pheromone deposited on the experimental substrata was obtained by multiplying the total number of cage cards used in the extraction by the number of days silk was deposited by each spider. This number was then divided by a dilution factor (since only a portion of the extract was deposited on filter paper for the arena tests, and the remainder used for chemical tests) to obtain our estimate of relative pheromone concentration, the number of "silk days." By pooling silk from several spiders and allowing three or more days for deposition of silk by each spider, behaviorally active pheromone concentrations were obtained. This method allowed us to compensate for potential differences both among and between spiders in silk deposition. Three concentrations were used: low, medium, and high, corresponding to 60, 147, and 694 silk days.

To examine the polar–nonpolar nature of the methanol extract, 60 silk day extract was applied on a Florisil column (0.5 × 5.0 cm; Fisher) and the flowthrough was collected. The material which remained bound to the column as a light brown ring was then eluted with methanol. The flowthrough and eluates were then prepared as above for bioassay. In addition, thin-layer chromatographic fractionation of 60 silk day extract was also carried out on silica gel (Eastman Kodak) to separate the polar and nonpolar lipid components in the extract. Two solvent systems (A: diethyl ether–benzene–absolute ethanol–acetic acid, 40 : 50 : 2 : 0.2; B: chloroform–methanol–acetic acid–water, 85 : 15 : 10 : 4) were used. Unfortunately the advanced age of our test animals and the relatively low concentration of the lipid components did not allow further bioassay of these samples.

Bioassay Procedure. Adult males were tested during their period of highest behavioral response (1500–2400 hr), and each male was tested only once per 24 hr. Behavioral responses were observed in an arena which consisted of two parts: A 0.5-cm-thick circular ring (43.2 cm diam × 5.0 cm height) made of nonreflecting nylon was used as the perimeter of the arena. The second part, a 1-mm-thick plastic sheet, served as the bottom of the arena. The top of the arena was open to the air. Both the arena and sheet were washed with water and thoroughly dried between experimental runs. Three experimental filter papers (with dried solvent extract or untreated silk) were placed equidistant from one another along the interior perimeter of the arena. Three additional control filter papers were interspaced among the three experimental substrata.

Individual male spiders were introduced into the center of the arena and allowed to wander freely until they contacted one of the experimental substrata. When exposed to the highest pheromone concentrations, males "froze" on introduction to the arena and, after a minimum of 5 min inactivity, were guided onto an experimental substratum with the aid of a camel's hair brush (such periods of inactivity were only observed in response to the high concentration).

In either case, the behavior of the test male was then recorded at 15-sec intervals for a 5-min test period. Four behavioral responses were recorded: (1) chemoexploration with the palps, (2) foreleg tapping, (3) stridulation, and (4) wave and arch. Descriptions and interpretations of these behaviors can be found in Aspey (1976) and Tietjen (1979a).

Male spiders respond to female silk by exhibiting courtship behavior having the following behavioral components: foreleg and abdominal vibration, and stridulation (Tietjen, unpublished data). In order to determine if the male silk extract had any effect on male courtship behavior, the males were transferred to a female's cage card and the frequency of courtship behavior in response to silk-deposited female sex pheromone was recorded for another 5-min period. Animals that did not court within the 5-min period were scored as "not courting." Since the data did not fit parametric assumptions, and sample sizes were relatively small, nonparametric tests were used for all analyses (Conover, 1971).

RESULTS

Bioassay. The frequencies of the behaviors were compared among treatments using the chi-square test (Table 1). Foreleg tapping decreased in the presence of high concentrations of extract, the column flowthrough, and the column eluate ($P < 0.01$). Similarly, wave and arch decreased with the high concentration and flowthrough treatments ($P < 0.001$). The frequency of chemoexploration was greatest for the 60 silk days extract ($P < 0.01$). Males exhibited neither chemoexploration nor stridulation in response to the high concentration of extract.

When males were tested on females' cage cards, those who had previously been exposed to high concentrations of male silk extract showed no courtship behavior (Table 1). The methanol eluate decreased the frequency of courtship to the same proportion as that observed with only male silk. The frequency of courtship following the methanol eluate differed from that of the flowthrough ($P < 0.001$).

Overall, a graded response dependent on pheromone concentration was observed. Foreleg tapping, wave and arch, chemoexploration, and courtship percent decreased with an increase in male pheromone concentration, indicating an inhibition of courtship behavior.

Preliminary Characterization of Pheromone. Methanol extracts in solution had an "oily" appearance on the surface of the solvent. When the extracts were deposited on filter paper and then dried, a light tan residue remained. These papers had a "pungent" odor, which decayed with time at room temperature.

The extract did not show free amino acids, polypeptides, sugars, or starch in detectable amounts as tested by biuret, ninhydrin, anthrone, and iodine tests

TABLE 1. BEHAVIORAL RESPONSES OF MALE *Schizocosa ocreata* TO EXPERIMENTAL TREATMENTS IN ARENA AND ON FEMALE CAGE CARDS FOLLOWING ARENA TESTS[a]

Treatments	(*N*)	Behavioral Responses				
		Foreleg tap	Wave and arch	Chemoexploratory behavior	Stridulation	Courtship % on female card
Methanol control	(12)	8.9 ± 1.4 (100.0%)	2.9 ± 1.0 (66.7%)	0.5 ± 0.2 (33.3%)	0.3 ± 0.3 (16.7%)	91.7%
Male silk	(10)	10.6 ± 1.2 (100.0%)	2.7 ± 0.1 (70.0%)	0.8 ± 0.4 (30.0%)	0.3 ± 0.2 (30.0%)	40.0%
Low conc.	(16)	10.0 ± 1.1 (100.0%)	4.8 ± 1.2 (100.0%)	1.6 ± 0.4 (75.0%)	1.3 ± 0.3 (75.0%)	75.0%
Medium conc.	(13)	9.2 ± 1.4 (100.0%)	3.4 ± 1.4 (84.6%)	0.2 ± 0.2 (7.7%)	1.8 ± 0.6 (61.5%)	69.2%
High conc.	(7)	2.4 ± 0.4 (100.0%)	1.1 ± 1.0 (28.6%)	0.0 (0.0%)	0.0 (0.0%)	0.0%
Flowthrough	(9)	4.9 ± 1.4 (100.0%)	1.0 ± 0.5 (55.6%)	0.7 ± 0.3 (44.1%)	0.9 ± 0.4 (50.0%)	77.8%
Methanol eluate	(10)	4.3 ± 1.3 (80.0%)	2.3 ± 1.3 (80.0%)	0.7 ± 0.4 (50.0%)	1.4 ± 0.4 (50.0%)	40.0%

[a]The responses to experimental substrata (foreleg tap, wave and arch, chemoexploration, and stridulation) are presented as the mean number of 15-sec intervals having one or more counts of the indicated behavior. The variation is the standard error of the mean and the data in parentheses indicate the percentage of runs with one or more instances of a behavior during the 5-min experimental period. The courtship measures are for tests on female cage cards following the responses to experimental substrata.

(60 days extract was used for these tests by mixing with an equal volume of water and agitating at 25°C for 15 min). The pH of the solution was 6.85, indicating the absence of significant amounts of free fatty acids.

The nonaqueous components of the solution were analyzed by extracting with carbon tetrachloride in a separatory funnel at room temperature for 15 min. The lower organic phase was collected and tested with bromine dissolved in carbon tetrachloride. Results suggest that the compound has a high degree of saturation. Tests for cholesterol and primary or secondary alcohol groups were negative (Libermann-Burchard test, oxidation tests).

Both polar and nonpolar components were present, as indicated by the lack of complete solubility of the dried methanol extract in benzene. This dual-component nature of the extract was also observed in thin-layer chromatographic fractionation, where two lipid spots were found; one a polar component (*Rf* = 0.6; system B, polar solvent system) and the other a nonpolar component (*Rf* = 0.8; system A, nonpolar solvent system).

DISCUSSION

A comparison of the behavioral responses to the low concentration (60 silk day) extract and unextracted male silk indicates that the actual efficiency of our extraction procedure was quite low. However, a significant portion of the inhibitory behavior associated with unextracted silk could be attributed to the mechanical cues provided by the silk (as is the case in other lycosid species; Tietjen 1977). It is important to note that the actual active pheromone concentrations are probably much lower than our estimates. We have recently developed an improved pheromone extraction procedure and have tested it on the related species, *Lycosa punctulata*. Using this method we have observed behavioral activity for concentrations less than 1 silk day.

Aspey (1976) used factor analysis to determine the function of display by male *S. ocreata* during staged agonistic encounters and determined that both foreleg tapping and wave and arch behaviors were indicative of agonistic display. Both behaviors occur spontaneously at a low level and, in addition to stridulation, may serve as an "announcement display." Thus, depending on context, the same behavior may serve for both announcement and agonistic display, with the agonistic displays usually being more vigorous. The present data indicate that the frequency of both behaviors decreases with increasing pheromone concentration and is lowest for the 694 silk days, flowthrough, and eluate. The decrease in announcement displays suggests that the males were intimidated" by high concentrations of pheromone. The observation that all males (100%) "froze" when introduced into the arena in the presence of high pheromone concentration supports our intimidation hypothesis.

The above observations, e.g., complete inactivity of the males on intro-

duction to the arena without contact with the extract, and the decrease in behavioral response toward the extract over several days, suggest that a volatile component of the extract is responsible for the observed behavioral effect. Thus, males confronted with the concentrated pheromone find themselves faced with a superior, but unseen opponent, and choose to abdicate in a potential dispute. Riechert (1984), working with staged contests among *Agelenopsis aperta*, suggested that pheromones could be employed in this way to affect the outcome of territorial disputes. In a similar vein, Tietjen (1979a, b) reported that male *L. rabida* also respond to a possible male-produced pheromone with a decrease in both courtship and chemoexploratory behavior when later tested on female silk. In addition, these spiders deposit greater quantities of silk when following female draglines (Tietjen and Rovner, 1982).

In the present study, high concentrations of extract showed a carry-over effect when males were tested for response to female pheromone following exposure to the stimulus. Males that previously were tested with 694 silk days concentrations exhibited no courtship in response to female silk. Similarly, methanol eluate (which is a further concentration of the 60-day extract) inhibited courtship to a greater degree than the flowthrough. These results and the independent observations in other species provide evidence for a pheromone-based mating interference strategy among male spiders, a behavioral strategy that has been recorded for several other arthropod species (Tietjen and Rovner, 1982).

The results of thin-layer chromatography suggest that both slightly polar and nonpolar lipids were extracted from male silk. Two other observations also suggest a multicomponent nature to the methanol extract: the incomplete solubility of the dried methanol extract in benzene, and the behavioral response (data not shown) seen with extracts of male silk with polar solvents (hexane and methylene chloride). Therefore, we suggest that the flowthrough and methanol eluate represent the nonpolar and slightly polar components of the extract since methanol can extract both components from the silk. We also suggest that these components are neutral lipids with small molecular weights and a volatile nature and are involved in a general way with male recognition. The polar component (methanol eluate) has been shown to affect courtship behavior by males, thus serving as a male inhibitory pheromone. However, further chemical characterization of both components is needed to determine the exact behavioral functions.

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ATTRACTANCY AND SPECIES SPECIFICITY OF 6-ACETOXY-5-HEXADECANOLIDE, A MOSQUITO OVIPOSITION ATTRACTANT PHEROMONE

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Abstract—Four stereoisomers of 6-acetoxy-5-hexadecanolide, a mosquito oviposition attractant pheromone, were bioassayed for their activity on mosquitoes. Only (−)-(5*R*,6*S*) isomer was active in attracting *Culex quinquefasciatus* Say females for oviposition at dosages of 0.5 μg/100 ml water and above with the floating-cap method. The activity of this isomer increased 50-fold when it was applied directly to the water surface. The other three isomers, (+)-(5*S*,6*R*), (+)-(5*R*,6*R*), and (−)-(5*S*, 6*S*), were not active. The active isomer was ovipositionally attractive not only to *C. quinquefasciatus* but also to *C. tarsalis* Coquillett; however, it was 100 times more active in the former than in the latter species. *Aedes aegypti* (L.) and *Anopheles quadrimaculatus* Say were not attracted to the pheromone, thus suggesting that the pheromone is genus-specific.

Key Words—(−)-(5*R*,6*S*)-6-Acetoxy-5-hexadecanolides, oviposition attractant pheromone, mosquitoes, *Culex quinquefasciatus*, *Culex tarsalis*, Diptera, Culicidae.

INTRODUCTION

The apical droplets of egg rafts of *Culex pipiens fatigans* Wiedemann contain an oviposition attractant pheromone which induces the oviposition of this and other *Culex* mosquito species (Bruno and Laurence, 1979). The major component of the pheromone was identified as *erythro*-6-acetoxy-5-hexadecanolide,

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and subsequently the racemates were synthesized from *erythro*-5,6-dihydroxy-hexadecanoic acid by treatment with acetic anhydride in dry pyridine (Laurence and Pickett, 1982).

The synthesis of the two enantiomers of this δ-lactone was accomplished by at least four groups. Fuganti et al. (1982) reported the synthesis of both enantiomers of *erythro*-6-acetoxy-5-hexadecanolide from a chiral C_4 aldehyde via a nonstereoselective route. Mori and Otsuka (1983) synthesized both enantiomers from (±)-1-tridecen-3-ol through its kinetic resolution by enantioselective epoxidation, thus achieving a stereoselective synthesis of the two isomers. Laurence et al. (1985) found that the synthetic (−)-(5*R*,6*S*) enantiomer attracted oviposition of mosquito egg rafts fourfold over the control, whereas the (+)-(5*S*,6*R*) enantiomer did not attract ovipositing females to any significant extent. The absolute configuration of the oviposition attractant pheromone was thus determined to be (−)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide. Subsequently, Laurence and Pickett (1985) reported the behavior of the gravid females of *C. quinquefasciatus* Say to the pheromone, the effect of modifying the structure of the pheromone on oviposition behavior, and the source and activity of the pheromone in nature. Sakakibara et al. (1984) reported the ovipositional activity of four stereomers of 6-acetoxy-5-hexadecanolide; however, the details of the synthesis of the isomers were not disclosed.

Independently, Lin et al. (1985) synthesized all four stereomers of 6-acetoxy-5-hexadecanolide including two *erythro* forms and two *threo* forms from (*Z*)-2-tridecen-1-01 and its *E* isomer via Sharpless asymmetric expoxidation. To reconfirm the findings of Laurence et al. (1985) and Sakakibara et al. (1984) and to obtain more information on the biological properties of all four isomeric compounds, we conducted the present investigation. Here we report the ovipositional attractancy of the four optical isomers of 6-acetoxy-5-hexadecanolide to *C. quinquefasciatus* (= *C. p. fatigans*) and the determination of the species specificity of the active (5*R*,6*S*) enantiomer among *Culex*, *Aedes*, and *Anopheles* mosquito species.

METHODS AND MATERIALS

Mosquitoes were obtained from our laboratory stock colonies at the University of California, Riverside. The *C. quinquefasciatus* colony was first established in 1950s with a strain obtained from Bakersfield, California. Since then the original strain has been reinforced by local strains from Riverside, California. The *C. tarsalis* colony was established in Riverside in 1984 with a strain from Chino Basin in California. *Aedes aegypti* (L.) and *Anopheles quadrimaculatus* Say were obtained from Gainesville, Florida. Gravid females, blood-fed and 7–10 days old, were chosen for bioassay studies.

Each of the olfactometers constructed and used for studying the oviposi-

tional attractancy of the four stereomers of 6-acetoxy-5-hexadecanolide consisted of: (1) a mosquito cage, (2) two 4-oz waxed paper cups, and (3) two polyethylene vial caps. The mosquito cage, 23 cm height, 23 cm width, and 32.5 cm depth, was constructed by framing it with 2 × 2.5-cm wood. The two sides and the rear were screened with Lumite® (32 × 32 mesh). The bottom was covered with a wood panel. The rear part of the top was covered with a 20.5 × 20.5-cm glass for viewing, and the front part of the top was screened with Lumite. The front was covered with a wood panel with a 15-cm-diameter porthole in its center. The hole is fitted with a muslin sleeve to allow access to the inside of the cage. All wood was painted white with Latex™. The waxed paper cups (7 cm diameter, Sweetheart® ice cream and food cups, No. 5304), each contained 100 ml distilled water and were used as oviposition sites for mosquitoes. The polyethylene vial caps (3 cm diameter) were inverted and used for holding test materials.

In conducting bioassay tests, a stock solution of each compound was made by dissolving the compound (1.0 mg) in 1,2-dichloroethane (10 ml). A predetermined volume of the solution was taken with a microsyringe and placed on the surface of a vial cap, and an equal volume of the solvent was applied onto another vial cap. After the solvent was allowed to evaporate, the two vial caps, one containing the predetermined amount of the compound and the other serving as a control, were each floated on water in the waxed paper cup. This method was termed the "floating-cap method." In some tests in which the so-called "direct method" was used, the solution or the solvent was directly applied on the water surface in the cup without using the vial cap. The two cups were separately placed at the rear corners of the mosquito cage.

Twenty gravid female mosquitoes were introduced into the cage and allowed to choose between the treated and control cups for oviposition. All tests were replicated at least six times in a ventilated room at a constant temperature of 25°C. After the start of the experiment, the room lighting was adjusted to simulate 1 hr dusk, 11 hr darkness, and 1 hr dawn with a combination of fluorescent and incandescent lights which were controlled by an adjustable timer (AMF Paragan-Electric Co., Inc., Two Rivers, Wisconsin, model 4003-05). The numbers of oviposition in the treated and control cups were recorded 1 hr after the resumption of normal lighting. The total duration of the bioassay was about 14 hr. The attractancy of the testing compounds was expressed in terms of an "attractancy ratio" which was the ratio of the number of egg rafts or eggs in the treated cup to those in the control cup. The data were analyzed for significance by the chi-square method.

The (5*R*,6*S*)-, (5*S*,6*R*)-, (5*R*,6*R*)-, and (5*S*,6*S*)-6-acetoxy-5-hexadecanolide, $[\alpha]_D^{20}$ − 37.4°, +37.2°, +14.6°, and −14.1° (c = 1.2 ~ 6, $CHCl_3$), respectively, were obtained by synthesis (Lin et al., 1985). All compounds had an enantiomeric excess of 96–97% and gave proper and required NMR, IR, and mass spectra.

RESULTS AND DISCUSSION

The bioassay tests (Table 1) revealed that, between the two *erythro* forms of 6-acetoxy-5-hexadecanolide, the (−)-(5*R*,6*S*) enantiomer was ovipositionally attractive to gravid females of *C. quinquefasciatus* in the floating-cap method from 0.5 to 50 μg. Laurence et al. (1985) tested the same enantiomers for activity at about 1 egg raft equivalent (0.3 μg) because they found that this level of dosage of the racemic mixture gave a high degree of response but was near the beginning of the steeply downward sloping part of the dosage–response curve. At this dosage, they found that the (−)-(5*R*,6*S*) enantiomer attracted 70 egg rafts, whereas only 17 egg rafts were laid in the control (equivalent to an attractancy ratio of 4.1). In the present study, we found that the attractancy ratio of the (−)-(5*R*,6*S*) enantiomer at 0.5 μg was 4.2. Our results were, therefore, quite comparable to those of Laurence et al. (1985). At higher dosages of 1–50 μg, the (−)-(5*R*,6*S*) enantiomer gave attractancy ratios of 2.3–3.5, all of which were significant at the 0.01 level.

The other *erythro* form, the (+)-(5*S*,6*R*) enantiomer, did not show any significant attractancy at all dosages tested, coinciding with the findings of Laurence et al. (1985). Likewise, neither of the two *threo* forms, (+)-(5*R*,6*R*) and (−)-(5*S*,6*S*) enantiomers, showed any attractancy in the floating-cap method. These results indicate that the interaction between the ovipositional receptor site of the *Culex* mosquito and the ovipositional attractant pheromone is stereospecific and that the receptor accepts only the (−)-(5*R*,6*S*) enantiomer. Not only is the (+)-(5*S*,6*R*) enantiomer not acceptable by the receptor as reported by Laurence et al. (1985), but the two *threo* forms are also unacceptable and elicit no ovipositional response in mosquitoes as shown in the present study.

When bioassayed in the direct method, the (−)-(5*R*,6*S*) isomer exhibited a higher level of attractancy than that in the floating-cap method (Table 2). In the direct method, the attractancy was significant at dosages from 10 through 0.01 μg/100 ml water. The lowest effective dosage in the floating-cap method was 0.5 μg as opposed to 0.01 μg/100 ml water in the direct method. Thus, the lowest effective dosage increased by 50-fold by adopting the direct method. The reason for this increase in activity might be due to the increase of surface area (from 28 to 154 cm^2), thus facilitating the evaporation of the pheromone.

Other stereoisomers were not attractive in the direct method except for the (+)-(5*S*,6*R*) and (−)-(5*S*,6*S*) isomers, which showed significant attractancy only at the high concentration of 10 μg/100 ml water. It should be noted that the enantiomeric excess for each isomer was found to be 96–97%; therefore it was possible that the (+)-(5*S*,6*R*) and (−)-(5*S*,6*S*) isomers contained a small fraction of the active isomer which was sufficient to show some activity. The (+)-(5*R*,6*R*) isomer was inactive at 10 μg; whether this inactivity is due to the absence of the active isomer as a contaminant is not clear at this time.

TABLE 1. ATTRACTANCY RATIOS OF FOUR STEREOISOMERS OF 6-ACETOXY-5-HEXADECANOLIDE TESTED AGAINST *Culex quinquefasciatus* IN LABORATORY OLFACTOMETERS BY FLOATING-CAP METHOD

Dosage	Attractancy ratio[a] (Mean No. ± SD)[b]			
(μg)	(−)-(5*R*,6*S*)	(+)-(5*S*,6*R*)	(+)-(5*R*,6*R*)	(−)-(5*S*,6*S*)
50.0	2.3* (8.2 + 2.2)	1.0 (5.8 ± 2.6)	1.3 (4.8 ± 1.9)	1.6 (7.8 ± 3.7)
25.0	2.9* (9.8 ± 2.9)	0.6 (2.7 ± 1.5)	1.0 (4.7 ± 3.1)	1.1 (4.2 ± 2.3)
10.0	2.5* (9.7 ± 3.2)	0.8 (4.0 ± 1.3)	1.0 (5.0 ± 2.5)	0.8 (3.8 ± 3.3)
1.0	3.5* (9.3 ± 2.3)	0.9 (5.2 ± 2.7)	0.8 (4.5 ± 2.3)	1.1 (5.8 ± 2.2)
0.5	4.2*(10.5 ± 1.4)	1.0 (4.5 ± 3.1)	1.1 (5.8 ± 2.7)	1.0 (5.8 ± 2.6)
0.1	1.2 (6.2 ± 2.3)	0.9 (3.0 ± 1.8)	0.9 (3.5 ± 1.6)	0.9 (5.5 ± 1.6)

[a] Attractancy ratios followed by asterisks indicate that the means of oviposition in the treated cups are significantly higher than those in the control cups at the 0.01 level by the chi-square test.
[b] Mean numbers of egg rafts in the treated cups with standard deviations.

TABLE 2. ATTRACTANCY RATIOS OF FOUR STEREOISOMERS OF 6-ACETOXY-5-HEXADECANOLIDE TESTED AGAINST *Culex quinquefasciatus* IN LABORATORY OLFACTOMETERS BY DIRECT METHOD

Dosage	Attractancy ratio[a] (Mean No. + SD)[b]			
(μg/100 ml H_2O)	(−)-(5*R*,6*S*)	(+)-(5*S*,6*R*)	(+)-(5*R*,6*R*)	(−)-(5*S*,6*S*)
10.0	5.5* (7.3 ± 1.5)	2.9*(9.2 ± 3.8)	1.0 (6.2 ± 3.2)	3.3*(10.8 ± 4.0)
1.0	5.4* (8.4 ± 3.3)	1.4 (3.9 ± 2.9)	0.8 (2.8 ± 2.5)	1.5 (4.1 ± 2.4)
0.5	2.4*(11.7 ± 2.3)	1.4 (3.8 ± 1.8)	1.1 (7.0 ± 2.5)	1.3 (8.0 ± 2.8)
0.1	5.1* (7.7 ± 3.5)	1.0 (5.5 ± 2.5)	0.8 (3.0 ± 1.3)	0.9 (5.3 ± 2.7)
0.05	7.0*(3.5 ± 1.9)	0.9 (2.5 ± 2.4)	0.8 (4.0 ± 1.3)	0.8 (5.3 ± 3.2)
0.01	2.5*(5.0 ± 1.8)	1.1 (3.0 ± 2.6)	0.8 (3.7 ± 3.4)	0.8 (4.5 ± 2.0)
0.005	1.5 (5.5 ± 1.9)	0.8 (2.8 ± 1.2)	1.3 (7.0 ± 2.7)	0.8 (4.7 ± 3.1)
0.001	0.8 (6.0 ± 1.3)	0.9 (3.7 ± 1.5)	0.9 (5.0 ± 2.5)	0.9 (3.8 ± 2.2)

[a] Attractancy ratio followed by asterisks indicate that the means of oviposition in the treated cups are significantly higher than those in the control cups at the 0.01 level by the chi-square test.
[b] Mean numbers of egg rafts in the treated cups with standard deviations.

TABLE 3. ATTRACTANCY RATIOS OF (−)-(5*R*,6*S*)-6-ACETOXY-5-HEXADECANOLIDE TESTED AGAINST *Culex*, *Aedes*, AND *Anopheles* MOSQUITOES IN LABORATORY OLFACTOMETERS BY DIRECT METHOD

Dosage (μg/100 ml H_2O)	Attractancy ratio[a] (Mean No. ± SD)[b]		
	C. tarsalis	*A. aegypti*	*A. quadrimaculatus*
10	2.1*(7.5 ± 3.3)	1.0 (247 ± 100)	0.8 (298 ± 156)
1	3.5*(8.7 ± 3.0)	1.1 (417 ± 74)	0.7 (360 ± 69)
0.1	1.3 (6.3 ± 2.1)	1.4 (236 ± 53)	1.2 (417 ± 204)
0.01	1.3 (6.6 ± 2.4)	1.1 (222 ± 64)	1.2 (347 ± 120)

[a] Attractancy ratio followed by asterisks indicate that the means of oviposition in the treated cups are significantly higher than those in the control cups at the 0.01 level by the chi-square test.
[b] Mean numbers of eggs (*A. aegypti* and *A. quadrimaculatus*) or egg rafts (*C. tarsalis*) in the treated cups with standard deviations.

Three other species of mosquitoes, *Culex tarsalis* Coquillett, *Aedes aegypti* (L.), and *Anopheles quadrimaculatus* Say, were used to study species specificity of the pheromone (Table 3). While *Aedes* and *Anopheles* mosquitoes were not attracted to the pheromone for oviposition, *C. tarsalis* mosquitoes were attracted at dosages of 1 and 10 μg/100 ml water but were not attracted to the lower dosages of 0.1 and 0.01 μg/100 ml water. Hence, the pheromone was at least genus-specific. However, in comparing the minimum effective dosages between the two *Culex* species in the direct method, the pheromone was 100 times more effective in attracting *C. quinquefasciatus* than *C. tarsalis*. Bruno and Laurence (1979) found that *C. tarsalis* responded to the presence of 10 egg rafts but, unlike *C. p. fatigans*, not to the presence of one egg raft. Our results are almost in line with these previous findings. It is therefore apparent that, among the four stereoisomers of 6-acetoxy-5-hexadecanolide, only (−)-(5*R*,6*S*) isomer is active in inducing a positive ovipositional response in *C. quinquefasciatus*. The other sources, (+)-(5*S*,6*R*), (+)-(5*R*,6*R*), and (−)-(5*S*,6*S*) isomers, show little or no activity. The ovipositional activity of the attractant pheromone is more prominent in the direct method than in the floating-cap method. In addition, the pheromone seems to be genus-specific, showing effectiveness only on *Culex* mosquitoes; however, it is much more attractive to *C. quinquefasciatus* than to *C. tarsalis*. Sakakibara et al. (1984) reported that the pheromone ovipositionally attracted *C. p. molestus* Forskal and *C. p. pallens* Coquillett but failed to attract *A. stephensi* Liston and *A. aegypti*, thus proving the pheromone to be genus-specific. Their findings were, therefore, in agreement with ours.

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CHEMISTRY AND FUNCTIONS OF EXOCRINE SECRETIONS OF THE ANTS *Tapinoma melanocephalum* and *T. erraticum*

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Abstract—Volatile constituents produced by ant workers belonging to the species *Tapinoma melanocephalum* and *T. erraticum* have been analyzed by gas chromatography–mass spectrometry. The pygidial (= anal) gland secretion of *T. melanocephalum* is fortified with 6-methyl-5-hepten-2-one and actinidine (the mass spectrum of which is corrected in this paper). An unidentified compound was detected in cephalic extracts. The pygidial gland secretion of *T. erraticum* was also dominated by 6-methyl-5-hepten-2-one, in addition to two isomers of iridodial, and iridomyrmecin. The sternal glands contained iridodial and C_{15}–C_{20} hydrocarbons. Workers of *T. melanocephalum* effectively utilize their pygidial gland secretions as an alarm–defense system during aggressive encounters with workers of *Solenopsis geminata*. 6-Methyl-5-hepten-2-one is active as a releaser of alarm behavior, and actinidine is repellent to workers of *T. melanocephalum*. Cephalic extracts possessed attractant and arrestant properties for workers of this species.

Key Words—*Tapinoma melanocephalum*, *Tapinoma erraticum*, pygidial gland, sternal gland, actinidine, chemistry, attractant, arrestant, repellent, alarm behavior, cephalic extract, *Solenopsis geminata*.

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INTRODUCTION

A key element in the high degree of ecological success enjoyed by ants in the genus *Tapinoma* may be their effective utilization of defensive secretions during encounters with other ant species. For example, *T. sessile*, an economically important North American species, employs secretions from its pygidial gland to ward off attacks from the pugnacious Argentine ant, *Iridomymex humilis* (Woodward, 1910, cited in Smith, 1928). Further, Weber (1961) described workers of *T. nigerrimum* discharging a substance from the gaster to repel workers of *Monomorium subopacum* during confrontations over food.

Mixtures of iridodial(s) and ketone(s) characterize the exocrine gland products of every species of ant belonging to the genus *Tapinoma* that has been examined so far. The pygidial glands of *T. sessile* produce iridodial and isoiridomyrmecin (McGurk et al., 1968), and the former compound also occurs in the pygidial glands of *T. nigerrimum* (Trave and Pavan, 1956) and *T. simrothi* (Hefetz and Lloyd, 1983). The widely distributed alarm releaser, 6-methyl-5-hepten-2-one, accompanies the cyclopentanoids in all three species. *Tapinoma nigerrimum* and *T. simrothi* also produce the apparently *Tapinoma*-specific alarm pheromone, 2-methyl-4-heptanone (Trave and Pavan, 1956; Hefetz and Lloyd, 1983). In addition, Hefetz and Lloyd (1983) found 4-heptanone and 4-hydroxy-4-methyl-2-pentanone in the pygidial gland products of *T. simrothi*.

Another highly successful, yet little studied, species of *Tapinoma* is *T. melanocephalum*. This ant, probably of African or Oriental origin, was introduced into the United States and now occurs in abundance in southern Florida. *Tapinoma melanocephalum* forms polygynous colonies that may contain large numbers of workers which nest opportunistically in soil, rotten wood, or other cavities. Its exploitative nesting habits and extensive foraging systems enable the ant to colonize a variety of habitats, including houses and other dwellings (Smith, 1965).

Chemical effrontery may also contribute to the success of *T. melanocephalum*, and in this report we describe the chemistry of exocrine products of this species as well as their utilization during interspecific encounters. We also examined the chemistry of the exocrine products of *T. erraticum*.

METHODS AND MATERIALS

Ants. Several colonies of *T. melanocephalum* and *S. geminata* were collected from Marathon Key, Bahia Honda Key, Big Pine Key, or Key West, Florida, and placed in separate, 35 × 16 × 9-cm plastic boxes, the sides of which were coated with Fluon GP-lR (I.C.I., United States, Inc.) to prevent the escape of ants. All colonies were composed of numerous workers, functional queen or queens, and brood in various stages of development. Ants were

allowed to nest in Petri dishes with moistened Castone® (Ransom and Randolph Co., Toledo, Ohio) flooring. The floor of each container served as a foraging arena where minced insects, honey, and water were provided ad libitum.

Chemical Analysis. Workers of *T. melanocephalum* (Florida Keys, U.S.A) were killed by freezing in Dry Ice for 5 min, extracted in 1–2 ml of methylene chloride or pentane, and then examined by gas chromatography–mass spectrometry (LKB-9000 equipped with a 2-m × 2-mm column packed with 1%, OV-17, 1% SP-1000, and 30-m capillary columns of SP-1000 and SE-30, with column temperature programmed from 30° to 240°C at 8°C/min. Whole-body methylene chloride extracts of *T. melanocephalum* workers from the Ivory Coast, Africa, were also analyzed by GC-MS.

To determine the source of the detected compounds, frozen workers were trisected into head, thorax, and gaster, and separately extracted in organic solvent. Preliminary bioassays suggested that cephalic preparations evoked behavioral responses. Thus, an additional 2000–3000 heads were collected as follows: five groups of 400–600 ants each in 20-ml glass vials were plunged in liquid nitrogen for 5 min. Vials were immediately placed on a vortex mixer and rapidly shaken for 2–3 min, thus producing separate head, thorax, gaster, and head–thorax sections. Since thoracic extracts did not have any effects on worker behavior, further efforts to separate heads from thoraces were not attempted. Head and head–thorax sections were combined, homogenized 5× in 250-μl aliquots of pentane, and then the extract fractionated by preparative GC (0.25-in. × 6-ft stainless-steel column packed with 10% OV-17 on Gas Chrom Q). Fractions were collected, redissolved in pentane, and bioassayed as subsequently described.

Secretions from the pygidial glands were collected on filter paper squares by prodding workers with forceps. Papers were then placed in organic solvent after they had been daubed by 3–5 workers. The exocrine gland products of approximately 100 ants were accumulated per vial.

The amounts of both 6-methyl-5-hepten-2-one and actinidine per ant were estimated. The GC peaks obtained from the analysis of five extracts, each containing 100 workers in approximately 1 ml of methylene chloride, were compared to calibrated GC peaks from dilutions of these compounds. Samples were run on a Gow-Mac Model 750P GC equipped with a 2-m × 2-mm ID glass column packed with 5% SP-1000 on 100/120 Supelcoport and temperature programmed from 40° to 200°C at 10°C/min.

Workers of *T. erraticum* were collected in Toulouse, France, and extracted in methylene chloride. Pygidial gland secretion was collected and extracted using methods described above. Sternal glands were also dissected from workers and extracted in the same solvent. Extracts were analyzed by GC-MS (LKB 9000) equipped with a 25-m column of SE-30, temperature programmed from 55° to 300° C at 10°C/min.

Confrontational Studies. Competitive encounters between foraging workers of *T. melanocephalum* and *S. geminata* were analyzed in the laboratory in order to determine the possible role of pygidial gland secretions in such contexts. The foraging area of a colony of each species was connected to a small plastic "confrontation" tray via 3.0-mm ID × 22-cm-long glass tubes. A piece of filter paper was placed in the bottom of the tray to retard static electricity, and the sides brushed with talcum powder to prevent the escape of ants. When the tray simultaneously contained one or two workers of each species, a small piece of mealworm (*Tenebrio molitor*) was placed near the tube entrance to stimulate recruitment from colonies of both species. Thereafter, the mealworms were moved to the center of the arena, thus promoting confrontations between similar numbers of ants from the two colonies.

Other Behavioral Investigations. Observations were made on the reactions of workers of *T. melanocephalum* to (1) stressed nestmates; (2) pentane extracts of head, thorax, or gastric sections (0.2 section equivalents per μl pentane); (3) GC fractions from head–thorax extracts (see below); and (4) synthetic 6-methyl-5-hepten-2-one (Aldrich Chemical Co., Inc.) and actinidine (Wuest et al., 1977) using methods similar to that of Howard et al. (1982). The responses of the workers to body section extracts were recorded using a Panasonic Omnipro PK-956 video camera equipped with an F/1.4 variable-speed zoom lens with macro function, and a Panasonic PV-5000 series video recorder with stop and slow action, and timing capabilities. Responses were quantified by counting the number of ants contacting a test substrate within the first minute, and the time that 10 randomly chosen ants remained within 1 c of it.

RESULTS

Chemical Analysis. Gas chromatography–mass spectrometry of methylene chloride extracts of workers of *T. melanocephalum* shows that they contain 6-methyl-5-hepten-2-one and actinidine as well as alkanes and alkenes. On all columns the earliest-eluting and most abundant compound had retention times and mass spectra (*m/z* 126, 111, 108, 43) identical to synthetic 6-methyl-5-hepten-2-one. The second component had retention times and mass spectra (*m/z* 147, 146, 132, 117; see below and Figure 1) identical to synthetic actinidine prepared according to the method of Wuest et al. (1977). A third compound, in greater concentration than actinidine, eluting shortly afterwards, is presumably an olefin corresponding to $C_{19}H_{38}$. The position of the double bond is unknown. This alkene was subsequently determined to be of cephalic origin. The natural and synthetic ketone and base had identical gas chromatographic retention times to their corresponding standards on two different columns (OV-25, SP-1000). In addition to these, iridodial and C_{28}–C_{30} normal and branched alkanes and alkenes were observed in quantities similar to the methyl heptenone.

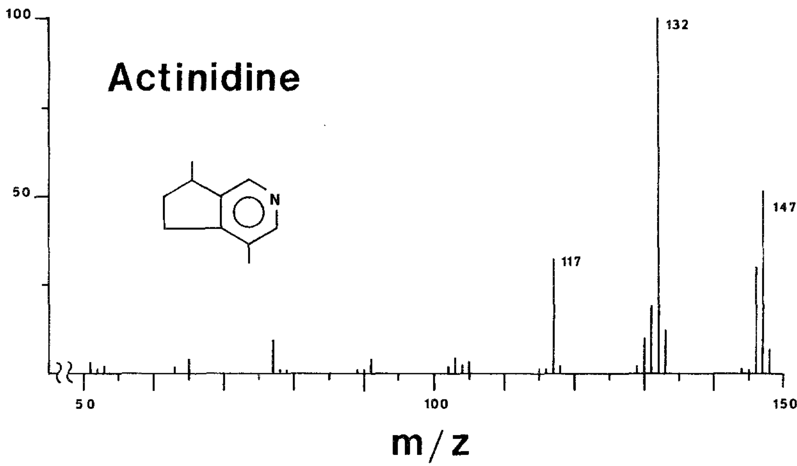

FIG. 1 Mass spectrum of synthetic (+)-actinidine that is identical to actinidine found in *T. melanocephalum*.

The mass spectrum of actinidine recorded in the literature is in error (Auda et al., 1967; Sastry, 1972). Speculations about *m/z* 89 which is not actually present must be discounted. The correct mass spectrum, reported here, was obtained using actinidine from *T. melanocephalum*, synthetic material, and a sample of *Actidinia polygama* extract from which it was originally found. All spectra were identical even when run at widely different times. No substance corresponding to the previously reported mass spectrum was detected. The spectrum of actinidine reported here resembles more closely that obtained from a different instrument by Wheeler et al. (1977).

Analysis of trisected ants and pygidial gland exudates demonstrated that the pygidial glands were the source of 6-methyl-5-hepten-2-one and actinidine. No volatiles were detected in the extracts of thoraces of this species.

We estimate that each ant contains approximately 0.53 μg $\pm$ 0.03 μg (mean $\pm$ SE) of 6-methyl-5-hepten-2-one and 0.29 μg $\pm$ 0.05 μg of actinidine.

Whole worker extracts and pygidial gland secretion of *T. erraticum* were dominated by 6-methyl-5-hepten-2-one. In addition, three iridoids were present: two isomers of iridodial, and iridomyrmecin. The later-eluting isomer, presumably the *trans,trans* isomer of iridodial, was also found in the sternal glands in admixture with C_{15}–C_{20} hydrocarbons.

Confrontational Studies. In the majority of skirmishes with *S. geminata*, workers of *T. melanocephalum* displayed alerting, alarm behavior, and the daubing of pygidial gland products. The initial contact between two ants often resulted in a condition of alertness whereby a *Tapinoma* worker with mandibles open slowly closed on the foreign ant while closely antennating the intruder's body. Movement by the alien ant at this time nearly always produced an in-

stantaneous alarm response which was characterized by frenzied running, with the head and sometimes gaster elevated, and the mandibles open. Contact with an alien ant at this juncture (and sometimes even their own sisters) elicited any one or a combination of the following aggressive behaviors: biting, assumption of a defensive posture, and the daubing of secretion from the pygidial gland. Biting was performed by a quick lunge forward and immediate recoil with a snapping of the mandibles at the point of contact with the intruder. Nipping, rather than grasping with the mandibles, appeared to be the preferred method of attack.

The most common form of aggressive behavior observed for workers of *T. melanocephalum* was the daubing of pygidial gland secretions. This maneuver was accomplished from a defensive stance in which the *Tapinoma* worker lowered its head and thorax while elevating its abdomen. The worker then turned its head to the side and quickly moved backward, guiding the tip of the gaster to make a fleeting contact with the body of the alien ant. The secretion was expressed as a clear droplet, which quickly became viscous and sticky upon exposure to air. Under low magnification (40–60 ×), the exudate appeared as a shiny, clear, convex globule on the surface of the cuticle.

Contact with the pygidial gland secretion prompted pronounced reactions from the affected ant. The alien worker immediately backed away while dragging the contaminated body part. It staggered prostrate with its legs extended outward while opening and closing its mandibles. Application of this secretion to the legs or antennae of a foreign ant often resulted in a hindrance of movement or in the limbs adhering together.

Further contact with *Tapinoma* workers seldom elicited aggressive responses from the daubed ants. In fact, some of these treated individuals, which were observed for as long as 10–15 min, exhibited no apparent responses after contact with *Tapinoma* workers.

The pygidial gland products may also be repellent, as indicated by the fact that the recruitment of *S. geminata* workers was halted when a *Tapinoma* worker daubed a fire ant while both were inside the tube connecting the arena with the *Tapinoma* colony. Ants of both species avoided passing through that spot in the tube for about 10 min.

Behavioral Assays. When individual workers held with forceps were positioned above and just out of reach of workers on a foraging trail, foragers quickly congregated beneath the restrained ant. The attracted workers moved at an accelerated and erratic pace while probing at the source with their antennae extended and their mandibles open. Dried or previously extracted workers, as well as small filter paper disks held in a similar manner, elicited weak responses at best.

Differences were observed between the ants' responses to paper disks containing head vs. gastric extracts. Gastric extracts released behaviors character-

istic of aggressive alarm behavior. Workers in the immediate vicinity of the disk promptly increased their rate of movement, and when encountering the disk, they pointed their gasters upward, nipped, and often dragged the filter paper about the foraging arena. Meanwhile numerous other workers ran in a frenzied and erratic manner over and around the test substrate. In contrast, workers that were attracted to cephalic extracts seldom molested the disks, but rather remained within a few centimeters of the cephalic extract for several seconds (Figure 2). The numbers of workers contacting the disks impregnated with 1 ant equivalent of cephalic or gasteric extract within the first minute of a trial were not significantly different (Table 1). However, duration of contact with the cephalic extract was over four times longer than that for the gaster and nearly twice as long as that spent at the controls. Behavioral responses to thoracic extracts were not detected.

Synthetic 6-methyl-5-hepten-2-one and actinidine induced different responses when presented to workers of *T. melanocephalum*. One to two micrograms of the methyl heptenone added to filter paper disks immediately released alarm reactions similar to those induced by gastric extracts. However, the same amounts of actinidine, while not eliciting any discernable alarm reactions, were repellent to ants within a 5- to 10-mm zone. An actinidine-treated disk gently

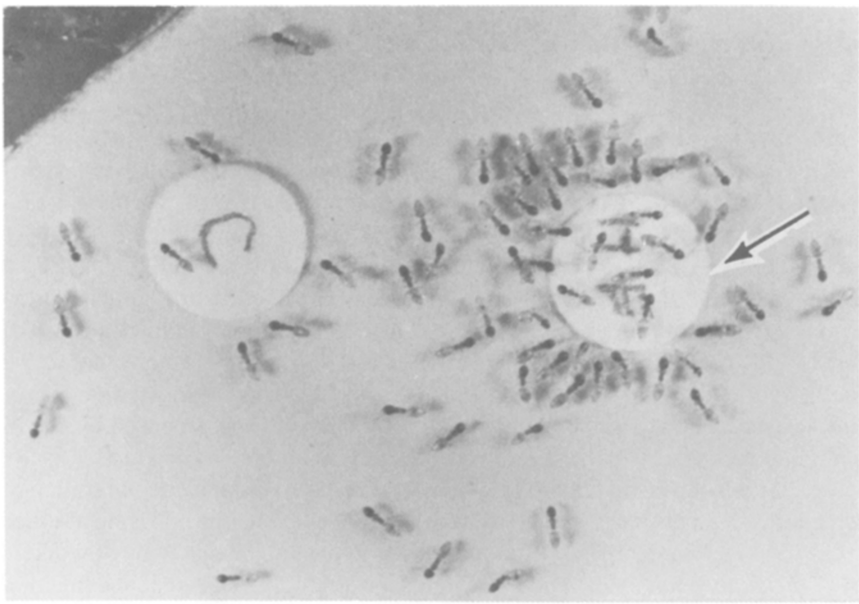

FIG. 2. The response of workers of *T. melanocephalum* to two ant equivalents of cephalic extract (arrow). The methylene chloride-treated control disk (C) is opposite.

TABLE 1. BEHAVIORAL RESPONSES OF WORKERS OF *T. melanocephalum* TO EXTRACTS OF CONSPECIFIC HEADS AND GASTERS

	Number of ants contacting disk within initial 60 sec (mean ±SE, N = 5 trials)	Time (sec) spent in contact with test disk (mean ±SE, N = 10 ants)
Control (pentane)	8.4 ± 2 a[a]	4.6 ± 0.8 b
Gastric extract	34.6 ± 7 b	1.7 ± 0.2 a
Cephalic extract	48.2 ± 7 b	9.0 ± 1.2 c

[a] Duncan's multiple-range test; values with the same letter are not significantly different (alpha = 0.05).

placed inside the nest resulted in an ant-free zone approximately 20 mm in diameter within 2 min. Pentane-treated disks elicited only transitory responses, and after 30 sec were ignored.

DISCUSSION

6-Methyl-5-hepten-2-one, one of the pygidial gland products of both *T. melanocephalum* and *T. erraticum*, is a typical dolichoderine natural product. However, the absence of 2-methyl-4-heptanone in both species, and the presence of actinidine in *T. melanocephalum*, and several isomers of iridodial in *T. erraticum*, lend the composition of the pygidial gland secretions of these two species somewhat distinctive chemical signatures. The acyclic alarm pheromone, 2-methyl-4-heptanone, has only been identified in other *Tapinoma* species (Trave and Pavan, 1956; Blum and Wheeler, 1975, unpublished data; Hefetz and Lloyd, 1983), but actinidine has not been previously detected as a *Tapinoma* natural product and has only been identified in the dolichoderine genera *Monacis* (Blum and Wheeler, 1978), *Iridomyrmex* (Cavill et al., 1980, 1982, 1984), and *Conomyrma* (Wheeler et al., 1977). Also interesting is the fact that iridodial was not detected in workers of *T. melanocephalum*. Thus, this key intermediate in the terpenoid biosynthetic pathway proposed by Cavill and Hinterberger (1960, 1962) is not coincident with the end product.

The well-developed pygidial glands of dolichoderine ants are the source of terpenoids that have been shown to have defensive and alarm-releasing abilities (Blum and Hermann, 1978). From our observations on the reactions of workers of *T. melanocephalum* to 1 and 2 μg of this alkaloid, it appears that one function of actinidine is to act as a repellent.

The cephalic extracts of dolichoderine species, although not well studied, are nevertheless known to be a source of volatile compounds. Three alkylpyr-

azines of unknown function have been identified in the cephalic extracts of *I. humilis* (Cavill and Houghton, 1974a, b). In this species, secretions from the mandibular gland are known to have a transitory releasing effect on trail-following behavior (Robertson et al., 1980). Our results with cephalic extracts of *T. melanocephalum* demonstrate the presence of a volatile component(s) having significant attractant and arrestant properties.

Trail-laying and mass-recruitment behaviors are well described in dolichoderine ants. The sternal gland (= Pavan's gland) is the source of volatile secretions that form the odor trail for all dolichoderine ants investigated (Wilson and Pavan, 1959; Hölldobler and Engel, 1978; Robertson et al., 1980, Cavill et al., 1979). In *T. melanocephalum*, workers rapidly tap the abdomen along the ground as they return to the nest from a food source. Once in the nest, the worker runs around erratically, rapidly antennating numerous sister workers (unpublished). This behavior, which we speculate occurs in combination with the release of attractant(s) from the head, may induce the recipient workers to stream out of the colony. Szlep and Jacobi (1976) have reported similar trail-laying, head-pushing, and accelerated antennal-beating behaviors for workers of *T. israelis* and *T. simrothi* returning from food sources. Further work is needed to characterize the role of cephalic and gastric secretions in the recruitment and mass foraging behaviors for workers of *T. melanocephalum.*

Wilson (1971) describes swift-running ant species that are more adept at locating and exploiting an ephemeral resource than are their competitors, as "opportunists." If a more aggressive species subsequently arrives in large numbers, the opportunist withdraws. Clark et al. (1982) observed that, although workers of *T. melanocephalum* displaced few other ant species from their baits, the high percentage of sole occupancy of *Tapinoma* workers at food stations may be attributable to their tendency towards rapid utilization of unoccupied baits rather than interspecific exclusion. Our observations confirm the existence of an opportunistic *modus operandi* for *T. melanocephalum*, and also suggest that potent defensive secretions may significantly contribute to the success of this species.

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NEW NATURAL ALIPHATIC ETHERS IN CUTICULAR WAXES OF GREGARIOUS AND SOLITARY LOCUSTS *Locusta migratoria cinerascens (II)*

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Abstract—An original homologous series of di-*n*-alkyl ethers has been revealed in the cuticular waxes of *Locusta migratoria cinerascens*. The mass spectrometry-electron impact, and positive and negative chemical ionization of these derivatives has been investigated in order to define the optimal conditions for structural determination. The quantitative analyses have shown that the ethers $C_{n1}H_{2n1+1}-O-C_{n2}H_{2n2+1}$ with an odd number of carbon atoms ($n1$ even and $n2$ odd) are clearly predominant. Furthermore, they have made a double phasal dimorphism appear: heavier ethers in the case of the solitary insects and majority participation of *n*-alkyl chains in C_{16} (60%) in the case of the gregarious locusts and in C_{18} (50%) in the solitary ones.

Key Words—Locusts, *Locusta migratoria*, cuticular waxes, aliphatic ethers, mass spectrometry, phase polymorphism, Orthoptera, Locustidae.

INTRODUCTION

The cuticular waxes which cover the surface of insects are made up mainly of aliphatic hydrocarbons. Among the other compounds present, the most common are primary fatty acids and alcohols; esters of these acids and alcohols; and acetates, ketones, and aldehydes, all saturated or unsaturated, linear or branched. Lastly, less frequently occurring compounds have been characterized in different species of insects: secondary alcohols and esters of secondary alcohols, α-hydroxy acids, diols, steroids, and terpenes (Blomquist and Jackson, 1979; Hadley, 1981).

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In *Locusta migratoria,* only the hydrocarbons have been studied so far. They comprise 54% of the extracts from the wings of these insects (Lockey, 1976) and 52–78% of the total cuticular extracts according to the age, sex, and phase (gregarious or solitary) of the locusts (Génin et al., 1985). A not insignificant portion of the cutical of these insects thus remained unknown. So we analyzed this as thoroughly as possible, bearing in mind the possible role of the polar compounds in the chemical communication between insects.

We also thought it would be interesting to find out if the phasal dimorphism that we had observed in hydrocarbons (Génin et al., 1985) could also be found in nonhydrocarbon compounds.

We describe here a homologous series of di-*n*-alkyl ethers, cuticular components of *Locusta migratoria cinerascens.* As far as we know, this is the first time that such compounds are reported in a biological environment. We determined them by mass spectrometry in the complex mixtures of which cuticular waxes consist. Their spectra were recorded under electron impact ionization and positive and negative chemical ionization conditions (at different temperatures and pressures in the ionization chamber) and compared.

Qualitative and quantitative analyses of the ether mixtures of the same mass, not resolved on the gas chromatograms, were carried out in order to determine the possible predominances and to pose the problem of the biosynthesis of these derivatives. Finally, comparisons were made between larvae and adult, male and female, and solitary and gregarious locusts.

METHODS AND MATERIALS

The insects used were last larval instar and mature adult, male and female, gregarious and solitary. They were raised at the Insect Biology Laboratory. The strain was *Locusta migratoria cinerascens* from Sardinia. Gregarious locusts were bred in groups of 200 individuals in cages of 40 × 40 × 60 cm. Solitary locusts were maintained from birth in individual 1 liter containers in a separate breeding room. Each room was submitted to a regular change of air 12 times an hour. The photo- and thermoperiods were 12/12 hr with temperatures of 25 ± 1°C (night) and 35 ± 1°C (day). Separate evaluation of characteristics such as behavior (Nicolas, 1972; Gillett et al., 1972), morphometrics (Nicolas, 1973; Minato et al., 1973), pigmentation and fecundity (Nicolas, 1972) had shown that, under our laboratory conditions, the locusts isolated from birth are conspicuously different from the crowded ones bred simultaneously. All locusts were fed on fresh corn shoots and bran every day of the week with no interruption.

The same numbers of animals of both sexes used were: 23 gregarious, last-instar larvae, 2–3 days old; 5 gregarious, mature adults, 34 days old; 10 soli-

tary, last-instar larvae, 2–3 days old; and 21 solitary, mature adults, 21 days old.

All the solitary locusts used were light beige as this color was close to that of the white paper surrounding them to keep them separated. Each insect was killed by freezing at −20°C, then extracted by stirring for 10 min in Mallinckrodt nanograde hexane (5 ml per adult and 3 ml per larva). The total extracts, after being weighed, were fragmented by thin-layer chromatography (Merck 10 × 20-cm plates of silica gel 60 F254 with concentration zone). A first elution with hexane made it possible to eliminate the hydrocarbons. A second elution with a mixture of hexane and ethyl acetate (50 : 1) eluted a band with the second solvent front. The latter turned out to comprise, among other things, a homologous series of lipid ethers, which are the object of the present study.

Analyses were carried out by combined gas chromatography–mass spectrometry using a Nermag R10-10 spectrometer interfaced to a PDP8 calculator (Digital Equipment) and coupled to a Girdel 31 chromatograph equipped with an all-glass injector. Fractions were temperature programmed from 160 to 300°C at 3°/min then held at 300° on a 25-m capillary column, 0.32 mm wide, coated with CpSil 5 CB Chrompack. The carrier gas was helium and inlet pressure 0.25 bar. Mass spectra were recorded in the electron impact ionization mode at 70 eV, in the ammonia positive chemical ionization mode (total source pressure: 0.03–0.2 torr; source temperature: 110–300°C) and in the negative chemical ionization mode using a mixture of N_2O–CH_4 (10 : 1) as a reagent gas (total source pressure: 0.1–0.2 torr; source temperature: 110–300°C). Integration of chromatographic peaks was carried out with a Hewlett-Packard integrator coupled to a Varian 3700 chromatograph with the 25-m column mentioned above, programmed from 40 to 300°C at 4°/min then held at 300° with a flow rate of helium of 18 cm/sec; an "on column" injector was used.

The retention indices, *I*, (Ettre, 1964) were calculated using the *n*-alkanes of C_{25} to C_{37} present on the chromatograms of the ether fragments.

The *n*-undecyl *n*-eicosyl ether, *n*-tridecyl *n*-octadecyl ether (Kobayashi, 1976), *n*-tetradecyl *n*-heptadecyl ether, *n*-pentadecyl *n*-hexadecyl ether, and di-*n*-hexadecyl ether (Senderens, 1925; Perron and Paquot, 1950) were synthesized according to the method described by Barry et al. (1984). The infrared spectra (CCL_4 as solvent) of these synthetic compounds all show a similar absorption in the 1112 ± 2 cm^{-2} region due to C—O—C antisymmetric stretching. In the same way, [^{1}H] NMR spectra (250 MHz, $CDCl_3$, TMS internal reference) show similar chemical shifts: δ R—$\underline{CH_2}$—O = 3.39 ± 0.01 ppm (triplet, 4H); δ R—$\underline{CH_2}CH_2$—O = 1.56 ± 0.01 ppm (multiplet, 4H); δ other methylenes = 1.26 ± 0.01 ppm (broad singlet, 50H; 52H for C_{16}—O—C_{16}); δ CH_3 = 0.88 ± 0.01 ppm (triplet, 6H).

All the bar charts (Figures 7 and 8) were constructed from the ether percentage columns shown in Table 1.

TABLE 1. CUTICULAR ETHERS $C_{n1}H_{2n1+1}—O—C_{n2}H_{2n2+1}$ OF *Locusta migratoria cinerascens*

GC peak No.	Total carbon number	F.G.L.[d]		M.G.L.[d]		F.S.L.[d]		M.S.L.[d]	
		%[a]	$n1$–$n2$	%	$n1$–$n2$	%	$n1$%$n2$	%	$s1$–$s2$
1	25	tr.	9–16	tr.	9–16				
2	27	4.5	9–18	4.6	9–18	0.2	9–18	0.5	9–18
3	27	4.0	11–16	4.7	11–16	0.1	11–16	0.4	11–16
4	28	tr.		tr.		0.1		0.3	
			9–20		9–20		9–20		9–20
5	29	24.7	11–18	23.2	11–18	5.4	11–18	8.2	11–18
			13–16		13–16		13–16		13–16
			15–14		15–14		15–14		15–14
			–		–		10–20		10–20
			–		–		11–19		11–19
6	30	4.1	–	4.3	–	3.2	12–18	2.6	12–18
			–		–		13–17		13–17
			14–16		14–16		14–16		14–16
			15–15		15–15		15–15		15–15
			–		–		11–20		11–20
7	31	45.0	13–18	44.8	13–18	37.6	13–18	38.2	13–18
			15–16		15–16		15–16		15–16
			17–14		17–14		17–14		17–14
			–		–		12–20		12–20
8	32	2.6	13–19	2.6	13–19	7.5	13–19	3.7	13–19
			14–18		14–18		14–18		14–18
			15–17		15–17		15–17		15–17
			16–16		16–16		16–16		16–16
			13–20		13–20		13–20		13–20
			15–18		15–18		15–18		15–18
9	33	11.6	17–16	11.9	17–16	34.9	17–16	34.9	17–16
			19–14		19–14		19–14		19–14
			13–21		13–21		13–21		13–21
			14–20		14–20		14–20		14–20
10	34	1.0	15–19	0.5	15–19	2.7	15–19	2.3	15–19
			16–18		16–18		16–18		16–18
			17–17		17–17		17–17		17–17

F.G.A.[d]			M.G.A.[d]		F.S.A.[d]			M.S.A.[d]	
%[a]	$n1 - n2$[b]	%[c]	%	$n1 - n2$	%	$n1 - n2$	%[c]	%	$n1 - n2$
tr.	9–16		tr.	9–16					
0.7	9–18	47	0.7	9–18	tr.			tr.	
0.8	11–16	53	0.8	11–16	tr.			tr.	
	–			8–20					
	–			9–19					
	10–18	9		10–18					
0.7	11–17	6	0.2	11–17	tr.			tr.	
	12–16	45		12–16					
	13–15	22		13–15					
	14–14	18		14–14					
	9–20	8		9–20		9–20	64		9–20
17.0	11–18	30	8.9	11–18	1.7	11–18	36	0.8	11–18
	13–16	42		13–16		–			–
	15–14	20		15–14		–			–
	9–21	tr.		9–21					
	10–20	tr.		10–20					
	11–19	tr.		11–19		11–19	23		11–19
2.8	12–18	1	2.7	12–18	1.6	12–18	27	0.7	12–18
	13–17	1		13–17		13–17	6		13–17
	14–16	65		14–16		14–16	32		14–16
	15–15	33		15–15		15–15	12		15–15
	7–24	tr.		7–24		–			–
	9–22	tr.		9–22		–			–
	11–20	12		11–20		11–20	24		11–20
36.8	13–18	10	45.2	13–18	22.2	13–18	41	16.0	13–18
	15–16	69		15–16		15–16	34		15–16
	17–14	9		17–14		17–14	1		17–14
	11–21	tr.		11–21					
	12–20	1		12–20		12–20	5		12–20
4.6	13–19	6	3.5	13–19	5.6	13–19	26	4.4	13–19
	14–18	22		14–18		14–18	41		14–18
	15–17	22		15–17		15–17	12		15–17
	16–16	49		16–16		16–16	16		16–16
	13–20	5		13–20		13–20	30		13–20
	15–18	19		15–18		15–18	54		15–18
27.6	17–16	66	32.8	17–16	43.3	17–16	13	50.1	17–16
	19–14	10		19–14		19–14	3		19–14
	13–21	tr.		13–21		13–21	1		13–21
	14–20	10		14–20		14–20	16		14–20
2.0	15–19	25	1.3	15–19	3.3	15–19	34	3.5	15–19
	16–18	59		16–18		16–18	43		16–18
	17–17	6		17–17		17–17	6		17–17

TABLE 1. (*Continued*)

GC peak No.	Total carbon number	F.G.L.[d]		M.G.L.[d]		F.S.L.[d]		M.S.L.[d]	
		%[a]	$n1$-$n2$	%	$n1$-$n2$	%	$n1$%$n2$	%	$s1$-$s2$
			15–20		15–20		15–20		15–20
11	35	2.5	17–18	3.4	17–18	8.3	17–18	8.9	17–18
			19–16		19–16		19–16		19–16
			21–14		21–14		21–14		21–14
12	36								
13	37								

[a]Percentages (±0.1%), scaled to 100, of ethers. tr.: <0.1%.
[b]Carbon number of ethers $C_{n1}H_{(2n1+1)}$-O-$C_{n2}H_{(2n2+1)}$.
[c]Percentages (±1%), scaled to 100, of structural isomers. tr.: <1%.
[d]See figure 7 for explanation.

RESULTS AND DISCUSSION

The aliphatic ethers identified in *Locusta migratoria cinerascens* comprise between 4% and 5% of total cuticular waxes. These results have been deduced from the percentages in weight, compared with the total extracts, of the elution bands taken off after thin-layer chromatography and the percentages of the ether peaks of the gas chromatograms obtained from these elution bands.

Their identification results from similarity of mass spectra and retention time of the mixture of the four synthetic ethers in C_{31} with the corresponding natural products. Moreover, the [^{1}H] NMR spectrum (250 MHz, $CDCl_3$/TMS) of the ether fraction of Figure 1 shows the same peak as the synthetic ethers with the same chemical shifts. The retention indices (C_{29} ethers: $I = 2943$; C_{31} ethers, synthetic and natural: $I = 3143$; etc.) and the differences in retention indices, dI, between these ethers and the n-alkanes with the same number of carbons ($dI = 43$) confirm the homogeneity of the series described here. They range from $C_{25}H_{52}O$ (M = 368) to $C_{37}H_{76}O$ (M = 536), those with odd numbers of carbons being predominant. Furthermore, each one is made up of one or several structural isomers (except for the C_{25} ethers) not resolved (except for the C_{27} ethers) on the chromatograms (flame ionization detector or mass spectrometer) (Figure 1).

F.G.A.[d]			M.G.A.[d]		F.S.A.[d]			M.S.A.[d]	
%[a]	*n*1 − *n*2[b]	%[c]	%	*n*1 − *n*2	%	*n*1 − *n*2	%[c]	%	*n*1 − *n*2
	15–20	18		15–20		15–20	31		15–20
7.0	17–18	49	3.9	17–18	17.2	17–18	65	18.2	17–18
	19.16	29		19–16		19–16	3		19–16
	21–14	4		21–14		21–14	1		21–14
						15–21	2		15–21
					1.8	16–20	32	2.1	16–20
						17–19	54		17–19
						18–18	12		18–18
	17–20	84		17–20		17–20	79		17–20
tr.	19–18	11	tr.	19–18	3.3	19–18	20	4.2	19–18
	21–16	5		21–16		21–16	1		21–16

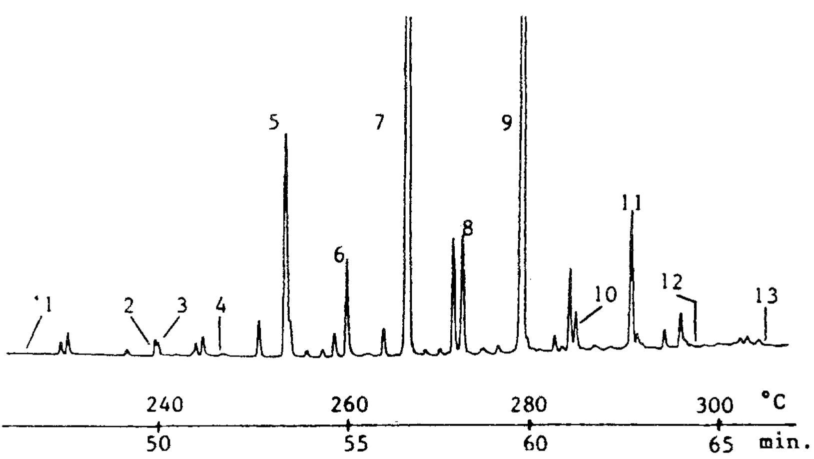


FIG. 1. Gas chromatogram of an ether fraction of male gregarious adult of *Locusta migratoria cinerascens*. CpSil 5 CB Chrompack capillary column of 25 m/0.32 mm, temperature programmed from 40 to 300° at 4°/min. then held at 300°; "on column" type injector.

Table 1 shows the relative percentages of the ethers compared with each other as well as the group of isomers identified, according to the age, sex and phase of the insects.

The complexity of the mixtures of ethers identified was determined by mass spectrometry. Different investigations carried out under 70 eV (McLafferty, 1957; Budzikiewicz et al., 1967; Beynon et al., 1968; Van de Sande, 1980) have established certain similarities between the spectra of the ethers R_1-O-R_2 and those of the alcohols $R_1-C(OH)-R_2$, the amines $R_1-N(R_3)-R_2$, and the sulfides R_1-S-R_2. Indeed, in all cases, the presence of the heteroatom determines the main fragmentation (Scheme 1).

```
                      +•      ipso
                Ri-O-R2  ---->  R1+  AND  R2+
            e-
Ri-O-R2  ---->                                    +
                        +•                α   R' 1-CH2-O=CH2
                R' 1-CH2-O-CH2-R' 2  ---->        +
                                              R' 2-CH2-O=CH2
```

SCHEME 1.

When the aliphatic chains lengthen, there is a rapid reduction of the α cleavages and the molecular ion disappears. As illustrated in Figure 2A for *n*-pentadecyl *n*-hexadecyl ether, this process continues beyond the di-*n*-decyl ether at which McLafferty stopped; it ends up with the complete disappearance of the α cleavages for out products and under our experimental conditions. The essential part of the ion current comes then from the fragments of the *n*-alkyl chains, the ipso cleavage rate maintaining itself at a constant level. Furthermore, the electron impact spectra of the ethers of *Locusta migratoria cinerascens* are complicated by the multiplicity and the weak intensity of the fragmentation ions R_1^+ and R_2^+ of the various structural isomers present. The spectrum of a mixture of ethers of the empirical formula $C_{31}H_{64}O$ (product No. 7 in Table 1) is shown in Figure 2B.

Spiteller-Friedman and Spiteller (1967) have shown elsewhere the advantages to be gained from lowering the source temperature (70–80°) and/or the ionization energy (12–20 eV) to maximize the ipso cleavages and the molecular ion of the ether. However, these operational conditions are not very compatible with the study of complex mixtures by GC-MS. In fact, reduction of ionization energy brings about a loss of sensitivity which is inconvenient when only a small quantity of extract is available. Moreover, the reduction of source temperature induces condensation in the source.

For all these reasons, identification of the ethers of *Locusta migratoria cinerascens* has led us to explore the techniques of chemical ionization, making it possible to identify and localize the functional groups, and to maximize the intensity of the quasimolecular ions.

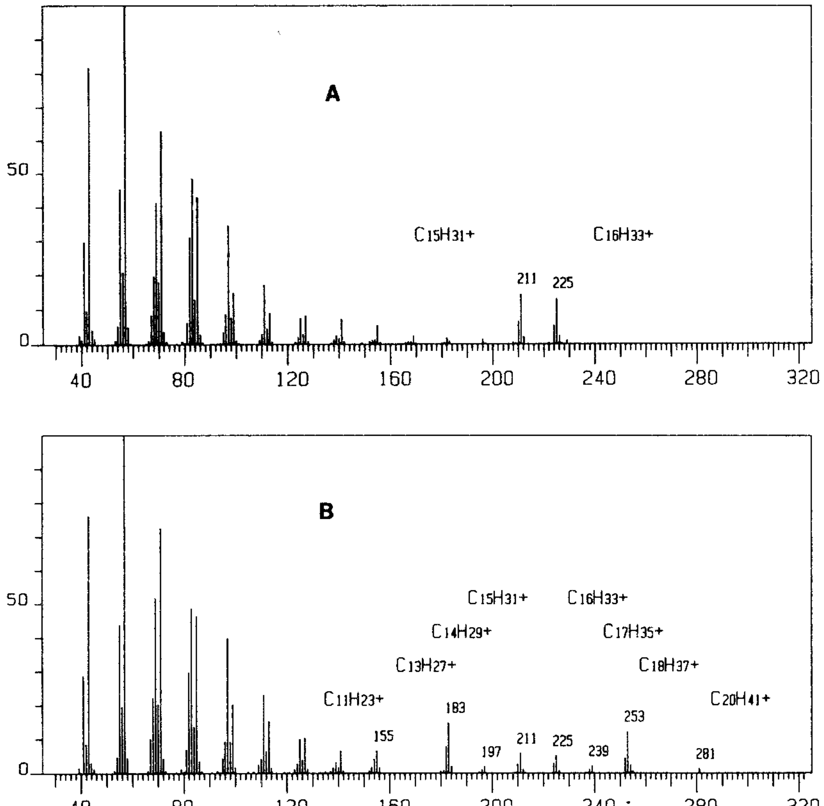


FIG. 2. (A) Electronic impact mass spectrum of pentadecyl hexadecyl ether. Ionization voltage: 70 eV; source temperature: 110°. (B) Electronic impact mass spectrum of GC peak 7 (solitary locusts: see Table 1). Ionization voltage: 70 eV; source temperature: 110°.

As yet, little has been done on the OH^- negative chemical ionization of the ethers. The spectra are characterized by RO^- type ions coming from nucleophilic attack by the hydroxyl ion on the carbons situated in the α positions in relation to the oxygen atom and the consecutive cleavage of the C—O bond (Scheme 2).

$$R'_1\text{-}CH_2 - O - CH_2\text{-}R'_2 \rightarrow R_1O^-,\ R_2O^- \quad (OH^-)$$

SCHEME 2.

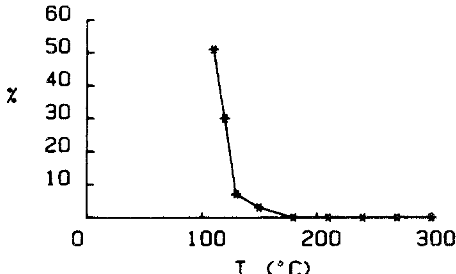
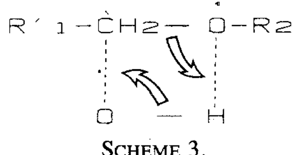


FIG. 3. Evolution of the relative intensity of the $(M + OH)^-$ ion in terms of the source temperature in the negative chemical ionization mass spectrum of pentadecyl hexadecyl ether.

Absence of the quasimolecular ion $(M - H)^-$ suggests weak acidity of the hydrogen atoms borne by these carbons. Lastly, ions of weak intensity of the $(M - 3H)^-$ and $RO - 2H)^-$ type can be observed and are explained by stabilization of the negative charge (Smit and Field, 1977; Budzikiewicz, 1981). In an effort to obtain a quasimolecular ion in the negative chemical ionization mode, we lowered the source temperature (Budzikiewicz, 1981) (Figure 3).

Beyond 150°C the latter is composed mainly of the R_1O^- and R_2O^- ions (Figure 4A). At 150° and below, a M + 17 peak appears; this reaches its highest intensity at 110° (Figures 3 and 4B). Kajdas (1971) had already noted the presence of an $(M + 17)^-$ ion in the negative ion current obtained by electron impact on saturated aliphatic hydrocarbons. This ion, resulting from an electrostatic interaction between OH^- and the initial molecule, increases in intensity with the molecular mass of the hydrocarbon. Furthermore, it appears only at low source temperature. The formation of a complex $(M + OH)^-$ is also very frequent in the case of alcohols (George, 1984). In the case of cyclic molecules, the $(M + OH)^-$ ion would seem to result from a nucleophilic attack of OH^- with a cleavage in the consecutive ring (Smit and Field, 1978). Dreifuss et al. (1983) suggest that the interaction of OH^- with the initial molecule is more likely to be produced through a hydroxyl–carbon bond than by an attachment to a hydrogen atom.

In the case of the aliphatic ethers studied here, the formation of a complex, as shown Scheme 3, can be envisaged. Such a complex could tend to cleave the C—O bond, depending on its internal energy (source temperature). The



SCHEME 3.

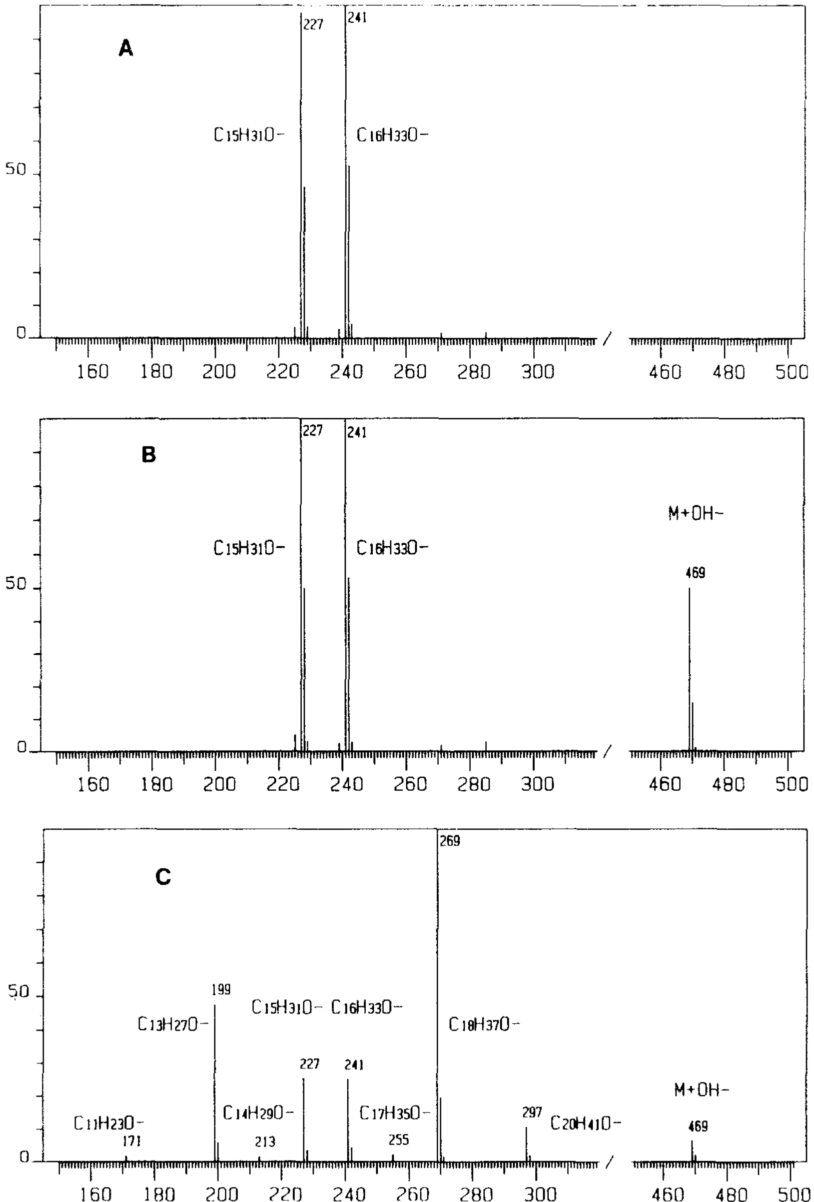


FIG. 4. (A) Negative chemical ionization mass spectrum of pentadecyl hexadecyl ether. Source temperature: 250°; source pressure: 0.1 torr. (B) Negative chemical ionization mass spectrum of pentadecyl hexadecyl ether. Source temperature: 110°; source pressure: 0.1 torr. (C) Negative chemical ionization mass spectrum of GC peak 7 (solitary locusts). Source temperature: 150°; source pressure: 0.1 torr.

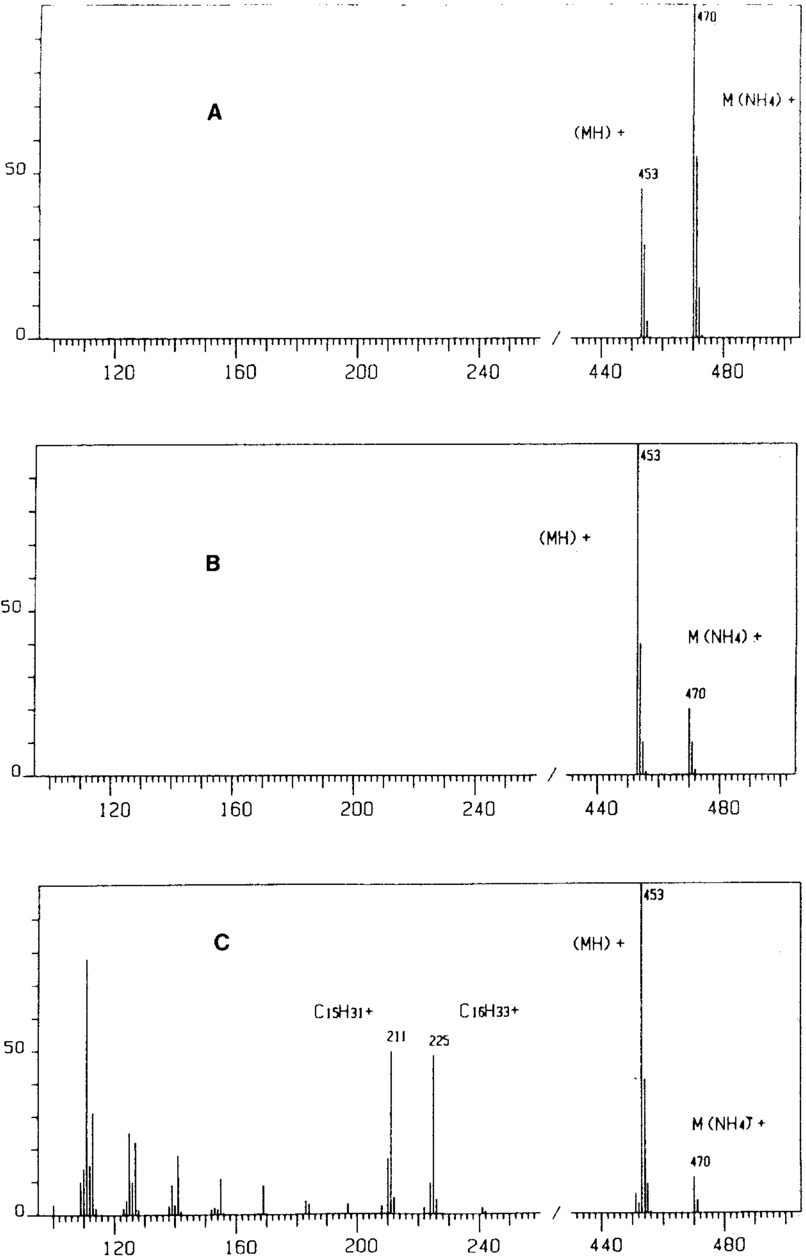


FIG. 5. NH_3 chemical ionization mass spectrum of pentadecyl hexadecyl ether. (A) Source temperature: 120°; source pressure: 0.2 torr. (B) Source temperature: 210°; source pressure: 0.2 torr. (C) Source temperature: 180°; source pressure: 0.04 torr.

analysis, under these experimental conditions, of an extract from *Locusta migratoria cinerascens* has shown that the formation of this M + 17 peak could be reproduced and generalized for all the ethers present in these insects (Figure 4C).

Finally, the presence of $(R_1O - 2H)^-$, $(R_2O - 2H)^-$, $(R_1O + 44)^-$, $(R_2O + 44)^-$ ions was observed; the last two could have resulted from the nucleophilic attack of the R_1O^- and R_2O^- ions on the nitrous oxide (N_2O). No dependency was observed between the intensity, always weak, of these ions and the source temperature. $(M - H + 44)^-$ type ions had already been pointed out for unsaturated hydrocarbons (Smit and Field, 1977; George, 1984).

In ammonia positive chemical ionization, as in Figure 5A for *n*-pentadecyl *n*-hexadecyl ether, the molecular mass can be immediately deduced from the only observed ions, $M(NH_4)^+$ and MH^+. We thought it would be interesting to know to what extent the variation of the experimental parameters could make it possible to observe fragmentation ions in the positive mode. Increasing the source temperature, with a constant ammonia pressure of 0.2 torr (Figure 5A and B) does not cause any fragmentation. This increase in source temperature shows only that the proportion of the intensities (M + 18)/(M + 1) varies in inverse ratio to the temperature. On the other hand, variations of pressure are decisive, as Figure 5C shows. Influence of the ammonia pressure on the degree of fragmentation of methoxy derivatives had already been observed in our laboratory (Péchiné et al., 1985). Figure 6 shows the evolution, depending on the ammonia pressure, of the relative intensity of the R_1^+ (*m/z* = 211) ion in relation to the base peak (generally MH^+ at the temperature used) of the spectrum of *n*-pentadecyl *n*-hexadecyl ether. An identical curve is obtained for the R_2^+ (*m/z* = 225 ion. At 0.1 tor of ammonia, the mechanisms of positive chemical ionization become weaker to the benefit of

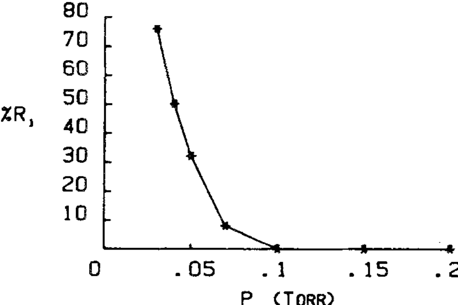


FIG. 6. Evolution of the relative intensity of the R_1^+ ion in terms of the source pressure in the NH_3 chemical ionization mass spectrum of pentadecyl hexadecyl ether. Source temperature: 180°.

an electron impact ionization, the R_1^+ and the R_2^+ ions appearing on the spectra, alongside the cleavage ions of the apliphatic chains. The abundance of these ions continues increasing as the pressure decreases; simultaneously, that of the $(M + 1)^+$ and (M + 18) diminishes. Thus, at 180° and 0.03 torr of ammonia, the $(M + 1)^+$ ion, the base peak at higher pressures, represents only 30% of the new base peak (m/z + 111) and the $(M + 18)^+$ ion has disappeared.

By these mass spectrometry techniques, we were able to obtain the results tabulated in Table 1. Examination of the global percentages of the isomer mixture reveals variations between gregarious and solitary insects (larvae and adults,

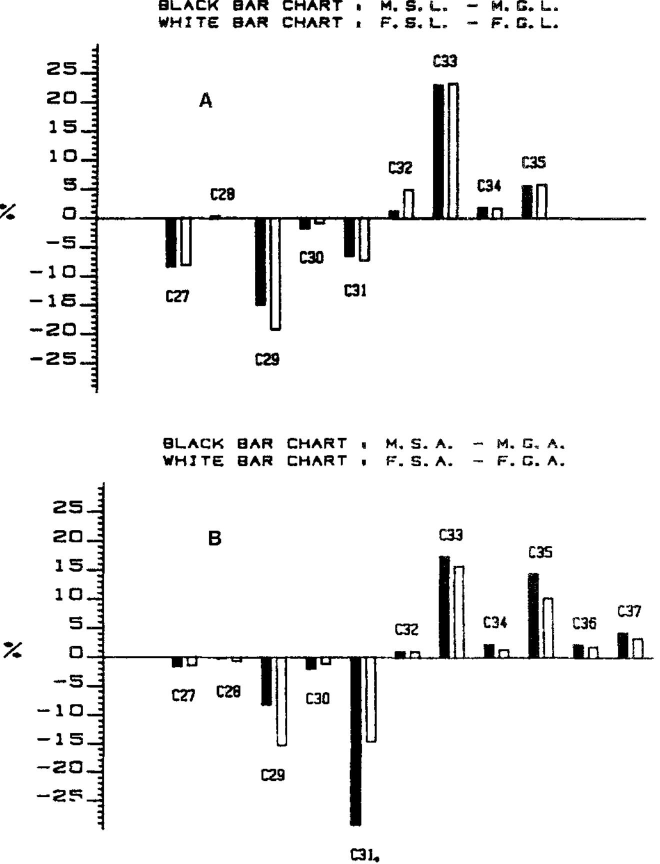


FIG. 7. (A) %MSL – %MGL (black bar chart); %FSL – %FGL (white bar chart). (B) %MSA – %MGA (black bar chart); %FSA – %FGA (white bar chart). MSL: male solitary larvae; MGL: male gregarious larvae; FSL: female solitary larvae; FGL: female gregarious larvae; MSA: male solitary adult; MGA: male gregarious adult; FSA: female solitary adult; FGA: female gregarious adult.

males and females) and between larvae and adults (gregarious and solitary, males and females). The differential histograms (Figures 7 and 8) show these results. They display, in the case of the solitary insects, an average reduction of 35% in the ethers from $C_{27}H_{56}O$ to $C_{31}H_{64}O$ and a same average augmentation in the ethers from $C_{32}H_{66}O$ to $C_{37}H_{76}O$ (Figure 7A and B). Displacement of the cuticular compositions of the solitary locusts, compared with the gregarious ones, towards the heaviest ethers, is quite parallel to that already observed in the case of hydrocarbons (Génin et al., 1985). It constitutes another example of phasal dimorphism connected with the lipid epicuticle or the insects, independent of their age and sex. Such an evolution towards the heaviest ethers also takes place when the locusts become mature. The histograms shown in Figure 8A and B establish the comparisons between the fifth instar larvae and

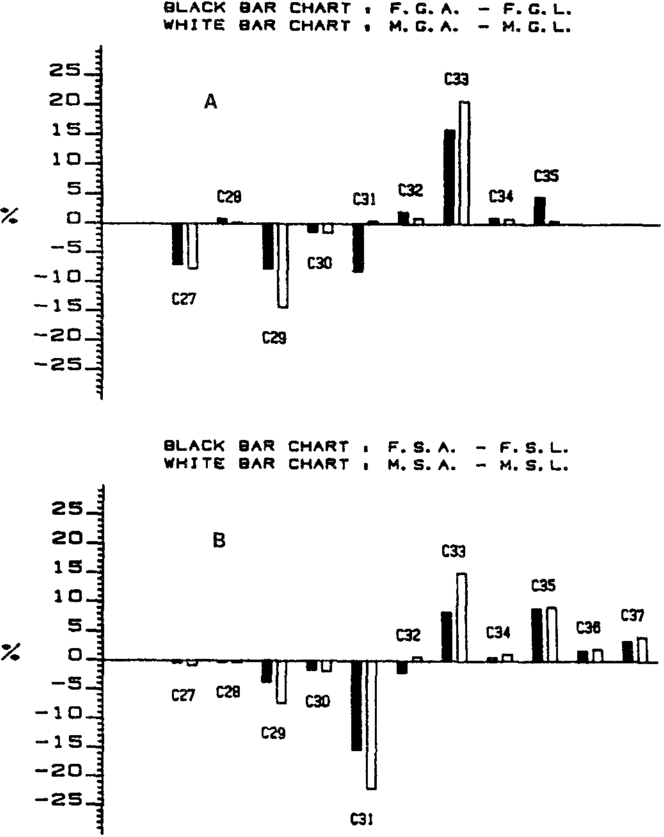


FIG. 8. (A) %FGA − %FGL (black bar chart); %MGA − %MGL (white bar chart). (B) %FSA − %FSL (black bar chart); %MSA − %MSL (white bar chart).

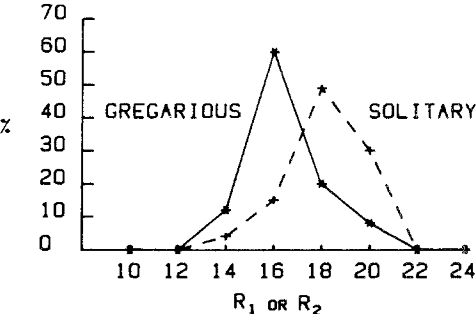


FIG. 9 Frequency of the even alkyl chains R_1 and R_2 in the gregarious and the solitary locusts.

the adults. Finally, the comparisons between sexes do not seem to imply noticeable differences, suggesting here a weak probability of a pheromonal role of a sexual kind for these aliphatic ethers.

These quantitative results have proved to be quite parallel in the cases of other populations also studied. Only slight variations in the absolute percentages of the ethers appeared.

Table 1 also gives the relative percentages of the structural isomers of the ethers identified. These results were obtained in negative chemical ionization (source temperature: 250°) by integration of the R_1O^- and R_2O^- ions of the various isomers present under a given total ion current peak (Figure 4C). The selected ion monitoring analyses have shown that the retention times of these isomers would seem to differ slightly from each other and increase in the dissymetry of the two chains R_1 and R_2, causing a lengthening of the retention time with broadening of the peak. Presented here for the adult solitary females and the adult gregarious females, these integrations can be generalized to apply to adult male locusts and to the larval stage and reveal, in all cases, a clear phasal dimorphism. It appears in particular (Figure 9) that the frequency of the chains R_1 and R_2 with an even number of carbons depends on the phase of the insects. Thus, in the case of the gregarious locusts, close to 60% (surface percentage) of the ethers have an R_1 or R_2 chain in C_{16} and 20% have a chain in C_{18}. The proportions are in inverse ratio in the case of solitary locusts: 17% of the ethers have a chain in C_{16} and 50% a chain in C_{18}. This phasal character diminishes for the heaviest ethers ($C_{35}H_{72}O$, $C_{36}H_{74}O$, and $C_{37}H_{76}O$), those with a chain in C_{17} becoming predominant, independently of the phase of the insects.

CONCLUSIONS

We have shown that negative chemical ionization of lipid ethers maximizes the R_1O^- and R_2O^- ions without an upper limit of source temperature. It allows

a quantitative analysis of the isomer mixtures by searching and integration of the different RO^- ions. Furthermore, molecular masses are detected due to $(M + 17)^-$ ion. A temperature of 150° has appeared to be the most adequate in the negative mode.

Ammonia positive chemical ionization has generated the quasimolecular ions $(M + 1)^+$ and $(M + 18)^+$ independently of the source temperature. Moreover, the appearance of the ions R_1^+ and R_2^+ at low ammonia pressure has made it possible to confirm the position of the functional group. Intermediate conditions (temperature: 180°, and ammonia pressure: 0.04 torr) have proved the most suitable for the various criteria sought: presence of the ions R_1^+, $(M + H)^+$, and $(M + NH_4)^+$, selectivity of the cleavages of the C—O bonds in relation to the C—C bonds; and absence of condensation in the source.

The quantitative analyses have brought out a double phasal dimorphism: on the one hand, in the overall distribution of the cuticular ethers of the locusts (these ethers are heavier in the case of the solitary locusts), and on the other hand, in the frequency of the structural isomers. Among them, those which are predominant have at least one branch in C_{14}, C_{16}, C_{18}, or C_{20}, the branches in C_{16} and C_8 remaining clearly predominant. This observation, connected with the fact that the majority ethers have a total odd number of carbons, is probably an indication for their biosynthesis which remains to be described.

Finally, these quantitative and qualitative results pose the problem of the possible role played by these ethers in the physiology and/or the behavior of *Locusta migratoria cinerascens*.

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EFFECTS OF DIET BREADTH ON AUTOGENOUS CHEMICAL DEFENSE OF A GENERALIST GRASSHOPPER

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Abstract—The lubber grasshopper, *Romalea guttata*, produces a metathoracic defensive secretion containing primarily phenolics and quinones. This insect feeds on a wide range of plant species. Insects reared on an artificial diet and a diet of onion, *Allium canadense*, had secretions that contained fewer compounds, lower concentrations of compounds, and markedly altered relative composition of components compared to insects reared on a varied diet of 26 plant species that included onion. The study demonstrates that diet breadth has a major effect on the quality and quantity of the autogenous defensive secretion of this generalist herbivore. The results are compared to diet effects known in chemically defended specialists. Two possible mechanisms explaining the effects of diet breadth are proposed: one involves changes in precursor availability with changing diet breadth; the other suggests that physiological stress due to diet restriction changes allocation of resources to chemical defense.

Key Words—Lubber grasshopper, *Romalea guttata (microptera)*, Orthoptera, Acrididae, generalist herbivore, dietary regime, host plant, artificial diet, autogenous defense, phenolics, quinones, allbomone, sequestration, physiological stress, plant–insect interaction.

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INTRODUCTION

Many plant-feeding insects use toxic or repellent chemicals as defenses against predators. These insects either make the chemicals themselves—autogenous defenses—or store, or sequester, chemicals directly from the diet (Duffey, 1980). Most studies on diet and chemical defense have focused on insects that feed on few plant species, such as the specialist monarch butterfly (e.g., Brower et al., 1982, 1984), and have not examined generalist insects that feed on many plants. We wanted to know if diet had any effect on the chemical defenses of a generalist.

The lubber grasshopper, *Romalea guttata* Houttuyn (Orthoptera; Romaleidae) (=*R. microptera* Beavois; Kevan, 1980), is a warningly colored generalist herbivore that is unpalatable to a number of different predators (Whitman, Jones and Blum, unpublished data). The insect produces a frothy, metathoracic spiracular secretion that is primarily comprised of phenolics and quinones (Jones et al., 1986) and is repellent to certain ants (Eisner et al., 1971). This defensive secretion shows extreme idiosyncratic chemical variation among individuals within wild populations, with certain phenolic and quinone compounds being absent in some individuals and present in others, and with concentrations varying over two to three orders of magnitude (Jones et al., 1986).

Although secretion composition has been examined (Eisner et al., 1971; Jones et al., 1986), the origin of the secretion components is not known. Since *R. guttata* may sequester certain compounds (Eisner et al., 1971; Berger, 1976), it was important to establish whether or not the compounds we previously analyzed were autogenous or were sequestered from the diet. We also wished to determine whether or not the extensive interindividual variation was diet-related. In the wild, each polyphagous individual insect consumes a different diet (Jones, Whitman and Blum, unpublished data). It was possible that the varied diet caused the variation in the defensive chemistry. To address these two questions, we needed to rear insects on a defined diet that lacked the compounds we analyzed, and a uniform diet that ensured that each individual consumed the same diet. Such defined and uniform diets inevitably lacked the diversity of plant material found in the wild and were of restricted diet breadth. We therefore reared grasshoppers on diets of one plant species and on an artificial diet, and compared the defensive secretion chemistry with secretions from insects reared on a varied diet of 26 species that included the same plant species as that used in one of the restricted diets.

METHODS AND MATERIALS

Rearing of Insects. R. guttata were reared from first instar to adult in cages, each containing about 30 insects. Cages were maintained at 30 ± 1°C with 65 ± 5% relative humidity and a 12 : 12-hr light–dark cycle. *Romalea* were reared

on three different diets: (1) A "natural diet" consisted of leaves of 26 species from 15 families of shrubs, herbs, forbs, and grasses known to be eaten by *Romalea* in the field (Jones, Whitman and Blum, unpublished data). This diet was selected to represent the varied generalist diet. Plant material was collected daily and the cut petioles or stems placed in water. The families and species of plants were: Apiaceae: *Daucus carota;* Asteraceae: *Lactuca sativa*, *Sonchus asper*, *Sonchus oleraceus*, *Taraxacum officinale*, *Erigeron canadensis;* Brassicaceae: *Lepidium virginicum;* Caprifoliaceae: *Lonicera japonica;* Chenopodiaceae: *Chenopodium album;* Cucurbitaceae: *Melothria pendula;* Euphorbiaceae: *Euphorbia supina;* Fabaceae: *Pueraria lobata*, *Trifolium repens;* Liliaceae: *Allium canadense;* Malvaceae: *Modiola virginicum;* Oxalidaceae: *Oxalis dillenii;* Plantaginaceae: *Plantago lanceolata;* Poaceae: *Sorghum halepense*, *Poa* sp., *Festuca* sp., *Cynodon dactylon*, *Digitaria sanguinalis;* Polygonaceae: *Rumex acetosella*, *Rumex crispus*, *Polygonum aviculare;* Solanaceae: *Solanum carolinianum.* (2) A single host-plant diet of freshly cut leaves of *Allium canadense* in water was chosen as the uniform plant diet of restricted breadth. This species was included in the natural diet and is a preferred food plant of *Romalea* in the field and in laboratory preference studies (Jones, Whitman and Blum, unpublished data). (3) An artificial diet formulated according to Dadd (1960) was selected to be a defined, restricted diet that lacked fresh plant material and the phenolic and quinonoid compounds. The dry powder diet contained cellulose, sucrose, dextrin, a salt mixture, cholesterol, linoleic acid, casein, peptone, egg albumen, ascorbic acid, and 10 water-soluble vitamins of the B complex. The diet was presented as a moist cake by addition of water.

Secretion Collection. The defensive secretion is eliminated at each molt and replenished thereafter. Metathoracic spiracular secretions were collected at the eighth day after molt to adult by milking with a microcapillary as described by Jones et al. (1986). Samples were stored at $-10°C$. We quantified secretions of 10 individuals of each sex on each diet, with the exception of males on natural diet ($N = 11$) and females on onion diet ($N = 11$).

Secretion Analysis. Compounds were identified by gas chromatography–mass spectroscopy (GC-MS). Secretions were analyzed by gradient reversed-phase high-performance liquid chromatography (HPLC) on a C_{18} bonded-phase silica column with a guard column and a convex elution gradient of methanol, water, and 1% H_3PO_4. Peaks were detected at 254 nm (UV). Full details on GC-MS and HPLC, including use of internal standards and detection thresholds, are reported in Jones et al. (1986).

RESULTS

The metathoracic secretion of lubber grasshoppers is chemically complex and contains over 40 components (Meinwald et al., 1968; Eisner et al., 1971; Jones et al., 1986). We restricted our analyses to seven compounds: hydro-

quinone, catechol, *p*-benzoquinone, phenol, guaiacol, 4-methoxybenzaldehyde, and a partially characterized unknown. These are the major HPLC/254-nm-detectable components, are repellent to ants (Eisner et al., 1971), and are the same compounds that were analyzed for interindividual variability within a wild population in a previous study (Jones et al., 1986). The absence or presence and quantity of these seven components in defensive secretions were determined for individuals of both sexes reared on the three diets.

Origin of Secretion Components. We reasoned that if the seven compounds we analyzed were directly sequestered from plants, they would be absent in all individuals reared on an artificial diet that did not contain these compounds. If these compounds were made by the insect, they would be present in at least some individuals, irrespective of diet. The compounds would not necessarily be expected to occur in all individuals because of the underlying qualitative variation (Jones et al., 1986). We looked at the frequency of occurrence of the seven compounds in individuals reared on the artificial diet. At least some individual males and/or females contained all the compounds when reared on the artificial diet (Figure 1). We infer from these data that these compounds are of autogenous origin and are not sequestered from the diet. However, such a result does not preclude the possibility that the same compounds could be sequestered as well as synthesized under some circumstances. Nevertheless, under these conditions, any effects of diet on chemical defense in *R. guttata* would be indirect.

Occurrence of Compounds in Individuals. The frequency of occurrence of the seven compounds in secretions of individuals reared on the natural diet was not significantly different from that of insects in wild populations (χ^2, $P > 0.1$) (see Jones et al., 1986). Consequently, the natural diet was equivalent to the wild situation from the perspective of defensive secretion quality. In contrast, insects reared on both the artificial diet and onion diet had reduced frequencies of occurrence of compounds in individuals (Figure 1). This was the case for two of seven compounds for females, and four of seven compounds for males on artificial diets, and six of seven compounds for females and two of seven compounds for males on onion diets. Since there was a considerable degree of qualitative variation between individuals on the natural diet, only eight of fifteen of the cases of reduced frequency on artificial and onion diets were significant (χ^2, $P \leq 0.05$).

Sexual differences in the frequency of occurrence of compounds were also evident. For example, on the onion diet all female secretions lacked catechol and *p*-benzoquinone, while at least some male secretions had these compounds. Similarly, on the artificial diet all male secretions lacked catechol and guaiacol, while some female secretions had catechol and all female secretions had guaiacol. Since there is an inherent tendency for secretion components to be absent in certain individuals (Jones et al., 1986), and since the biosynthetic origins of, and relationships between, compounds are not known, it is not possible at

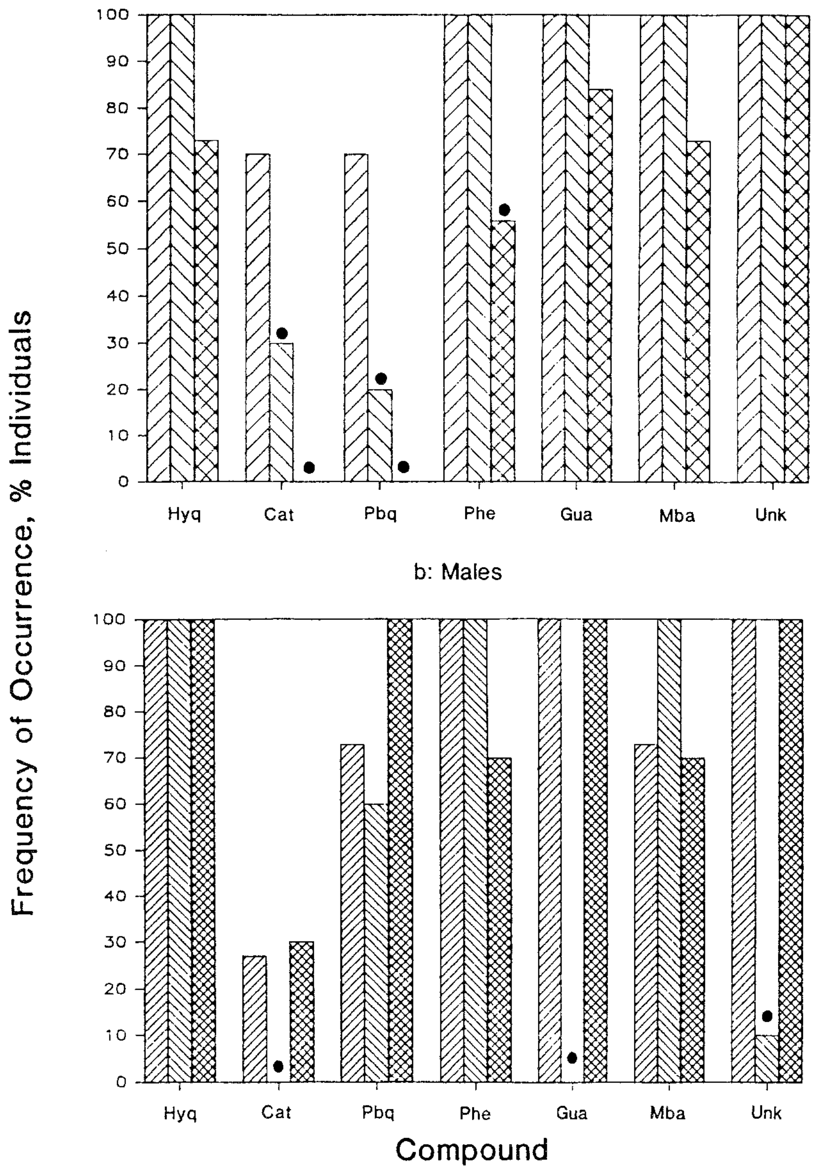

FIG. 1. Frequency of occurrence of phenolics, quinones, and an unknown compound in secretions of individual adult *R. guttata*. (a) Females: ▨ = natural diet; ▧ = artificial diet; ⊠ = onion diet. (b) Males: ▨ = natural diet; ▧ = artificial diet; ▩ = onion diet. Hyq = hydroquinone; Cat = catechol; Pbq = *p*-benzoquinone; Phe = phenol; Gua = Guaiacol; Mba = 4-methoxybenzaldehyde; Unk = unknown. ●: Significantly lower frequency than expected (χ^2; $P \leq 0.05$), compared to the natural diet for the same sex.

this juncture to understand specifically why certain compounds are absent for different diets and sexes. However, it is clear that a reduction in diet breadth results in a reduced probability of an individual containing a given compound, with the strongest effects occurring with females on a single-plant diet.

Effects on Overall Secretion Complexity. A direct consequence of a change in the probability of occurrence of a given compound was a change in secretion complexity. There were significant reductions in the mean number of secretion components for females reared on artificial and onion diets and males on artificial diets (Figure 2). Consequently a reduction in diet breadth resulted in a reduced number of secretion components. Compounds that had a ubiquitous distribution in individuals of both sexes on natural diets became sporadically distributed on restricted diets. Compounds that were sporadically distributed in females on natural diets became more so on restricted diets.

Variation in Secretion Complexity within Treatments. A reduction in the mean number of secretion components due to restricted diet breadth did not reduce the amount of variation in the number of secretion components. The range of values for the number of secretion components on natural diets more

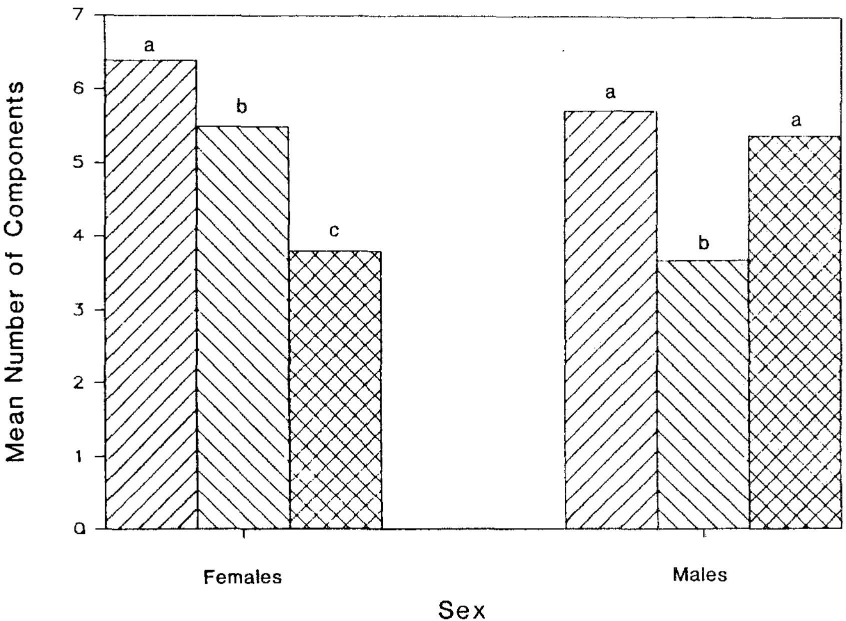

FIG. 2. Mean number of components (phenolics, quinones, and an unknown) in the secretion of adult female and male *R. guttata* reared on three different diets. [natural pattern] = natural diet; [artificial pattern] = artificial diet; [onion pattern] = onion diet. [a,b,c]Means followed by different letters, are significantly different within a sex between diets (t test, $P \leq 0.05$).

than doubled on onion diets, despite a 60% reduction in the mean number of components. This increase in variance was most noticeable in the pronounced skewness of the frequency distribution values for the number of compounds on onion diets and in increased values for the coefficients of variation (Figure 3).

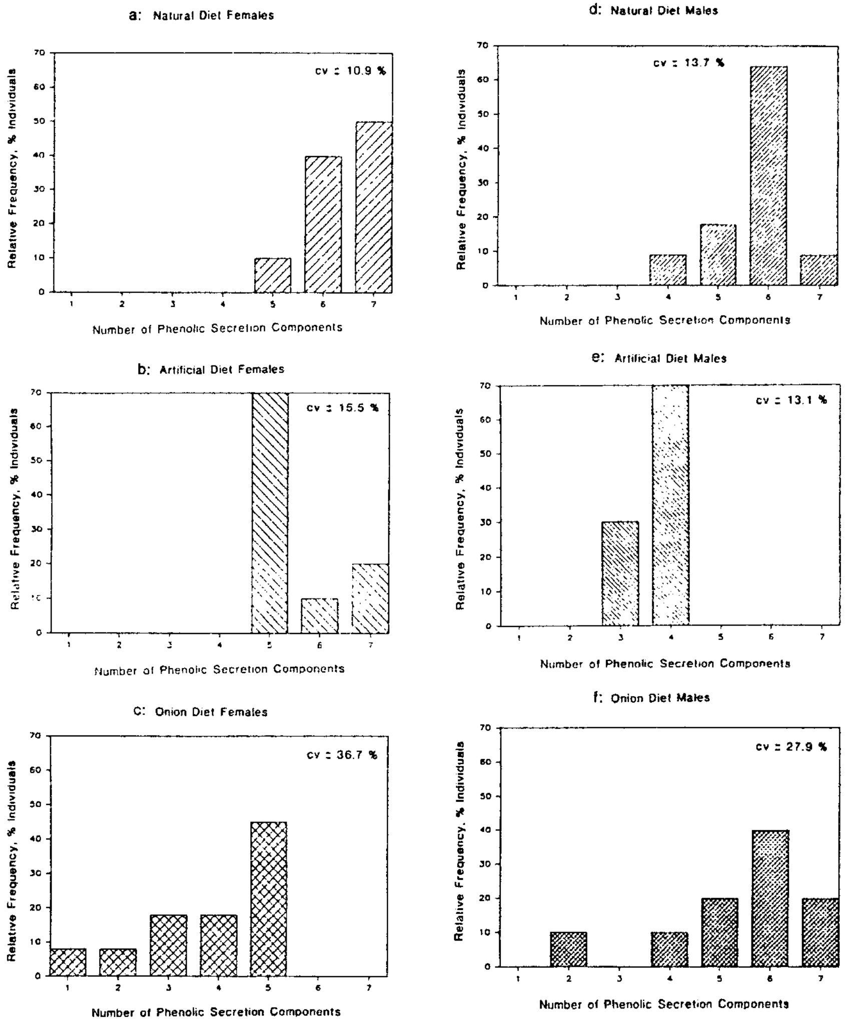

FIG. 3. Relative frequency distributions, as percent individuals with a given number of phenolic secretion components (= phenolics, quinones, and an unknown), for the secretions of adult *R. guttata*. a, ▨: natural diet females; b, ▧: artificial diet females; c, ⊠: onion diet females; d, ▨: natural diet males; e, ▨: artificial diet males; f, ▩: onion diet males. cv = coefficient of variation ($s/\bar{x}$, %).

Thus, a population of insects reared on restricted diets not only had fewer secretion components than the natural diet but were also as much or more variable in regard to the number of compounds likely to be found in a given individual.

Effects of Diet on Secretion Component Quantity. Concentrations of all seven compounds in secretions of insects reared on the natural diet were not significantly different from those found in wild insects (t tests, $P > 0.1$) (see Jones et al., 1986). As with secretion component quality, it appears that the natural diet was equivalent to wild diets from the quantitative viewpoint. Secretions from females reared on onion diets had significantly lower concentrations of all the compounds detected in secretions from females on natural diets (Figure 4). The same was true for all but two compounds (p-benzoquinone and phenol) for female secretions from artificial diets. There was a tendency for female secretions from onion diets to have the lowest combined concentrations of these phenolics and quinones. While similar trends occurred for male secretions, differences were not significant for detected compounds. The lack of significant differences in these cases, despite considerable reductions in concentration (e.g., one order of magnitude difference for catechol in males) are likely due to the large amounts of natural variation in concentration (Jones et al., 1986, and see later) superimposed on generally low concentrations.

The lack of significant differences should not be taken as implying equality of the means, but rather as inadequate sample sizes to detect significant differences, given the exceedingly large variation. The significant differences in concentrations between sexes found on natural diets (t tests, $P \leq 0.05$; except for catechol and p-benzoquinone) disappeared on both artificial (t tests, $P > 0.05$; except for phenol, $P \leq 0.05$; and catechol and guaiacol, which were absent in males) and onion diets (t tests, $P > 0.05$; except catechol and p-benzoquinone, which were absent in females). Thus a reduction in diet breadth reduced the concentrations of defensive components and eliminated the sexual differences in concentrations that were noticeable on natural diets.

Variation in Secretion Component Quantity. Variation among individuals in concentrations of specific components is considerable in wild lubber populations, ranging up to three orders of magnitude (Jones et al., 1986). Coefficients of variation for compound concentrations in individuals reared on natural diets were not obviously higher or lower than those for artificial or onion diets (Figure 4) (F tests, $P > 0.5$). Since, however, the restricted diets had compound concentrations that were lower by one to two orders of magnitude, it is surprising that there was no noticeable reduction in relative variation. Thus a reduction in dietary breadth may affect concentration of defensive components, but does not have a major effect on the considerable amounts of variability between individuals.

Changes in Secretion Composition. The overall composition of a chemical defense may be relevant to signal recognition by predators (Pasteels et al., 1983a; Pasteels and Grégoire, 1984; Jones et al., 1986). The changes in per-

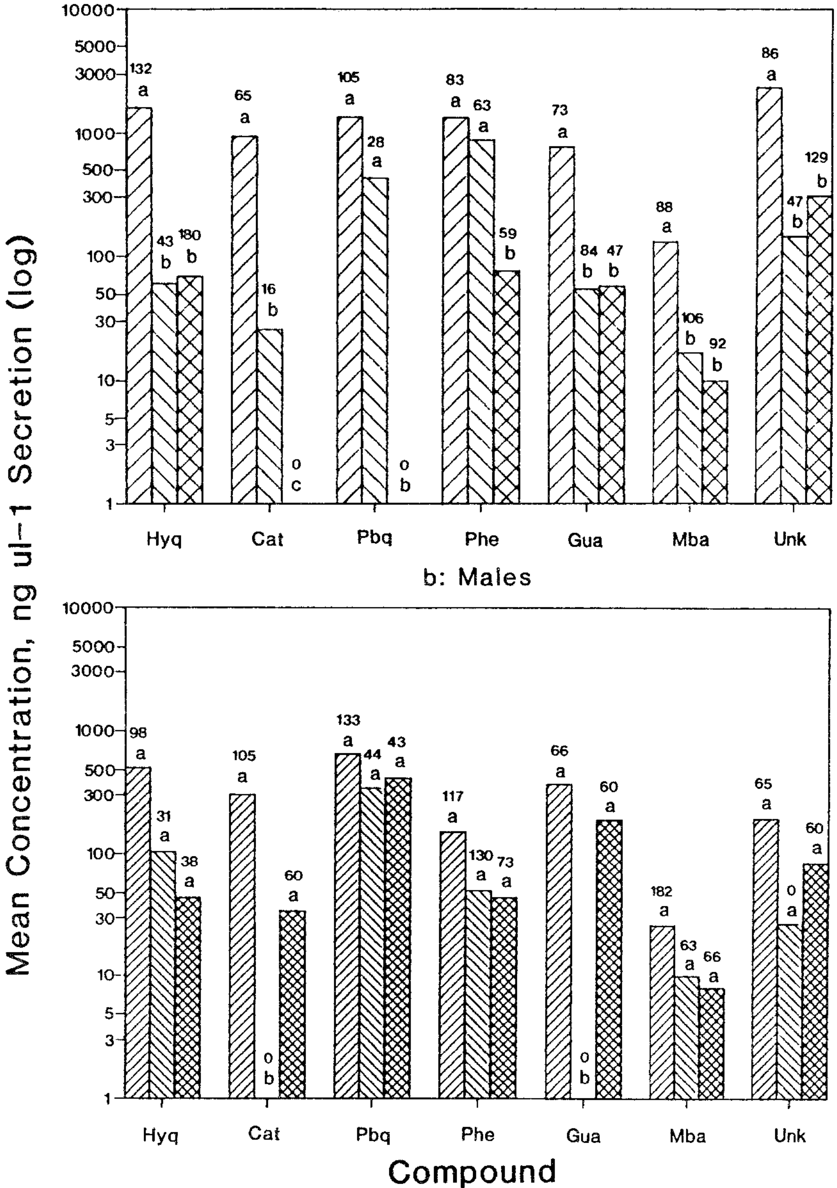

FIG. 4. Mean concentration of phenolics, quinones, and an unknown compound (as equivalents of the internal standard, 4-hydroxybenzoic acid) (log scale) in secretions of adult *R. guttata*. For abbreviations of compounds, see Figure 1. (a) Females: [pattern] = natural diet; [pattern] = artificial diet; [pattern] = onion diet. (b) Males: [pattern] = natural diet; [pattern] = artificial diet; [pattern] = onion diet. [a,b,c]Means followed by different letters are significantly different from each other, within a sex and compound, between diets (t tests, $P \leq 0.05$). Numbers above histogram bars are coefficients of variation ($s/\bar{x}$, %) in concentrations.

cent composition due to restriction of diet breadth were considerable (Figure 5). It is clear that secretions from insects on natural diets had a more even composition than those from insects on artificial or onion diets. For example, the deviation in value for percent composition for each compound from an ex-

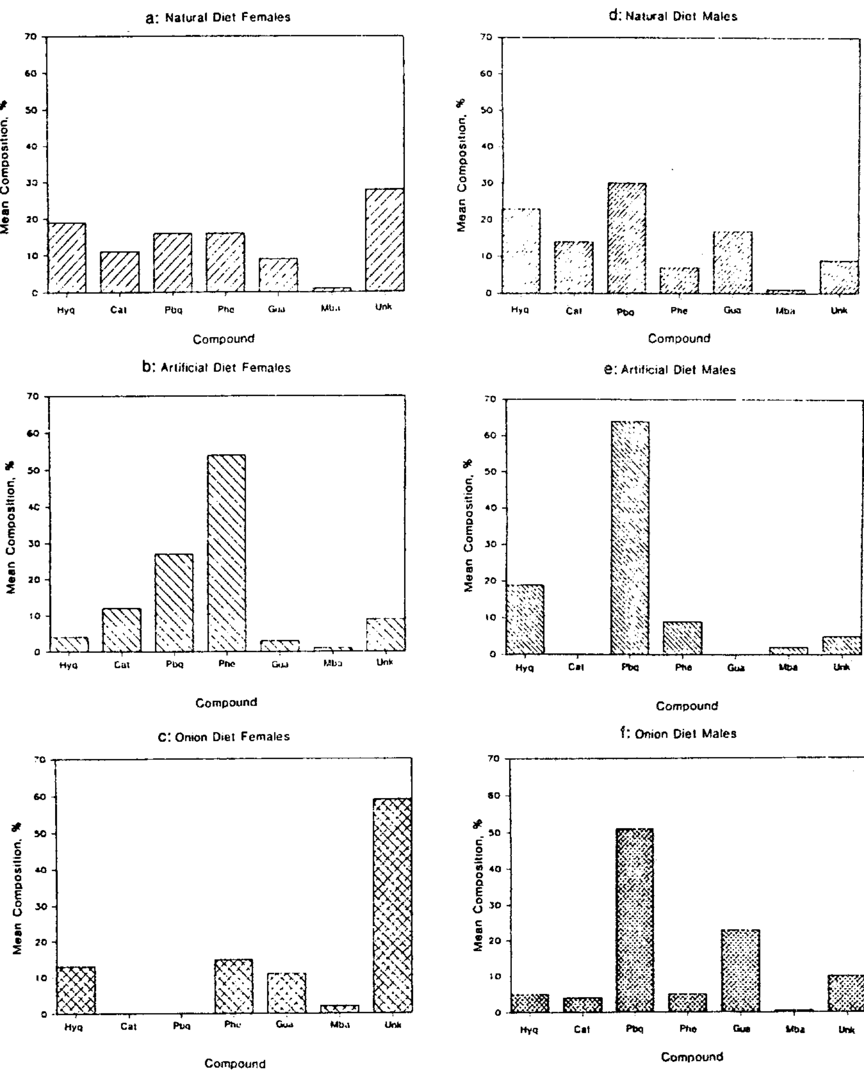

FIG. 5. Mean composition of defensive secretions, based on seven compounds (phenolics, quinones, and an unknown) for *R. guttata*. For abbreviations of compounds, see Figure 1. a, [pattern]: natural diet females; b, [pattern]: artificial diet females; c, [pattern]: onion diet females; d, [pattern]: natural diet males; e, [pattern]: artificial diet males; f, [pattern]: onion diet males.

pected value (expected = 100/7 = 14%), when averaged for all seven compounds, was 6% for females and 8% for males on natural diets, 13% (female) and 16% (male) for artificial diets, and 13% (female) and 13% (male) for onion diets. So, restricting diet breadth changed the balance of components.

Relative Importance of Dietary Effects. Variation among individuals (Jones et al., 1986), effects of diet and sex, and the interaction of diet and sex were all possible factors that contributed to variation in the concentration of defensive secretion components. A previous study showed that interindividual variation accounted for 60–88% of the total quantitative variation in concentrations of compounds in defensive secretions of a wild population, and sex accounted for 9–39% (Jones et al., 1986). Diet was assumed to be the same for all individuals in that population. We applied the same type of analysis to these data (Table 1). The average importance of the three factors and their interaction were: individuals, 57%; diet, 21%; sex, 9%; diet/sex interactions, 9%. Thus the reduction in secretion component quantity due directly or indirectly to diet was a major factor accounting for an average of 30% of the total quantitative variation.

Similar estimates for variation in secretion quality were more difficult to derive. The same type of analysis was applied to the number of compounds in the defensive secretion (see Figure 2). While this was by no means a complete representation of secretion quality (e.g., it ignores which compounds are present or absent in which individuals), the variation was distributed as follows: individuals, 49%; diet, 25%; sex, 1%; diet/sex interactions, 25%. Again, direct and indirect effects of diet emerge as a major factor, accounting for 50% of variation in this characteristic of secretion quality. We concluded that direct

TABLE 1. DISTRIBUTION OF VARIANCE, FROM PARTIAL SUM OF SQUARES, OF CONCENTRATION OF PHENOLICS, QUINONES, AND UNKNOWN (AS EQUIVALENTS OF THE INTERNAL STANDARD 4-HYDROXYBENZOIC ACID) FOR ADULT MALE AND FEMALE *R. guttata* REARED ON THREE DIFFERENT DIETS[a]

Compound	Diet	Sex	Diet × sex interaction	Individuals	Analytical error
Hyq	20	3	7	70	<1
Cat	32	16	—	47	2
Pbq	7	3	2	77	3
Phe	12	21	9	50	1
Gua	39	3	9	50	3
Mba	21	7	12	55	1
Unk	19	8	13	50	<1
AVERAGE	21	9	9	57	2

[a] For abbreviations of compounds, see Figure 1.

and indirect effects of dietary restriction on autogenous defensive secretion quality and quantity were considerable and second only to interindividual variability. However, the large portion of the variation that was still attributable to individuals indicated that a substantial amount of the quantitative and qualitative variation between wild individuals was not due to diet, but was an innate characteristic of *R. guttata*.

DISCUSSION

Our study clearly demonstrates that diet can have a major effect on the quality and quantity of autogenous defensive secretions of *R. guttata*. The effects are indirect and are not due to sequestration of the phenolics and quinones from the diet, although sequestration of these compounds might occur under different circumstances. Furthermore, the effects of diet were relatively independent of the extensive interindividual variability. Rather, the major effects of restricting diet breadth were to reduce the probability of compounds occurring in individuals, thereby reducing the number of secretion components, reduce the concentration of secretion components, and change the composition of the secretion. While all of these effects occurred for both artificial and single host-plant diets, restriction to a single host-plant produced the more dramatic effects. Similarly, effects of dietary restriction affected females more than males.

To our knowledge, this study is the first to investigate the effects of diet on autogenous defensive secretions for a generalist insect. The focus on the influence of diet breadth permits comparison of our data to some analogous studies with specialist insects producing autogenous defenses on their host plants. Pasteels and Daloze (1977; Daloze and Pasteels, 1979) showed that the cardenolide defenses of the chrysomelid beetles *Chrysolina* and *Chrysochloa* were autogenous and apparently unaffected by the host plant. Presumably the host plants did not vary significantly in their provision of sterol dietary precursors from which the insect cardenolides were synthesized (Pasteels and Daloze, 1977).

In contrast, Pasteels et al. (1983b) showed that larvae of certain chrysomelids feeding on *Salix* or *Populus* used the host-plant phenylglucoside salicin as the sole source of a defensive compound, salicylaldehyde. In this case there is obviously a very close biochemical coupling between the precursor and the defense and is similar to direct sequestration of plant compounds. Larvae and adults of other, related chrysomelid species feeding on other host plants produce autogenous methylcyclopentanoid monoterpenes, with no obvious host-plant effects. Similarly, Nahrstedt and Davis (1983) showed that the cyanogenic glucoside defenses linamarin and lotaustralin found in neotropical Heliconiini (Lepidoptera, Nymphalidae) were synthesized from valine and isoleucine of the host plant (Wray et al., 1983). Interestingly, these host plants also contained

cyanogenic glucosides, but of a different structural type and biogenetic origin (Conn, 1980).

Many studies on biosyntheses of defensive compounds, using administration of pure radiolabeled compounds, reveal a dependence on specific precursors that presumably do occur in the host (for reviews, see Duffey, 1976; Blum, 1981). It seems reasonable that variation in precursor type and abundance in the diet and biochemical similarity of precursor and defense could affect defensive quality and quantity in some cases, although experimental tests of these phenomena are lacking. The above studies clearly show that diet can have a major effect on autogenous defenses in specialists under some circumstances, and have no obvious effect in others.

The generalist feeding behavior of *R. guttata* may create a somewhat different situation from that found in specialists. We think there are two possible explanations for our results, one relating to precursor availability, the other to physiological effects of restricted diets. First, in contrast to extreme specialists where the single host plant is the sole source of precursors for autogenous defenses, a generalist such as *R. guttata* has a potentially large number of sources of precursors for its defenses. This assumes relative ubiquity of common precursors in host plants. Consequently, a lack of precursors—and detrimental effects on the defensive secretion—are unlikely to occur when the diet consumed is a varied one. However, the probability of consuming a diet that lacks adequate quantities of precursors should increase with decreasing diet breadth. This would be especially true if host-plant precursors are not ubiquitous, but are nonuniformly distributed and restricted to only some food plants. Restricted diets may therefore lead to an autogenous defense reduced in complexity and concentration because of a lack of adequate precursors on some single host-plant diets. Since we do not yet know what precursors are involved, it is not yet possible to determine whether or not this hypothesis is correct.

A second, and equally plausible, explanation is that restricting diet breadth causes a physiological stress on the insect. Under these conditions, allocation of energy and resources by the insect to growth and maintenance may take precedence over allocation to defense, biosynthesis of defensive compounds is reduced, and therefore the quality and quantity of the defensive secretion decreases. This hypothesis is compatible with our data showing that the greatest effects of diet restriction on defense occur with females. Females have a larger body size and much faster growth rate than males. Females triple the body weight following the molt to adult because they must allocate considerable resources to egg production. Male body weight only increases about 20% (Whitman, Jones and Blum, unpublished data). Consequently, under conditions of restricted diets, females are probably under considerable metabolic stress following the molt to adult, while males simply need to maintain their weight and may have sufficient resources to allocate to defense.

While the exact mechanisms must await further study, the effects of diet

that we observed raise some immediate methodological concerns. Many studies on arthropod defenses have examined a multitude of factors that might cause variation in the composition of autogenous defenses. These include age, sex, season, instar, caste, population, and genotype or colony (see reviews by Blum, 1981; Pasteels et al., 1983a; Prestwich, 1983). In many of these studies, the possible influence of diet due to local specialization (sensu Fox and Morrow, 1981) within or between insect populations on particular hosts has not been taken into account. In those studies in which dietary sources of variation have been examined, it is usual to investigate the influences of direct sequestration (e.g., Brower et al., 1982, 1984) or the direct role of specific host precursors in vitro (e.g., Nahrstedt and Davis, 1983). If the results we found for *R. guttata* hold for other insects, it may necessitate a reexamination of variation previously attributed to nondietary factors. In addition, while it is expected that dietary effects would be more noticeable in phytophages, it is quite possible that diet may also affect autogenous defenses in predaceous arthropods. The factors that influence the quality and quantity of autogenous chemical defenses in insects are likely to be complex. However, a better understanding of the ecological role of these defenses cannot be achieved without elucidating these factors and their interrelationships. In the case of *R. guttata*, it would appear the breadth of the diet is one such major factor.

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ALLELOPATHIC EFFECTS OF *Chrysanthemum morifolium* ON GERMINATION AND GROWTH OF SEVERAL HERBACEOUS PLANTS

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Abstract—Aqueous extracts obtained from young green tops of *Chrysanthemum morifolium* inhibited the germination of six flowering plants, including chrysanthemum itself, provided for experiments. The same phenomenon was also clearly observed when powder made from young green tops and old leaves of chrysanthemum was used. Moreover, the growth of seedlings planted again in garden soil which was once used for the culture of chrysanthemum was greatly interrupted. Chrysanthemum cultured in used garden soil showed far less dry weight than that cultured in fresh garden soil. The weight of chrysanthemum cultured using its root exudates was also less than that cultured with water leachate of fresh garden soil, and therefore these results may be considered to indicate allelopathic effects. In order to find the allelochemicals related to this phenomenon, benzoic acid and phenolic acids such as salicylic, *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, gallic, ferulic, and caffeic acids were identified by gas chromatography.

Key Words—*Chrysanthemum morifolium*, Allelopathy, aqueous extract, used garden soil, phenolic acids, gas chromatography, continuous cropping, germination inhibition rate, autointoxication.

INTRODUCTION

From the interference viewpoint, allelopathy is an important phenomenon in plant–plant interaction. Since Molisch (1937) coined the word, allelopathy has been studied by many scholars. There have been numerous reports of the production by plants of phytotoxic substances which inhibit seed germination and seedling growth of the same and other plants (Muller, 1969; Chou and Lin,

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1976; Dalrymple and Rogers, 1983; Rice, 1984). It has also been shown that continuous cropping causes a harmful effect on the growth of some crops such as tomato, red pepper, Korean ginseng, and chrysanthemum, and therefore crop rotation is essential. Tukey (1969) reported that chrysanthemum cannot grow for several years in the same soil, Ballantyne (1962) demonstrated that growth inhibitors are released from the roots of chrysanthemum, and Lee and Chang (1969) stated that soil-sickness is caused by toxic substances secreted from the roots of chrysanthemum. Kozel and Tukey (1969) identified phenolic compounds as the toxic substances by the foliage of chrysanthemum, and Osawa et al. (1973) identified chlorochrymonin, a sesquiterpene lactone from chrysanthemum.

Although these studies were carried out in various ways, few comprehensive reports have dealt intensively with allelopathy of chrysanthemum or investigated minutely its phytotoxic substances. Therefore, it was intended in this study to (1) investigate the effects of young green tops, leaves, and garden soil of chrysanthemum on seed germination and growth of some selected plant species and (2) identify phytotoxins of chrysanthemum using chemical methods.

METHODS AND MATERIALS

Aqueous Extracts of Chrysanthemum and Garden Soil. One hundred grams of chrysanthemum green tops were put into 3000 ml of distilled water and ground fine, so that aqueous extracts could be obtained. After two sheets of filter paper (11 cm in diameter) were spread on Petri plates (12 cm in diameter) and 100 grains of sample seeds, that is, *Chrysanthemum morifolium*, *Callistephus chinensis*, *Cosmos bipinatus*, *Petunia hybrida*, *Celosia cristata*, or *Coleus Blumen*, were sown on it, 100 ml of aqueous extracts were applied to each Petri plate and then water was given to control plates. These plates were sealed and kept in a 25°C growth chamber. The aqueous extract and water were applied to each plate every two days and seed germination was recorded every day. This experiment was repeated three times.

It was intended in this study to introduce the concept of percent germination inhibition rate (GIR) in order to evaluate quantitatively the effect of phytotoxic substances of chrysanthemum according to concentration gradient on the germination of experimental species. The following formula was employed for the calculation of GIR.

$$\text{GIR} = \left(1 - \frac{\text{Germination rate of experimental species}}{\text{Germination rate of control species}}\right) \times 100$$

About 20 days after chrysanthemum bloomed in the previous year, its leaves and young green tops 10–15 cm in length were collected, dried, powdered, and passed through a #40 mesh sieve. After one sheet of filter paper (14

cm in diameter) was placed in each Petri plate (12 cm in diameter), 0.5 or 1 g of the powder was spread over each plate and 20 ml of distilled water added. The plates were sealed, kept for 20 h at 25°C, and filtered. Aqueous extracts thus obtained were used for germination experiments, 10 ml added to each plate, and water was used for the control. One hundred each of the sample seeds of *Callistephus chinensis*, *Cosmos bipinnatus*, *Tagetes electa*, *Petunia hybrida*, *Celosia cristata*, *Salvia splendens*, and *Portulaca grandiflora*, was planted in each pot, covered with 20 g of vermiculite, and distilled water was added. Ten days after germination, 200 ml of Boysen Jensen's nutrient solution were supplied once every four or five days. The plants were harvested in 55 days, washed clean, and dried in an oven at 80°C. This experiment was repeated three times.

Root exudate was obtained by culturing chrysanthemum to a height of about 30 cm in the same flowerpots used the previous year and pouring distilled water into them for 24 h. Root exudate thus gathered was poured continuously over fresh garden soil in flowerpots and distilled water was used for the control. Plastic pots (each 12 × 7 cm) were filled with 300 g of vermiculite and 50 seeds of *Callistephus chinensis*, *Cosmos bipinnatus*, *Tagetes electa*, *Petunia hybrida*, *Celosia cristata*, *Salvia splendens*, or *Portulaca grandiflora*, were sown in each pot. After germination, 200 ml of the root exudate were supplied every two days. The same procedure as above was applied in supplying nutrient solution and harvesting after germination and growth. This experiment also was repeated three times.

Identification by Gas Chromatography. The analysis of various extracts was performed using a Varian 2440 gas chromatograph equipped with 6-ft × $\frac{1}{8}$-in. (inside diameter) stainless-steel column packed with 2.5% SE-30 on ABS 90–100 mesh sieve. The flow rate of helium carrier gas was 40 ml/min. The flame ionization detection was set at a sensitivity of 2 × 10 mv. Injection port and detector temperature were held at 230°C and 205°C, respectively. The column temperature was programmed at a rate of 6°/min from 100°C to 250°C. One microliter sample injection was made with a 5-ml SGE syringe employing the solvent flush technique. Otherwise, the extraction and procedures of gas chromatography were as before (Kil and Yim, 1983).

RESULTS

Germination Experiment. The germination of six species, including *Chrysanthemum moriflorium*, was determined using aqueous extracts of young green tops of chrysanthemum (Table 1). All of these showed a significant inhibition of germination rate compared with the control at the 5% level. Although chrysanthemum itself showed germination inhibition by its own extract, the degree of its harmful effect was not so severe. However, the germination inhibition

TABLE 1. EFFECTS OF AQUEOUS EXTRACTS OF CHRYSANTHEMUM SEEDLING GREEN TOPS ON PERCENT GERMINATION

Species	Germination (%)		GIR
	Control	Test	
Chrysanthemum morifolium	61.3	52.0[a]	15.2
Callistephus chinensis	65.7	52.0[a]	20.9
Cosmos bipinnatus	78.3	65.7[a]	16.7
Petunia hybrida	76.0	65.7[a]	13.6
Celosia cristata	81.8	72.7[a]	11.1
Coleus Blumen	65.0	54.7[a]	15.9

[a]Significantly different from control at 5% level by *t* test comparison.

rate (GIR) of *Petunia hybrida* and *Celosia cristata* showed less effect of the extracts than the other five species. *P. hybrida* belongs to the Solanaceae and *C. cristata* to the Amaranthaceae, while the remaining five species belong to the Compositae.

Aqueous extracts of young green tops and old leaves of chrysanthemum inhibited seed germination of all species compared with the control (Table 2). Moreover, the germination rate was lower in the high concentrations of the extracts (B in Table 2) than in low concentrations. Especially severe inhibitory effects were observed in the germination of treated *Callistephus chinensis* and *Cosmos bipinnatus*. According to the results of Duncan's multiple-range test, such germination inhibitory effects were significant at the 5% level.

Seedling Growth. The seedling growth of all species used in the present

TABLE 2. EFFECTS OF AQUEOUS EXTRACTS OF CHRYSANTHEMUM LEAF POWDER ON PERCENT GERMINATION[a]

Species	Control	Young green tops		Old leaves	
		A	B	C	D
Callistephus chinensis	62.7a[b]	49.7bc	36.3e	53.7b	45.3cd
Cosmos bipinnata	73.3a	61.3bc	51.3de	65.7b	58.3cd
Tagetes electa	34.7a	24.3bc	11.3e	27.0b	19.7cd
Petunia hybrida	78.3a	69.3bc	56.9de	72.3ab	63.7cd
Celosia cristata	86.3a	79.7abc	71.0de	82.7ab	78.3bcd
Salvia splendens	76.3a	64.3bc	55.0de	69.7ab	59.7cd
Portulaca grandiflora	40.3a	34.7bc	28.7de	35.3ab	30.3cd

[a]A and C: aqueous extracts 0.5 g/H_2O 20 ml; B and D: aqueous extracts 1.0 g/H_2O 20 ml.
[b]Means within rows followed by the same letter do not differ significantly at the 5% level of probability according to Duncan's multiple-range test.

TABLE 3. DRY WEIGHTS (MG) OF DIFFERENT SPECIES GROWN IN FRESH GARDEN SOIL (CONTROL) AND USED SOIL (TEST)

Species	Control	Test	%
Callistephus chinensis	800.00	475.56[a]	54.0
Cosmos bipinnatus	1976.67	1188.67[a]	60.0
Tagetes electa	1073.50	634.60[a]	59.1
Petunia hybrida	846.32	436.24[a]	51.5
Celosia cristata	935.36	414.04[a]	44.3
Salvia splendens	1103.20	645.00[a]	58.5
Portulaca grandiflora	864.38	490.00[a]	56.7

[a] Significantly different from control at 1% level by *t* test comparison.

experiment was markedly lowered when they were cultured in garden soil used in the previous year (Table 3), indicating an allelopathic phenomenon. Dry weight of the seedlings of *Celosia cristata* harvested from the experimental plot 55 days after sowing was 414.04 mg and that of the control was 935.36 mg, thereby showing only 44.3% growth of the control. Similar results could also be observed in *Petunia hybrida*, i.e., 846.32 mg of the control and 436.24 mg of the experimental plot in dry weight or 51.5% of the control.

Seedling growth of the seven experimental species cultured with water leachate obtained by washing the roots of chrysanthemum was markedly inhibited (Table 4). The growth of *Celosia cristata* and *Petunia hybrida* was severely

TABLE 4. COMPARISON OF DRY WEIGHTS (MG) OF DIFFERENT SPECIES GROWN IN WATER LEACHATE OF FRESH GARDEN SOIL (CONTROL) AND OF CHRYSANTHEMUM ROOTS (TEST)

Species	Control	Test	%
Callistephus chinensis	902.35	395.77[a]	43.9
Cosmos bipinnatus	1883.45	936.80[a]	49.7
Tagetes electa	957.60	478.25[a]	49.9
Petunia hybrida	932.50	342.40[a]	36.7
Celosia cristata	842.19	303.00[a]	36.0
Salvia splendens	1061.12	518.81[a]	48.9
Portulaca grandiflora	788.50	374.38[a]	47.5

[a] Significantly different from control at 1% level by *t* test comparison.

TABLE 5. IDENTIFICATION OF PHYTOTOXINS FROM *Chrysanthemum morifolium* BY GAS CHROMATOGRAPHY

No.	Phytotoxins	Relative retention time	Flowers	Leaves	Roots	Young green tops	Old leaves
1	Benzoic acid	0.28	+	+	+	+	+
2	Salicylic acid	0.75	+	+	–	–	–
3	*p*-Hydroxybenzoic acid	1.00	–	+	+	–	–
4	Vanillic acid	1.33	–	–	+	–	+
5	Gentisic acid	1.38	+	+	+	+	+
6	Protocatechuic acid	1.48	+	+	+	+	+
7	Syringic acid	1.63	–	+	–	–	–
8	Gallic acid	1.80	+	–	–	–	+
9	Ferulic acid	2.03	+	+	–	+	+
10	Caffeic acid	2.15	+	–	–	+	–

[a]Minus = absence; plus = presence.

affected by the leachate of chrysanthemum, whereas the other five species were less affected in their growth.

Identification of Phytotoxic Substances. The leaves, roots, young green tops, and old leaves of *C. morifolium* were analyzed by gas chromatography (Table 5, Figures 1 and 2). Benzoic acid and nine kinds of phenolic acids were detected; i.e., salicylic, *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic,

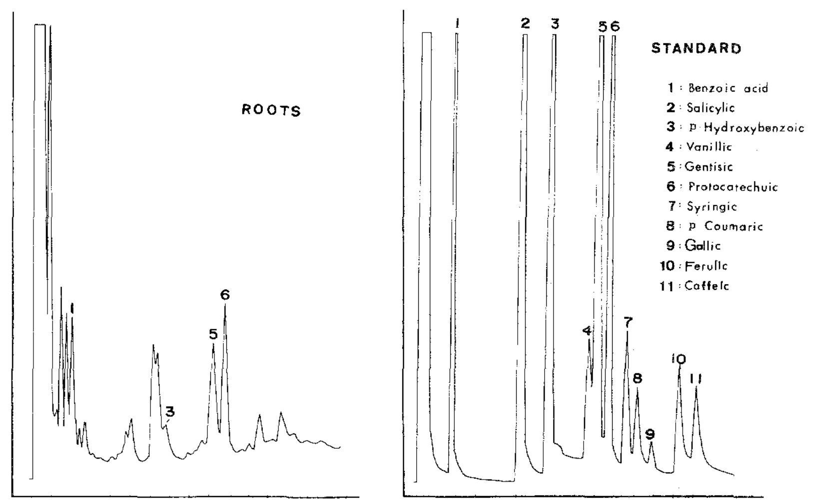

FIG. 1. Gas chromatograms of chemical substances from *Chrysanthemum morifolium* roots.

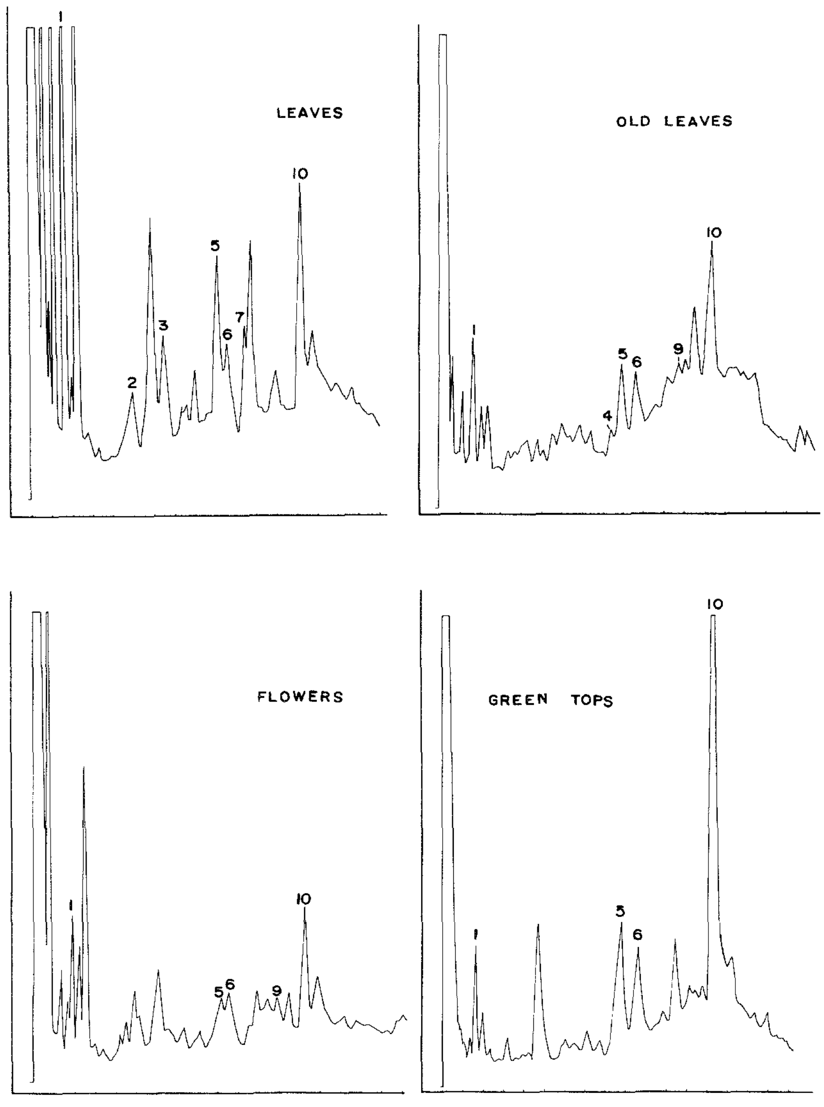

FIG. 2. Gas chromatograms of chemical substances from *Chrysanthemum morifolium* shoots.

syringic, gallic, ferulic, and caffeic acids. It was also confirmed that benzoic, gentisic, protocatechuic, and ferulic acids were contained abundantly in all materials used for the analysis (Figures 1 and 2). Seven kinds of compounds were detected from the leaves and flowers of chrysanthemum, six from old leaves and five from roots and young green tops.

DISCUSSION

The experimental data given in Table 1 show that the aqueous extracts of chrysanthemum had an inhibitory effect on the germination of other experimental species. The aqueous extract of young green tops of chrysanthemum was harmful to all test species. This fact suggests that both the young leaves and stems of chrysanthemum contain phytotoxic substances.

Considering results of the experiments, it is apparent that seed germination of chrysanthemum is inhibited by chemical substances contained in chrysanthemum itself, indicating an autointoxication phenomenon as reported by other researchers (Chou and Lin, 1976; Hussain et al., 1983). The experimental species *P. hybrida* and *C. cristata*, which are different from the chrysanthemum family, were lower in GIR than those belonging to the same family. This finding agrees with the results reported by other researchers (Hayashi and Takiyo, 1959; Lee and Lee, 1982).

On the other hand, the seed germination of every experimental species was severely inhibited by the aqueous extract of the leaves of chrysanthemum. Now, there remains a question of which chemical substances contained in chrysanthemum exert an inhibitory action on the seed germination of plants. In order to solve this problem, different experiments were carried out, and the results obtained are presented in Table 2. Aqueous extracts of chrysanthemum in low and high concentration were used in this experiment. The germination rate of the experimental plot showed far greater inhibition than that of control. Moreover, the germination inhibition rate was statistically significant in all experimental species, and therefore it seems that the young green tops of chrysanthemum also contain certain germination inhibitors, a fact already reported (Ashraf and Sen, 1978; Datta and Chattergee, 1980; Chou and Yang, 1982; Schon and Einhellig, 1983; Kil and Yim, 1983).

Chrysanthemum apparently secreted phytotoxic substances, and therefore the growth of its seedlings was inhibited when cultured in used garden soil. There have been many reports proving that plants secrete phytotoxic substances into the soil in which they grow (Guenzi and McCalla, 1966; Wang et al., 1967; Patrick, 1971; Carballeira, 1980). It is well known that when a farmer cultures chrysanthemums again in previously used garden soil, the yield is reduced. This result has not been shown to be attributable to any inorganic nutrient contained in the soil or to other factors, but the evidence is strong that it is due to an allelopathic effect. It is clearly proven by the result of the present experiments that even the roots of chrysanthemum produce phytotoxins (Table 4). It may further be interpreted that some toxic substances produced by chrysanthemum are increasingly accumulated and harm other plants.

The results of this growth experiment with seven species show that phytotoxic substances of chrysanthemum had greater inhibitory effects on plants of distant families than on Compositae. This finding agrees with the report that

toxins secreted from the roots of eggplant, tomato, wheat, barley, and rice are harmful to the growth of plants. In other words, chemical substances secreted from a species are usually more harmful to distant plants that those of close relationship (Takiyo and Hayashi, 1959).

Nine kinds of phenolic acids and benzoic acid were identified in the chrysanthemum with gas chromatography. Many researchers have already reported that germination, shoot elongation, and total seedling growth are inhibited by phenolic acids (Olmsted and Rice, 1970; Kaputska and Rice, 1976; Tinnin and Muller, 1972; Rice et al., 1980). Such chemical substances are secreted during the decaying process of plants (Shindo and Kuwatsuka, 1977) or foreign chemical substances exert inhibition only in low nutrient concentrations (Stowe and Osborn, 1980). It is also reported that ferulic and caffeic acid inhibit nitrification (Rice and Pancholy, 1974). In conclusion, the phytotoxins of chrysanthemum have inhibitory effects on the germination of various herbaceous plants including chrysanthemum itself and growth of young plants. The nine phenolic acids and benzoic acid are considered to be important allelochemicals.

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PLANT SECONDARY COMPOUNDS AS OVIPOSITION DETERRENTS FOR CABBAGE BUTTERFLY, *Pieris rapae* (LEPIDOPTERA: PIERIDAE)

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Abstract—Oviposition by *Pieris rapae* butterflies was deterred by spraying the plant secondary compounds coumarin and rutin on cabbage plants in greenhouse choice tests. In no-choice tests ranging from 5 min to 24 hr, acceptance of rutin-treated plants for oviposition increased with trial duration. Both coumarin and rutin deterred oviposition primarily by affecting prealighting rather than postalighting behavior, indicating that deterrence was mediated by noncontact cues.

Key Words—Oviposition deterrence, oviposition behavior, *Pieris rapae*, cabbage butterfly, Lepidoptera, Pieridae, coumarin, rutin, plant secondary compounds, insect-plant interactions, cabbage, Cruciferae.

INTRODUCTION

For insects such as Lepidoptera, host selection by mobile adults is crucial in determining the fitness of their less mobile offspring (Scriber, 1984; Tabashnik and Slansky, 1987). Egg-laying females identify suitable plants by chemical and physical stimuli that characterize such plants (Miller and Strickler, 1984). Plant secondary compounds (allelochemicals) typical of a given plant may be oviposition stimulants for insects which feed on that plant (e.g., Gupta and Thorsteinson, 1960; Rodman and Chew, 1980; Renwick and Radke, 1983). In contrast, oviposition by a particular insect may be deterred by compounds that occur at high levels in unsuitable plants, but not in suitable plants (Tingle and Mitchell, 1984; Mitchell and Heath, 1985). Plants suitable for a particular insect may also contain compounds that deter oviposition by that insect (Renwick and Radke 1981, 1985). Oviposition deterrence by plant compounds could have significant consequences for management of crop pests.

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Previous work showed that chemical extracts from noncruficerous plants deter oviposition by the crucifer-feeding cabbage butterfly, *Pieris rapae* L. (Lundgren, 1975; Renwick and Radke, 1985). However, relatively little is known about the effects of individual compounds as oviposition deterrents. The plant secondary compounds coumarin and rutin are potential oviposition deterrents for *P. rapae* because they do not occur in crucifers at substantial levels, but they are present at high concentrations in many noncruciferous herbs. Coumarin occurs in members of several plant families (e.g., Compositae, Lauraceae, Leguminosae, Umbelliferae), in some cases as the major aromatic constituent (Leung, 1980). Rutin is a nonvolatile flavonoid pigment found in numerous plant families, with concentrations of up to 24% of leaf dry weight recorded for some species (Leung, 1980). Both coumarin and rutin deterred oviposition by the crucifer pest, *Plutella xylostella* L. (Tabashnik, 1985).

The study reported here examined how *P. rapae* oviposition is affected by application of coumarin and rutin to cabbage plants. The effects of the compounds on oviposition, prealighting behavior, and postalighting behavior were investigated using choice and no-choice behavioral assays.

METHODS AND MATERIALS

Insects. Adults were obtained from a laboratory colony 5–8 generations removed from field populations at the Pearl City Instructional Facility community garden, Oahu, Hawaii. Crucifers available for oviposition at the Pearl City field site include broccoli, cabbage, and watercress; larvae were fed broccoli in the laboratory colony (Haji-Mamat, 1984).

Plants. Cabbage plants were grown from seed ("C-G Cross," Takii and Co., Ltd.) in the greenhouse in plastic pots (9.5 × 10 × 10 cm) filled with vermiculite. Seedlings were fertilized with dilute Ortho-Gro® Liquid Plant Food 12-6-6 (1 ml : 200 ml H_2O) within one week after planting. Plants used in behavioral assays were treated with either a control solution of 0.5% Tween 80 (polyoxyethylene sorbitan monooleate, Sigma) in water or a solution of 0.5% Tween 80 in water plus the test material. Solutions tested were coumarin (Kodak) and rutin (Sigma). Solutions were thoroughly shaken in a 235-ml polyethylene jar, then sprayed on upper and lower plant surfaces for about 3 sec using aerosol propellant (Sigma spray kit). The amount of solution applied to leaves was estimated by weighing 10 leaves immediately before and after spraying. The estimated mean volume of solution sprayed on leaves was 0.294 ml (±0.022 SE)/g leaf fresh weight, indicating that leaves sprayed with coumarin solutions of 0.1 M and 0.01 M had approximately 4.3 mg and 0.43 mg coumarin/g leaf fresh weight, respectively. Estimated values for applied rutin concentration were 19.6 and 1.96 mg/g leaf fresh weight, for 0.1 M and 0.01 M solutions, respectively.

Behavioral Assays. All behavioral assays were conducted between February and June 1984 in the Entomology Department greenhouse at the University of Hawaii Manoa Campus. Daily maximum temperatures were 29 ± 2°C and daily minimums were 22 ± 2°C; the natural photoperiod (ca. 12 hr light) was used. Groups of females (3–10) with approximately equal numbers of males were tested in screen cages (50 cm on each side) in all experiments. Cotton dental wicks immersed in dilute honey-water (1 ml : 5 ml) solutions in open vials were suspended from the cage tops to provide food for adults. Within the constraints imposed by confinement, behavior of females in the assays was similar to their field behavior (Chew 1977); females laid their eggs singly and usually flew off the plant after oviposition.

Choice Tests. One plant treated with control solution and another treated with either a coumarin or rutin solution were placed in diagonally opposite corners of each cage. Trials lasted 2–3 days. Eggs were counted after each trial, and plant positions in the cage were alternated in successive trials.

No-Choice Tests. Females were offered either two control plants or two treated plants (either coumarin or rutin at 0.1 M). Plants were placed in diagonally opposite corners of the cage. In the 5-min no-choice tests, each group of females was tested for 10 trials, consisting of an alternating sequence of control and treated plants offered for 5-min periods, with less than 1 min between trials. For no-choice tests lasting 15 min, 60 min, and 24 hr, groups of females were paired so that one group was offered two control plants while, at the same time, a matched group of equal numbers and age was offered treated plants. In the next trial, the plants were switched so that the first group received treated plants while the second group received controls.

Pre- and Postalighting Behavior. Females were observed during behavioral assays to determine how coumarin and rutin influenced their pre- and postalighting behavior. "Contacts" were recorded when females touched a plant with one or more legs.

Analysis. G tests for goodness of fit, adjusted for sample size as necessary (Sokal and Rohlf, 1969), were used throughout. The statistical significance of deterrence was determined by contrasting observed frequencies of behaviors (ovipositions or contacts) on control vs. test plants with the 1 : 1 frequency of behaviors expected if butterflies did not differentiate between treatments. Ratios of behaviors (eggs laid/contact) on control vs. test plants were contrasted by testing for independence in 2×2 contingency tables (Sokal and Rohlf, 1969). The 2×2 contingency analysis was also used to compare frequencies of eggs on control vs. plants treated with rutin (0.1 M) in no-choice tests of various duration. Means (±SE) for percentages of eggs on treated plants were calculated to provide a rough index of variation among replicates. Statistical inferences from ANOVA on the percentages were generally consistent with the G tests.

RESULTS

Choice Tests. Both coumarin and rutin deterred oviposition by *P. rapae* (Table 1). At both concentrations tested, coumarin was a weak deterrent. Rutin at 0.1 M caused a fivefold reduction in oviposition, but 0.01 M rutin did not deter oviposition.

No-Choice Tests. Responses to cabbage plants treated with 0.1 M rutin in no-choice tests depended on the trial duration, with observed deterrence inversely related to trial duration (Table 2). Deterrence was strongest in the 5-min no-choice test, intermediate in 15- and 60-min no-choice tests, and weakest in the 24-hr no-choice test.

Pre- and Postalighting Behavior. Coumarin and rutin deterred oviposition primarily by affecting prealighting rather than postalighting behavior. Direct behavioral observations showed that both compounds significantly reduced the number of contacts with plants (Table 3). Analysis of the ratio of eggs laid/contact showed that after contacting a plant, females were somewhat less likely

TABLE 1. EFFECTS OF COUMARIN AND RUTIN ON OVIPOSITION BY *Pieris rapae* IN CHOICE TESTS

Treatment		Trials	Total eggs laid		% of eggs on treated
			Control	Treated	($\bar{X} \pm$ SE)
Coumarin	0.1 M	7	1155	605[a]	40.9 ± 5.2
Coumarin	0.01 M	5	918	668[a]	42.5 ± 2.5
Rutin	0.1 M	6	1928	375[a]	18.5 ± 4.1
Rutin	0.01 M	6	949	886	44.8 ± 6.1

[a] $P < 0.001$ by G tests on pooled data.

TABLE 2. EFFECT OF RUTIN (0.1 M) ON OVIPOSITION BY *Pieris rapae* IN NO-CHOICE TESTS OF VARIOUS DURATIONS

Trial duration	Trials	Total eggs laid[a]		% of eggs on treated
		Control	Treated	($\bar{X} \pm$ SE)
5 min	10	27	0 a	0.0 ± 0.0
15 min	4	46	19 b	28.4 ± 2.1
60 min	4	116	48 b	29.0 ± 3.6
24 hr	16	924	752 c	38.7 ± 7.7

[a] Ratios of eggs (control:treated) followed by different letters are significantly different from each other by analysis of 2 × 2 contingency tables ($P < 0.05$).

TABLE 3. EFFECTS OF COUMARIN AND RUTIN (0.1 M) ON PRE- AND POSTALIGHTING BEHAVIOR OF *Pieris rapae*

			Contacts		Eggs/contact	
Assay	Treatment	Trials	Control	Treated	Control	Treated
Choice	Coumarin	10	75	35[c]	0.21	0.14
Choice	Rutin	10	25	4[c]	0.76	0.51
No-choice[a]	Coumarin	10	42	14[c]	0.31	0.00[d]
No-choice[a]	Rutin	10	76	14[c]	0.36	0.00[d]
No-choice[b]	Rutin	4	142	61[c]	0.41	0.39

[a] 5 min.
[b] 60 min.
[c] $P < 0.001$ by G tests on pooled data.
[d] $P < 0.05$ by analysis of 2×2 contingency tables.

to oviposit on treated plants than on control plants (Table 3). However, the effects of coumarin and rutin on postalighting behavior were small compared to the prealighting effects.

DISCUSSION

The results of this study support the view that host acceptance by insect herbivores is mediated by positive and negative stimuli from plants and by internal levels of satiation (Dethier, 1982). Both coumarin and rutin deterred oviposition by *P. rapae*, yet females laid some eggs on treated plants in nearly all experiments. These results suggest that the cabbage plants provided strong positive stimuli, whereas the applied compounds were negative stimuli that reduced the acceptability of treated cabbage plants significantly, but not completely.

In no-choice tests, acceptance of rutin-treated plants relative to control plants increased with trial duration. It is unlikely that the reduced deterrence of rutin seen in prolonged no-choice tests was due to rutin concentration declining through time. Rutin's deterrence remained effective in choice tests lasting 2–3 days (Table 1) and plants treated with rutin 24 hr before testing did not show reduced deterrence compared to plants treated with rutin immediately before testing. Another possibility is that the control plants became less acceptable as the number of eggs per control plant increased in no-choice tests of increasing duration. Egg-load assessment is reported to ocur for some *P. rapae* populations (Rothschild and Schoonhoven, 1977) but not others (Ives, 1978; Traynier, 1979; Shapiro, 1981). Results from a no-choice test in the present study show that the rate of oviposition on a clean control plant (9.3 eggs laid/female/hr) was not greater than the rate of oviposition on a control plant with 56 eggs (10.0 eggs

laid/female/hr), suggesting that acceptance of control plants in no-choice tests did not decline due to egg-load assessment. It seems that increased acceptance of rutin-treated plants occurred because females were deprived of the opportunity to oviposit on untreated plants. This type of behavior is analogous to increased acceptance of nonpreferred host-plant species when preferred species are unavailable (e.g., Singer, 1982, 1983; Tabashnik, 1983a, b; Tabashnik et al., 1985).

Time available for oviposition may be a limiting factor for butterflies like *P. rapae*, which lay their eggs singly (Tabashnik 1983a). Thus, single egg-layers may accept nonpreferred plants for oviposition more readily than cluster egg-layers. The results show that during 5-min trials, *P. rapae* consistently rejected treated plants, while consistently accepting untreated plants (Table 2). During trials of 15 min or longer, however, females accepted treated plants, indicating that the "discrimination phase" (Singer, 1982) for *P. rapae* in these experiments was between 5 and 15 min. These results are consistent with earlier findings (Tabashnik, 1983a) supporting the hypothesis that single egg-laying butterflies have relatively short discrimination phases.

Analysis of pre- and postalighting behavior revealed that the compounds reduced oviposition primarily by lowering the rate at which females contacted plants, indicating that deterrence was mediated by noncontact cues. These observations conflict with the notion that host-plant chemistry is most important in determining postalighting rather than prealighting behavior. However, secondary compounds were applied topically in this study, and thus their impact on behavior may not mimic the effects of compounds incorporated in leaves. The volatile compound, coumarin, probably deterred oviposition by alteration of olfactory cues. The noncontact effects of the nonvolatile pigment, rutin, were probably mediated by vision.

The results suggest that plants producing a secondary compound typical of nonhosts might gain some protection from specialist herbivores by reducing their acceptance to ovipositing females. If the observed responses to coumarin and rutin by *P. rapae* and *Plutella xylostella* (Tabashnik, 1985) are indicative of general trends, then this protection would only be partial—even when a compound was produced in relatively high quantities. If the compound reduced the plant's suitability, selection would be expected to increase discrimination against it. Alternatively, if the compound had a neutral or positive effect on suitability, selection would act to reduce the deterrent effect of the compound.

The results imply that nonhost-plant secondary compounds might confer partial crop protection by deterring oviposition. Due to increasing acceptance of treated plants when untreated plants are absent, treatments would be most effective when some plants were left untreated as a "trap crop." Application of plant secondary compounds to protect crops poses many of the same problems and hazards as do conventional pesticides, yet this technique offers potential as a tool to be integrated with other pest control measures (Schoonhoven,

1982). Attempts to deter oviposition by mobile adults may be more fruitful than tactics aimed at reducing consumption by immatures.

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SEPARATION AND IDENTIFICATION OF PHENOLIC ACIDS AND RELATED COMPOUNDS BY GAS CHROMATOGRAPHY AND FOURIER TRANSFORM INFRARED SPECTROSCOPY[1]

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Abstract—Phenolic acids and related compounds were separated by gas chromatography using three separate columns. One of these columns was coupled to a Fourier transform infrared spectrometer. The trimethylsilyl derivatives could be separated and identified by comparing the relative retention times of the three different columns. However, where there was overlap, the accompanying infrared data clearly distinguished between the questionable derivatives, thus enabling characterization of all derivatives.

Key Words—Phenolic acids, gas chromatography, Fourier transform spectroscopy.

INTRODUCTION

Phenolic acids and related compounds constitute one of the largest groups of secondary metabolites in plants. They are found as esters or as glycosides when combined with sugar and are water soluble and easily leached from plant tissue. These compounds also have been shown to be allelopathic to plant growth (Rice, 1974; Horsley, 1977). Humic acids, produced by oxidation of phenolic acids, increase the production of the superoxide radical O_2^-. This radical is involved in the destruction of cell membranes and is thus highly toxic to biological systems (Vaughan and Ord, 1982). Phenolic acid compounds also have been found

[1] The use of trade, firm, or corporation names in this article is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture or the Forest Service of any product or service to the exclusion of others that may be suitable.

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to be inhibitory to certain mycorrhizal fungi that are essential for the establishment and growth of trees on harsh environments such as surface mined areas (Melhuish and Wade, 1985; Schramm, 1966). Therefore, a relatively quick and easy technique for separating and identifying phenolic acids and related compounds would aid in studying their role in vegetational systems.

Gas chromatography is a common and relatively inexpensive method used to separate organic compounds. The separation of phenolic compounds by gas chromatography, using either one or two columns, has been reported (Vande Casteele et al., 1976; Dallos and Koeppl, 1969; Horvat and Senter, 1980). In gas chromatography, however, two or more compounds may have similar retention times on a given column. This peak overlap makes qualitative and quantitative analysis of any compound difficult. It would be advantageous to use more than one column because compounds that are not resolved on one column may be resolved on a column of different chemical composition (Patterson, 1971). If the information obtained from multicolumn gas chromatography can be supplemented by the additional information given by coupled Fourier transform infrared spectroscopy (FTIR), a technique not previously used in these studies, then the indentification of these phenolic compounds can be produced more quickly and accurately than by one or two columns alone. This paper reports on the results of using such a coupling technique.

METHODS AND MATERIALS

Instrumentation. Gas chromatographic analysis was performed using two separate gas chromatographs: a Beckman GC-65 with Beckman 10-in. recorder and Spectra-Physics intergrator, and a Varian 3700 with Varian 9176 recorder and CDS 111 integrator. Two columns were used in the Beckman GC: (1) glass (1.8 m × 6.0 mm OD) with Gas Chrom WHP (80–100 mesh) packed stationary phase and 3% SE-52 liquid phase and (2) stainless steel (4.6 m × 9.6 mm OD) with Gas Chrom Q (100–120 mesh) packed stationary phase and 1% SE-30 liquid phase. One column was used on the Varian GC: stainless steel (2.0 m × 3.2 mm OD) packed with Gas Chrom WHP (80–100 mesh) stationary phase and 3% OV-17 liquid phase. Both the Beckman and Varian instruments were equipped with flame ionization detectors.

Infrared analysis was performed using the Varian 3700 GC described above with an OV-17 column and thermal conductivity detector attached to a Digilab FTS-15C Fourier transform infrared spectrometer. The data were handled using a Data General Nova 3 computer.

Materials. The 25 phenolic and organic compounds analyzed were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin), Sigma Chemical Co. (St. Louis, Missouri), Eastman Kodak Co. (Rochester, New York), and ICN Pharmaceuticals (Cleveland, Ohio). The derivatizing agent was Tri-Sil/

BSA in dimethylformamide manufactured by Pierce Chemical Co. (Rockford, Illinios).

Procedure. Trimethylsilyl (TMS) derivatives of the compounds were formulated using Tri-Sil/BSA because of its one-step simplicity. The derivatizing procedure was followed as described by the manufacturer; 8 mg of the pure compound was heated at 67°C for 8 min to ensure complete silylation. The sensitive nature of some of the compounds required extra care to minimize light and moisture exposure.

For gas chromatography, operating conditions were maintained as similar as possible on all columns. The hydrogen and air flow rates were the same on both instruments—30 cc/min and 300 cc/min, respectively. The helium flow rates were slightly different by column: 20 cc/min (SE-52), 24 cc/min (OV-17), and 30 cc/min (SE-30). The injector and detector temperatures were also slightly different on the two instruments, 230°C and 280°C for the Beckman injector and detector temperatures, and 220°C and 250°C for the Varian injector and detector temperatures. The programmed column temperatures were identical for all columns. The initial temperature of 155°C was held for 2 min, then programmed to rise at 5°C/min to a final temperature of 265°C. One-microliter preparations were injected into the OV-17 and SE-52 columns, and 5-μl injections were used in the SE-30 column because of lack of sensitivity. All derivatives were analyzed three times and standard deviations were calculated.

The complete set of 25 derivatives was analyzed individually by GC-IR to give identifying spectra. A thermal conductivity detector was used in the GC and had a temperature of 250°C, a filament temperature of 310°C, and a filament current of 210 mA. The carrier gas flow rate, injector temperature, and column temperature program were the same as those in the gas chromatographic analysis for the OV-17 column. However, because of the less sensitive nature of the FTIRs liquid nitrogen-cooled, mercury–cadmium telluride photodetector (MCT), aliquots of 7 μl were injected. The FTIRs parameters were set to a resolution of 8, a sensitivity of 16, and a scan range of 4000 cm^{-1} to 650 cm^{-1}. Upon elution from the GC, the FTIR was manually triggered, and 10 scans were taken and averaged to give the spectra. Each derivative was run three times.

RESULTS

Triplicate runs of each derivative on each column resulted in the mean relative retention times given in Table 1. The relative retention times are relative to benzoic acid, which had actual retention times of 0.78 min (OV-17), 1.17 min (SE-52), and 2.55 min (SE-30). The standard deviations of all retention times were between 0.01 and 0.05 min. The longest actual analysis time was 50.57 min for chlorogenic acid on the SE-30 column.

TABLE 1. GAS CHROMATOGRAPHIC RELATIVE RETENTION TIMES OF TMS DERIVATIVES BY COLUMN RELATIVE TO BENZOIC ACID

TMS derivatives	OV-17	SE-52	SE-30
Benzoic acid	1.00	1.00	1.00
Catechol	1.04	1.40	1.35
2-Hydroxybenzyl alcohol	1.55	2.19	1.85
Salicylic acid	2.50	2.95	2.24
8-Hydroxyquinoline	3.54	3.38	2.29
trans-Cinnamic acid	3.59	3.42	2.46
Phloroglucinol	3.64	4.86	3.28
4-Hydroxybenzoic acid	3.81	4.76	3.24
Vanillin	3.82	3.34	2.32
Coumarin	4.05	2.94	1.94
3,4-Dimethoxybenzyl alcohol	4.32	4.08	2.63
Vanillic acid	6.81	6.85	4.17
Photocatechuic acid	7.04	7.91	4.76
alpha-Resorcylic acid	7.29	7.97	4.74
Guaiacol glycerol ether	8.24	7.73	4.68
2,3,4-Trihydroxybenzoic acid	8.68	9.55	5.57
Gallic acid	9.92	10.78	5.99
4-Hydroxycinnamic acid	10.38	9.64	5.61
Syringic acid	10.46	9.09	5.27
4-Hydroxyphenylpyruvic acid	12.04	11.87	6.64
Caffeic acid	14.03	13.26	7.36
Ferulic acid	14.42	12.28	6.76
Chrysophanic acid	26.63	20.76	10.61
Emodin	30.67	25.35	13.71
Chlorogenic acid	33.09	33.63	19.83

The FTIR with GC attachment enabled the vaporized derivatives to be scanned directly from the GC at a rate of 4 scans/sec for 2.5 sec. The computing and averaging of these scans was done by the Data General computer. The major infrared peaks are listed in Table 2 in units of wavenumbers (cm^{-1}). These values are within the operating error of four wavenumbers for the Digilab FTS-15C instrument.

DISCUSSION

The analysis of the gas chromatographic data revealed some general trends in the retention times of the phenolic derivates. With all three columns, and especially the two SE columns, the retention times were generally linear with molecular weight, that is, the compounds with the lowest molecular weight eluted first. The exception is that the more polar compounds, such as the al-

TABLE 2. INFRARED PEAK DATA OF TMS DERIVATIVES IN WAVENUMBERS (cm^{-1})[a]

TMS derivatives	COOTMS	$SiOC_6H_5$	$Si(CH_3)_3$	$Si(CH_3)_3$
Benzoic acid	1721s	—	*	856s
Catechol	—	934s	*	849s
2-Hydroxybenzyl alcohol	—	934s	1258s	849s
Salicylic acid	1721m	926s	1250s	856s
8-Hydroxyquinoline	—	918m	1258m-sh	864s
trans-Cinnamic acid	1713s	—	1265m-sh	856s
Phloroglucinol	—	*	1265m	864s
4-Hydroxybenzoic acid	1713s	918ms	1250w	856s
Vanillin	1713s	910s	1258vw	849s
Coumarin	1775vs	926w	1265w	826mw
3,4-Dimethoxybenzyl alcohol	—	—	1265s	849s
Vanillic acid	1713mw	903s	1258vw	864s
Photocatechuic acid	1713m	918s	1265m	856s
alpha-Resorcyclic acid	1721m	*	1250s	856s
Guaiacol glycerol ether	—	—	1258s	849s
2,3,4-Trihydroxybenzoic acid	1713m	*	1265s	856s
Gallic acid	1713m	895m	1258s	856s
4-Hycroxycinnamic acid	1713m	926m	1265s	856s
Syringic acid	1713m	918m	1258m	864s
4-Hydroxyphenylpyruvic acid	1705m	926m	1258m	856s
Caffeic acid	1713m	926m	1265s-sh	856s
Ferulic acid	1713m	910w	1265s	856s
Chrysophanic acid	1728w	895w	1258vw	856w
Emodin	1728w	926vw	1258vw	856w
Chlorogenic acid	1728w	926vw	1258vw	856w

[a]vs = very strong, s = strong, m = medium, w = weak, sh = shoulder, — = no peak, * = peak not resolved from adjacent peaks.

cohols, had the lowest retention times on the SE-30 column. This is expected with a nonpolar column and derivatives that are of intermediate polarity. In fact, this intermediate polarity caused the OV-17 and SE-52 columns to separate the phenolic TMS derivatives more efficiently than the nonpolar SE-30 column. The relative retention times of the derivatives also tended to follow the order: (lowest to highest retention times) benzoics, phenols, coumarins, cinnamics, and anthraquinones. Also because of the placement of substitution on the aromatic ring, there was a trend for the retention time to increase in order of ortho, meta, and para substitution. Other trends in retention times involve the number and type of substituent. Nonsubstituted compounds eluted first followed by mono-, di-, and tri-substituted ones. The type and number of functional groups tended to alter the retention time with 2 OCH_3, 1 OH + 1 OCH_3, and 2 OH eluting in increasingly longer times, respectively. This latter relationship occurs

because the greater the number of OH groups attached to the ring the greater the amount of derivatization at these sites. The TMS radical is much heavier than the OCH_3 radical, resulting in longer retention times.

FTIR data peak assignments for the silylated compounds were in accordance with other researchers (Vande Casteele et al., 1976). The only deviation from normal peak assignment came in the carbonyl peak region where the values were 20 cm^{-1} to 30 cm^{-1} higher than previously reported values. This increase in wavenumber, due to reduced hydrogen bonding at the carbonyl site, occurred because the compounds were in the vapor phase, a result of GC-IR coupling, instead of in the solid or liquid phase. A 20–30 wavenumber shift is normal for the hydrogen bonding effect (Bellamy, 1975).

Each of the three GC columns generally gave good separation of the compounds tested. Tentative identification of most of the reference compounds could be made by using the three relative retention times for each compound. An example of this is the derivatives of 4-hydroxybenzoic acid and vanillin. On the OV-17 column, the relative retention time difference was very close (0.01 min), whereas the difference on the SE-52 column was 1.42 min and on the SE-30 column 0.92 min. The OV-17 column did not separate the two derivatives, but by injecting the derivatives into each of three columns, both separation and tentative identification were performed. There were some compounds with GC retention times so close that, regardless of the column used, further information was needed for identification. Examples of this are the derivatives of 8-hydroxyquinoline and *trans*-cinnamic acid. The greatest difference in the relative retention times was 0.17 min on the SE-30 column. Although this difference is larger than the 0.01- to 0.05-min standard deviation for the column, identification was unambiguous when the FTIR spectra were considered. 8-Hydroxyquinoline lacked a carbonyl peak in the 1600 cm^{-1} to 1800 cm^{-1} region, whereas *trans*-cinnamic acid showed a strong peak at 1713 cm^{-1}. Use of FTIR data in conjunction with GC data enabled us to separate and identify all the standards we used.

Because of the importance for the study of phenolic acids and related compounds in different biological disciplines, quick and accurate methods for separating and identifying these acids are needed. Although NMR and/or MS would be desirable to more exactly establish the identity of these compounds, our techniques may be a useful substitute for those laboratories not having the more expensive equipment.

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SYNTHESIS OF RACEMATE AND ENANTIOMERS OF 15-METHYLTRITRIACONTANE, SEX-STIMULANT PHEROMONE OF STABLE FLY *Stomoxys calcitrans* L.

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Abstract—The synthesis of the racemate and two enantiomers of 15-methyltritriacontane (**1**), an active component of the sex-stimulant pheromone of *Stomoxys calcitrans* L., is described. Racemic 15-methyltritriacontane was synthesized in four steps from 1-hexadecene with a 72% overall yield. Both the *R*- and *S*-enantiomers were synthesized in eight steps, respectively, starting from optically pure (*R*)-(+)-pulegone.

Key Words—Stable fly, *Stomoxys calcitrans*, Diptera, Muscidae, sex-stimulant pheromone, methyl-branched hydrocarbon, 15-methyltritriacontane, pulegone, enantiomer.

INTRODUCTION

A number of methyl-branched hydrocarbons have recently been isolated from cuticular lipids of various fly species (Nelson, 1978; Carlson et al., 1984). Several hydrocarbons, selected as targets, have been synthesized as racemates, meso forms, or enantiomers, and their biological activities evaluated. It is of interest to note that only the meso form of 17,21-dimethylheptatriacontane, a component of the contact sex pheromone of *Glossina morsitans morsitans*, showed a pheromone activity (Ade et al., 1980; Helmchen and Langley, 1981), while all of the four stereoisomers of 15,19,23-trimethylheptatriacontane were equally active on the fly species (Helmchen and Langley, 1981). An active principle of the sex-stimulant pheromone of the stable fly, *Stomoxys calcitrans* L., 15-methyltritriacontane (**1**), was synthesized either as a racemate by Sonnet et al. (1977) or as two enantiomers by Sonnet (1984). There was no description

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of the overall yield of the synthesized racemate or of the optical activity of the two synthesized enantiomers.

A matter of interest is the pheromone activities for the stereoisomers of methyl-branched hydrocarbons such as **1** (Scheme 1) in terms of the relationship

$$n\text{-}C_{14}H_{29}\text{CH=CH}_2 \longrightarrow n\text{-}C_{14}H_{29}\text{CH(CH}_3\text{)OH} \longrightarrow n\text{-}C_{14}H_{29}\text{C(CH}_3\text{)=O}$$

1-hexadecene 2 3 4

$$\xrightarrow{n\text{-}C_{18}H_{37}P^{+}Ph_3Br^{-}} n\text{-}C_{14}H_{29}\text{C(CH}_3\text{)=CH-}n\text{-}C_{17}H_{35}$$

5

$$\xrightarrow{H_2\ /\ Pd\text{-}C} n\text{-}C_{14}H_{29}\text{CH(CH}_3\text{)-}n\text{-}C_{18}H_{37}$$

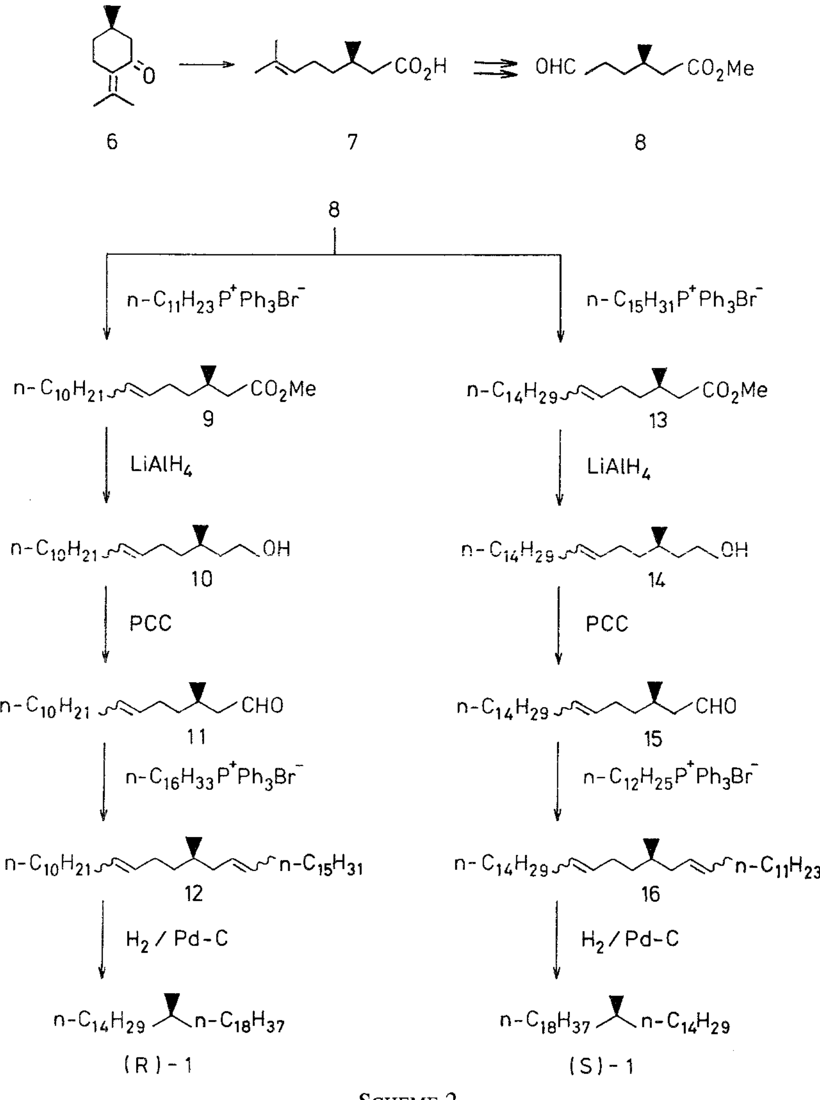
racemic 1

SCHEME 1.

between absolute configuration and pheromone activity. Our synthetic approach is based on the Wittig reaction aimed at the synthesis of racemic **1** and the *R*- and *S*-enantiomers of **1** and includes the use of 1-hexadecene (**2**) and optically pure (*R*)-(+)-pulegone (**6**), respectively, as the starting materials (Scheme 2).

METHODS AND MATERIALS

Column chromatography was carried out employing 70–230 mesh silica gel (Merck Kieselgel 60 Art 7734). All solvent systems were expressed in ratios by volume (v/v). IR spectra were determined on a Hitachi model 270-30 spectrometer. [^{1}H]- and [^{13}C]NMR spectra were obtained on a Hitachi model R-24B spectrometer and on a Hitachi model R-90H Fourier transform NMR spectrometer, respectively, in $CDCl_3$ solutions using Me_4Si as an internal standard. EI and CI mass spectra were recorded on a JEOL model JMS-D300 double-focusing mass spectrometer at 70 eV and 200 eV, respectively, using a direct insertion probe. Optical rotations were determined on a JASCO model DIP-140 polarimeter. GLC analysis for the determination of the purity of the products was made isothermally at 250°C (N_2, 60 ml/min), employing a Hitachi model 163 gas chromatograph equipped with a 1-m × 3-mm glass column of 3% OV-1 on 80–100 mesh Chromosorb W AW DMCS. HPLC was performed on a Gasukuro Kogyo model 572 liquid chromatograph equipped with a UV detector. A Unisil Q 100-5 4.6-mm × 25-cm column was used with *n*-hexane–THF



SCHEME 2.

(10:1, 0.5 ml/min) as solvent. Solvents were purified and dried by standard methods.

15-Methyl-15-tritriacontene (5). Octadecyltriphenylphosphonium bromide, which was freshly prepared from triphenylphosphine (2.2 g, 8.4 mmol) and 1-bromooctadecane (2.7 g, 8 mmol), was dissolved in dry 1,2-dimethoxyethane (50 ml). The cooled solution was stirred while 5.2 ml of 1.6N *n*-BuLi in hexane (Aldrich) was added dropwise, and the mixture allowed to stir for 1

hr at room temperature. After the resulting phosphorus ylid solution had been cooled to $-30°C$, a solution of **4** (1 g, 4.2 mmol) in dry 1,2-dimethoxyethane (15 ml) was added dropwise over a period of 20 min. The reaction mixture was allowed to warm gradually to room temperature with stirring, and stirring was continued for 5 hr at 40°C. The mixture was quenched with a saturated NH_4Cl solution under ice-cooling, diluted with water, and extracted with ether. The aqueous layer was saturated with NaCl and then extracted with additional ether. The combined ethereal solutions were washed with water and brine and dried over Na_2SO_4. After evaporation of the solvent, the semicrystalline residue was triturated with *n*-hexane. The solid thus obtained was removed by filtration, and the filtrate was concentrated in vacuo. The resulting slightly yellow liquid was purified by column chromatography on silica gel (100 g) with *n*-hexane followed by removal of the low boiling impurities in vacuo giving **5** as a colorless viscous liquid (1.6 g, 81.4% from **4**), which solidified during storage in a refrigerator. IR (neat): 2930, 2850, 1470, 1380, 720 cm^{-1}. [^{1}H]NMR δ: 0.88 (distorted t, 6H, J = 5 Hz), 1.27 (broad s, 48H), 1.6 (m, 6H), 1.75–2.25 (m, 7H), 5.17 (t, 1H, J = 5 Hz). EI-MS *m/z:* 476 (M^+, 3), 294 (7), 279 (5), 57 (100). CI-MS (isobutane) *m/z:* 477 [$(M + H)^+$, 100].

(R,S)-15-Methyltritriacontane [(R,S)-1]. Hydrogenation of **5** (1.5 g, 3.15 mmol) in a mixture of ethanol (10 ml) and *n*-hexane (10 ml) was carried out in the presence of 10% Pd/C catalyst (0.15 g). After hydrogenation had been completed, the catalyst was filtered off and washed with *n*-hexane. The filtrate and washings were combined and concentrated in vacuo, and the slightly yellow residue was then dissolved in ether. The ethereal solution was washed with brine and dried over Na_2SO_4, and the solvent was evaporated in vacuo to obtain a crude semisolid of racemic **1.** The solid was purified by column chromatography on silica gel (10 g) with *n*-hexane giving (*R,S*)-**1** as a white solid (1.41 g, 94%), which was recrystallized from *n*-hexane: mp 37.5–38°C. IR (KBr): 2925, 2850, 1475, 1380, 720 cm^{-1}. [^{1}H]NMR δ: 0.8–1.0 (m, 9H), 1.25 (broad s, 61H). EI-MS *m/z:* 463 (M-15, 1), 281 (M-$C_{14}H_{29}$, 8), 225 (M-$C_{18}H_{37}$, 11), 57 (100). CI-MS (isobutane) *m/z:* 477 [$(M-H)^+$, 100]. GLC: R_t 16 min (99%), impurity at 10 min (1%). Analysis: Calcd. for $C_{34}H_{70}$: C, 85.27; H, 14.73. Found: C, 84.97; H, 15.06.

Methyl (3R)-6-oxo-3-methylhexanoate (8). Compound **8** was synthesized from (*R*)-pulegone with a 36% yield by the procedures described previously: bp 59–60°C at 0.4 mm Hg; $[\alpha]_D^{20}$ + 5.24° (neat, d[d:]$_4^{20}$ 0.9747) {$[\alpha]_D^{20}$ + 5.00° ± 0.06° (neat), Jackman and Lange, 1983}. IR (neat): 2740, 1735–1745 cm^{-1}. [^{1}H]NMR δ: 0.95 (d, 3H, J = 6 Hz), 3.67 (s, 3H), 9.77 (t, 1H, J = 1.5 Hz). [^{13}C]NMR δ: 19.452, 28.507, 29.849, 41.222, 41.435, 51.375, 172.877, 201.720.

Methyl (R)-3-methyl-6-heptadecenoate (9). Undecyltriphenylphosphonium bromide, which was freshly prepared from triphenylphosphine (4.4 g, 16.8 mmol) and 1-bromoundecane (3.8 g, 16 mmol), was dissolved in dry 1,2-di-

methoxyethane (60 ml) and treated with 10 ml of 1.6N *n*-BuLi in hexane (Aldrich). After the resulting ylid solution had been cooled to −50°C, a solution of **8** (1.33 g, 8.4 mmol) in dry 1,2-dimethoxyethane (20 ml) was added dropwise over a 30-min period. The reaction mixture was allowed to warm slowly to room temperature and left stirring for 4 hr. The mixture was worked up to obtain a pale yellow viscous residue. The residue was purified by column chromatography on silica gel (150 g) with *n*-hexane–ether (30:1) to give a colorless liquid of **9** (2.3 g, 92% from **8**): bp 135–137°C at 0.4 mm Hg. IR (neat): 2930, 2870, 1750, 1205, 1160, 1010 cm^{-1}. [^{1}H]NMR δ: 0.9 (apparent d, 6H, J = 6.5 Hz), 1.25 (broad s, 19H), 1.65–2.37 (m, 6H), 3.6 (s, 3H), 5.27 (t, 2H, J = 5 Hz). [^{13}C]NMR δ: 14.086, 19.604, 22.653, 24.635, 27.227, 29.331, 29.544, 29.636, 29.727, 30.032, 31.892, 36.709, 41.527, 51.222, 129.063, 130.161, 173.365. EI-MS *m/z:* 296 (M^+, 4), 264 (M-CH_3OH, 21), 222 (73), 68 (100). CI-MS (isobutane) *m/z:* 297 [$(M + H)^+$, 100].

*Methyl (*R*)-3-methyl-6-heneicosenate (13).* According to the procedure described above, compound **8** (1 g, 6.3 mmol) was condensed with a Wittig reagent which was prepared from pentadecyltriphenylphosphonium bromide and *n*-BuLi. Column chromatography on silica gel (120 g), with *n*-hexane–ether (30:1), of the product gave **13** as a colorless liquid (2.17 g, 98% from **8**): bp 152–155°C at 0.3 mm Hg. The IR and NMR spectra were identical with those reported (Kuwahara and Mori, 1983).

*(*R*)-3-Methyl-6-heptadecen-1-ol (10).* A solution of **9** (1.56 g, 5.27 mmol) in dry ether (5 ml) was added dropwise to a stirred and cooled suspension of lithium aluminum hydride (0.2 g, 5.26 mmol) in dry ether (10 ml). The mixture was stirred for 2 hr at room temperature. Work-up of the reaction mixture gave a slightly yellow liquid, which was purified by column chromatography on silica gel (20 g) with *n*-hexane–ether (5:1) to give **10** as a colorless liquid (1.36 g, 96%). IR (neat): 3350, 2950, 2870, 1475, 1380, 1070, 720 cm^{-1}. [^{1}H]NMR δ: 0.91 (apparent d, 6H, J = 6 Hz), 1.26 (broad s, 21H), 1.75–2.35 (m, 5H), 3.67 (t, 2H, J = 6 Hz), 5.25 (t, 2H, J = 5 Hz). [^{13}C]NMR δ: 14.086, 19.543, 22.684, 24.696, 27.257, 29.331, 29.636, 30.794, 31.922, 37.167, 39.911, 61.071, 129.551, 129.917. EI-MS *m/z:* 250 (M-H_2O, 4), 80 (100). CI-MS (isobutane) *m/z:* 266 [$(M + H)^+$, 100].

*(*R*)-3-Methyl-6-heneicosen-1-ol (14).* As described for **9**, compound **13** (2.17 g, 6.2 mmol) was reduced with lithium aluminum hydride (0.24 g, 6.2 mmol). Column chromatography on silica gel (20 g) with *n*-hexane–ether (5:1) gave **14** as a colorless liquid (1.95 g, 97%). IR (neat): 3350, 2940, 2850, 1470, 1380, 1060, 720 cm^{-1}. [^{1}H]NMR δ: 0.90 (apparent d, 6H, J = 6 Hz), 1.25 (broad s, 29H), 1.8–2.3 (m, 5H), 3.62 (t, 2H, J = 6 Hz), 5.25 (t, 2H, J = 5 Hz). [^{13}C]NMR δ: 14.086, 19.513, 22.684, 24.666, 27.227, 29.239, 29.331, 29.666, 31.892, 37.136, 39.911, 61.101, 129.520, 129.886. EI-MS *m/z:* 306 (M-H_2O, 5), 81 (100). CI-MS (isobutane) *m/z:* 325 [$(M + H)^+$, 100].

*(*R*)-3-Methyl-6-heptadecenal (11).* A solution of **10** (1.35 g, 5 mmol) in

dry dichloromethane (5 ml) was added dropwise to a stirred and cooled suspension of pyridinium chlorochromate (2.44 g, 11.3 mmol) in dry dichloromethane (20 ml). The mixture was allowed to stir for 1 hr at room temperature. Work-up of the reaction mixture gave a yellow liquid, which was purified by column chromatography on silica gel (10 g) with *n*-hexane–ether (30 : 1) to give **13** as a colorless liquid (1.14 g, 85%). IR (neat): 2945, 2855, 2710, 1730, 1470, 1380, 720 cm^{-1}. [^{1}H]NMR δ: 0.8–1.02 (m, 6H), 1.25 (broad s, 18H), 1.8–2.35 (m, 7H), 5.3 (t, 2H, J = 5 Hz), 9.67 (t, 1H, J = 1.5 Hz).

(R)-3-Methyl-6-heneicosenal (15). As described for **10**, compound **14** (1.6 g, 4.9 mmol) was oxidized with pyridinium chlorochromate (2.44 g, 11 mmol). Column chromatography on silica gel (15 g) with *n*-hexane–ether (30 : 1) gave **15** as a colorless liquid (1.35 g, 85%). IR (neat): 2950, 2860, 2710, 1735, 1470, 1385, 720 cm^{-1}. [^{1}H]NMR δ: 0.85–1.0 (m, 6H), 1.25 (broad s, 28H), 1.75–2.4 (m, 7H), 5.28 (t, 2H, J = 5 Hz), 9.63 (t, 1H, J = 1.5 Hz).

(R)-15-Methyl-11,17-tritriacontadiene (12). Hexadecyltriphenylphosphonium bromide, which was freshly prepared from triphenylphosphine (2 g, 7.6 mmol) and 1-bromohexadecane (2.3 g, 7.15 mmol), was dissolved in dry 1,2-dimethoxyethane (40 ml) and treated with 5 ml of 1.6N *n*-BuLi in hexane (Aldrich). After the resulting ylid solution had been cooled to −30°C, a solution of **11** (1 g, 3.8 mmol) in dry 1,2-dimethoxyethane (15 ml) was added dropwise over a 20-min period. The reaction mixture was allowed to warm slowly to room temperature and left stirring for 4 hr. Work-up of the mixture gave a slightly yellow viscous liquid, which was purified by column chromatography on silica gel (100 g) with *n*-hexane followed by removal of the low-boiling impurities in vacuo giving **12** as a colorless liquid (1.47 g, 82% from **11**), which was solidified during storage in a refrigerator. IR spectral data indicated it to be a mixture of geometric isomers. IR (neat): 3020, 2930, 2850, 1655, 1470, 1380, 970 (very weak), 720 cm^{-1}. [^{1}H]NMR δ: 0.8–0.95 (m, 9H), 1.25 (broad s, 45H), 1.74–2.36 (m, 8H), 5.28 (m, 4H). EI-MS *m/z:* 474 (M^+, 2), 320 (3), 250 (5), 81 (100). GLC: R_t 14 min (98%), impurity at 10.5 min (2%).

(R)-15-Methyl-12,18-tritriacontadiene (16). According to the procedure described above, compound **15** (0.96 g, 3 mmol) was treated with a Wittig reagent which was prepared from dodecyltriphenylphosphonium bromide and *n*-BuLi. Column chromatography on silica gel (100 g) with *n*-hexane gave **16** as a colorless liquid (0.96 g, 70% from **15**), which solidified during storage in a refrigerator. IR spectral data showed a mixture of geometric isomers. IR (neat): 3020, 2950, 2865, 1660, 1470, 1380, 970 (very weak), 720 cm^{-1}. [^{1}H]NMR δ: 0.8–0.95 (m, 9H), 1.25 (broad s, 45H), 1.8–2.28 (m, 8H), 5.3 (m, 4H). EI-MS *m/z:* 474 (M^+, 2), 306 (4), 264 (6), 81 (100). GLC: R_t 13.8 min (98.5%), impurity at 9.2 min (1.5%).

(R)-15-Methyltritriacontane [(R)-1]. Hydrogenation of **12** (1 g, 2.1 mmol) in a mixture of ethanol (5 ml) and *n*-hexane (5 ml) was carried out in the pres-

ence of 10% Pd/C catalyst (0.1 g). Column chromatography on silica gel (10 g) with *n*-hexane gave (*R*)-**1** as a white solid (0.95 g, 95%), which was recrystallized from *n*-hexane: mp 46–46.5°C. GLC analysis showed the recrystallized sample to be 97.6% pure [R_t 15.8 min, impurities at 11 min (2%) and 11.8 min (0.4%)]. (*R*)-**1** was identical with racemic **1** in its spectral data. Analysis: Calcd. for $C_{34}H_{70}$: C, 85.27; H, 14.73. Found: C, 85.62; H, 14.92.

(S)-15-Methyltritriacontane [(S)-1]. As described for **12,** catalytic hydrogenation of **16** (0.87 g, 1.83 mmol) gave (*S*)-**1** as a white solid, with almost quantitative yield, which was recrystallized from *n*-hexane: mp 46.5–47°C. Spectra matched those of both racemic **1** and (*R*)-**1.** GLC: R_t 16 min (98.2%), impurities at 12 min (1.2%), 3 min (0.3%), and 1.5 min (0.3%). Analysis: Calcd. for $C_{34}H_{70}$: C, 85.27; H, 14.73. Found: C, 85.12; H, 14.85.

DISCUSSION

As shown in Scheme 1 for the synthesis of racemic 15-methyltritriacontane, inexpensive 1-hexadecene (**2**) was chosen for the starting material. The alkene **2** was easily converted to the known ketone **4** (Naoshima et al., 1986), which was subjected to a Wittig reaction with octadecyltriphenylphosphonium bromide and subsequently hydrogenated over palladium charcoal. Thus racemic pheromone (*R,S*)-**1** was synthesized in four steps from **2** with a 72% yield. GLC analysis of the synthesized racemate showed 99% chemical purity. Sonnet et al. (1977) reported a three-step synthesis for racemic **1** employing a Wittig reaction, although the overall yield was not given. Chemical purity of the racemate was reported as 97%.

Readily available (*R*)-(+)-pulegone (**6**), $[\alpha]_D^{20}$ + 22.33° (neat, $d[d:]_4^{20}$ 0.9373), was used for the synthesis of the two enantiomers of **1** as shown in Scheme 2. A basic feature in our synthetic design of the enantiomers is that the chiral center at C-15 comes from pulegone (**6**) which will lead to **1** through ring cleavage and Wittig olefination.

First, **6** was converted via (+)-citronellic acid (**7**), $[\alpha]_D^{20}$ + 8.39° (neat, $d[d:]_4^{20}$ 0.9255), to the formyl ester **8** by known procedures (Overberger and Weise, 1968; Jackman and Lange, 1983; Kuwahara and Mori, 1983). For the synthesis of (*R*)-**1,** the formyl ester **8** underwent a Wittig reaction with undecyltriphenylphosphonium bromide giving the olefinic ester **9** in a 92% yield. In the IR spectrum of **9,** a strong absorption in the region of 965 cm^{-1} was absent, consistent with the major product being the *Z*-isomer. The ester **9** was converted by reduction and subsequent oxidation to the aldehyde **11,** followed by Wittig olefination with hexadecyltriphenylphosphonium bromide to give the long-chain unsaturated hydrocarbon **12** in a 67% yield from **9.** Finally, catalytic hydrogenation of the diene **12** gave the pheromone (*R*)-**1** in a 95% yield and in a 21% overall yield from **6.**

Employing the same procedure described for the synthesis of (*R*)-**1** with the exception that pentadecyltriphenylphosphonium bromide and dodecyltriphenylphosphonium bromide were used for Wittig reaction, the pheromone (*S*)-**1** was synthesized in a 19.5% overall yield from **6.** Although we were unable to determine the optical purities of the two present enantiomers, the pheromones (*R*)- and (*S*)-**1** appeared to be enantiomerically pure, allowing the evaluation of biological activities within the limits of accuracy. The assumption of optical purity is based on the observation that the starting material, (*R*)-pulegone, was found to be highly enentiomerically pure, about 100%, by the HPLC analysis of diastereomeric amides derived from (+)-citronellic acid (**7**) and configurationally pure (*R*)-(+)-1-(1-naphthyl)ethylamine (Mori et al., 1981), and it was assumed that no racemization at the chiral center had occurred during the reaction sequence described above (Overberger and Weise, 1968; Mori and Kuwahara, 1982). It is reasonable that both (*R*)- and (*S*)-**1** exhibited no measurable optical rotation at the sodium D-line, since this finding was consistent with Brewster's empirical calculations relating to optical rotations (Brewster, 1959; for the optical activity of chiral hydrocarbons, see Wynberg et al., 1965; Hoeve and Wynberg, 1980). There was no description of the optical rotation for the two enantiomers synthesized by Sonnet (1984), while the configurational purity for both these enantiomers was determined by GLC analysis of precursors as diastereomeric amides.

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RESPONSES OF PARASITE *Edovum puttleri*[1] TO KAIROMONE FROM EGGS OF COLORADO POTATO BEETLE, *Leptinotarsa decemlineata*[2]

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Abstract—When females of the eulophid parasite *Edovum puttleri* were exposed to an *n*-hexane wash of eggs of the Colorado potato beetle, *Leptinotarsa decemlineata*, their responses indicated that the eggs contained a kairomone which elicited host-finding and host-acceptance behaviors. The host-finding responses included an increased amount of time searching glass beads treated with kairomone, and drumming on the beads with the flagellum of the antennae. Host-acceptance responses included grasping of the beads with the legs, raising of the abdomen, and probing the kairomone-treated surface with the ovipositor. None of these responses were noted in females on beads treated only with hexane, or in males exposed to kairomone-treated beads. When kairomone was applied to a flat surface, filter paper disks, few episodes of drumming and no episodes of probing with the ovipositor were noted. Responses of *E. puttleri* to eggs of Colorado potato beetle were similar to those on kairomone-treated beads, but females normally drilled into eggs and fed on host fluids after retracting the ovipositor. The kairomone is volatile, and responsiveness was reduced in parasites exposed to beads treated several hours previously.

Key Words—Kairomone, parasite, host finding, host acceptance, behavior, *Edovum puttleri*, Hymenoptera, Eulophidae, Colorado potato beetle, *Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae.

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INTRODUCTION

The importance of the role of kairomones as chemical cues to orient parasites to their prey has been demonstrated for an increasing number of species of parasitic Hymenoptera. Kairomones have been shown to function as stimuli to orient parasites to host habitats (reviewed by Vinson, 1981), to locate hosts within the habitat of the host (reviewed by Weseloh, 1981), and to facilitate discrimination of acceptable hosts (reviewed by Arthur, 1981). Herein, we describe host-finding and host-acceptance responses of *Edovum puttleri* Grissell (Hymenoptera: Eulophidae) to a kairomone extracted in *n*-hexane from eggs of Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae).

Edovum puttleri was described from specimens emerging from eggs of the chrysomelid *Leptinotarsa undecemlineata* (Stal) from Columbia, South America. Grissell (1981), in his description, noted the potential of *E. puttleri* as a possible biocontrol agent for the Colorado potato beetle. The host range of *E. puttleri* was examined by Puttler and Long (1983). Of 18 species of chrysomelid eggs exposed to *E. puttleri*, the favored host was the Colorado potato beetle. With the exception of a few eggs of *Labdometa clivicolis* (Kirby), no other species were parasitized.

One important factor that will influence the utilization of *E. puttleri* in biological control strategies is the inability of the parasite to overwinter in temperature areas of the United States (Shroder et al., 1985). Shroder and Athanas (1985) noted consistent performance of the parasite in the field over the past four years and suggested that *E. puttleri* can be an effective integral part of management systems for Colorado potato beetle control. The inability of the parasite to overwinter, however, will require rearing of sufficient numbers of adults for release during each field season.

The kairomone could enhance the use of *E. puttleri* as a biocontrol agent. Several authors (e.g., Lewis et al., 1975a,b; Gross et al., 1975) discuss the possibility of using kairomones to manipulate behavior and increase the effectiveness of parasites. The use of kairomones in situations requiring inundative releases of parasites was reviewed by Gross (1981). In addition to the potential importance of the kairomone in enhancing host finding and host acceptance in field conditions, the kairomone could have utility in the development of methods to facilitate mass rearing of *E. puttleri*.

METHODS AND MATERIALS

Extraction of Kairomone. Preliminary tests were conducted to determine if washes of Colorado potato beetle eggs with various solvents produced a response when presented to female *E. puttleri*. Of the initial solvents tested, dis-

tilled water, methanol, and *n*-hexane, females responded to the *n*-hexane wash of Colorado potato beetle eggs on filter paper by aggregating near and tapping the treated area with their antennae. These responses, however, were weaker than we had observed in previous experiences with kairomonal responses in other species of parasites. Strand and Vinson (1983), and our colleague, H.P. Lee (personal communication) demonstrated that size and shape of treated objects can be important in eliciting kairomonal responses in species of hymenopteran egg parasites. We found that 1-mm-diameter, round, clear glass beads treated with the hexane wash of eggs elicited strong kairomonal responses in *E. puttleri* females.

The *n*-hexane wash of Colorado potato beetle eggs was prepared from fresh egg masses from our colony reared on potato, *Solanum tuberosum*. One thousand eggs were removed from potato leaves, placed in a clean, glass vial, and covered with 2.5 ml of *n*-hexane. After 5 min, the hexane wash was decanted into a vial and stored at 0°C.

Parasite Culture. Edovum puttleri were obtained from the Mission Biological Control Laboratory, USDA, APHIS, PPQ, Edinburg, Texas. Parasites were received as pupae in parasitized eggs of *Leptinotarsa texana* (Schaeffer) and were held at 25°C and 16-hr photophase. On emergence, parasites were transferred to screened, glass jars and provided with distilled water and with honey. Males and females were kept together for mating. Parasites were provided fresh egg masses of Colorado potato beetle to obtain host-experienced females for bioassays, since previous ovipositional experience has been shown to influence responses in some parasites (Lenteren, 1976; Strand and Vinson, 1982).

Bioassays. The test arena was a culture microscope slide with a glass ring 3 mm high and 16 mm in diameter fused to a 76 × 25-mm glass slide. A piece of plastic screening with a mesh opening of 0.8 mm was cut with a cork borer to the inner diameter of the glass ring and placed on the bottom of the arena. Clear glass beads, selected for uniformity in size (1.0 mm) and roundness, were placed on the screened bottom of the test arena in a 5 × 5 bead array in the center of the arena. The screen bottom provided a uniform array of beads and prevented beads from touching. The beads were treated by placing 25 in a clean depression slide. The hexane wash was pipetted on the beads, and the beads were stirred with forceps to aid in coating each bead. After several minutes, the hexane had evaporated and the beads were transferred with forceps to the screened base of the test arena.

Bioassays were conducted in a room maintained at 25 ± 1°C. We found in preliminary tests, however, that parasites responded poorly on some days when exposed to our standard concentration of hexane wash. We have not examined the possible causes of this variation in responsiveness to kairomone, although it appears related to periods of inclement weather or to changes in weather patterns. To maintain as much uniformity as possible in our bioassays,

we first tested several female parasites with our standard concentration of kairomone; if few or no responses were noted, bioassays were not conducted on that day.

For the initial test to compare responses of *E. puttleri* to untreated and to kairomone-treated beads, the control beads were prepared by pipetting 5 μl of *n*-hexane over the beads. After several minutes, the beads were transferred to a test arena in a 5 $\times$ 5 bead array. The test beads received 5 μl of our standard concentration of hexane wash of Colorado potato beetle eggs as detailed above, and, after several minutes, the beads were transferred to the test arena. The arena was covered with a glass cover slip. A mated, host-experienced *E. puttleri* female, 4–5 days old, was placed in the control arena and allowed to acclimate for 1 min. The female was then observed for 5 min with a dissecting microscope illuminated from the microscope base. The number of times the female contacted (walked on) the beads, the time spent on the beads, and behaviors on the beads were recorded. Directly after the 5-min exposure to the control beads, the parasite was transferred on the cover slip to the arena containing treated beads. During the 5-min exposure to treated beads, the data recorded were as above. After exposure to the treated beads, the parasite was discarded, and another female was used. Both the control and test arenas were used only for 1 hr, after which females were exposed to newly prepared arenas.

Some females spent much time walking on the top and sides of the arena, and we wanted to compare differences in behavior of parasites on control and treated beads. Only females that had two or more episodes of walking over the control beads were transferred to the arena containing kairomone-treated beads. A total of 20 such females were tested.

The initial test demonstrated that host-finding and host-acceptance responses occurred with females exposed to kairomone-treated beads. We were interested in eliciting more pronounced responses, particularly host-acceptance responses, and ran preliminary tests to determine whether age of females or increasing the amount of the hexane wash of eggs would influence the level of response. One-day-old females showed no response to treated beads, whereas older females responded to kairomone-treated beads throughout their ovipositional period, which in our laboratory is ca. 30 days. By increasing the dosage and time of exposure, we could increase the incidence of probing responses on beads with the ovipositor. For subsequent tests we utilized host-experienced females of 11–12 days of age, increased the level of kairomone to 10 μl of hexane wash of eggs applied to 25 glass beads, and used an exposure time of 10 min. We also found that females were strongly attracted to white surfaces and that we could conduct our bioassays on a white surface with the light source (cool white, fluorescent) from above, rather than using the reflecting base of a microscope.

To determine whether the attractiveness to kairomone-treated beads declined over time, four arenas were prepared using 25 beads covered with 10 μl

of our standard concentration of hexane wash and were left uncovered. Each hour, starting with hour 1 after the beads were treated, 12 parasites from a cohort of 50 were tested individually. Each parasite was observed for antennal drumming or probing with the ovipositor on the beads. If probing with the ovipositor did not occur within the 10-min test period, the test was ended. Females were returned to the cohort, and the arenas were left uncovered until the next test period.

A reduction in response to kairomone-treated beads over time could be a function of beads exposed previously having been "marked" by previous females to deter other females from searching eggs. To test whether this occurred, an arena of 25 treated beads was prepared, and five 12-day-old, experienced females were placed singly into the arena for a period of 10 min each. Each female was observed and the course of movement over each bead was charted. When antennal drumming responses were noted, the time the female remained on the bead was recorded. After 10 min, the female was replaced with a new female, and data were collected as above. A total of five females were exposed to each arena, and a total of four replicates was run.

To test the responses of females to reduced concentrations of kairomone, 25 beads were treated with 10 μl of kairomone wash, beginning with the standard concentration and in serial dilutions to 6.2% of the standard concentration. The test arenas were set up as above, and a total of 20 *E. puttleri* from a cohort of ca. 100 females were tested for each concentration. Data recorded were antennal drumming and probing with the ovipositor on the treated beads. If probing with the ovipositor did not occur within 10 min, the test was ended.

RESULTS

The exposure of 4- to 5-day-old host-experienced, mated *E. puttleri* females to hexane-treated and subsequently to kairomone-treated glass beads showed about a 10-fold marked increase in the amount of time that females remained on the kairomone-treated beads (Table 1). Of the 20 females tested, two spent less time on the beads treated with kairomone and one spent about the same amount of time on the untreated and treated beads during the 5-min exposure periods. The remaining 17 females spent much more time on the beads coated with kairomone, and 12 females displayed one or more episodes of probing the beads with the ovipositor. Two females were engaged in drumming and probing at the end of the five-min test period, but the additional time spent on the beads is not included in the data.

Antennal drumming and the probing of the beads with the ovipositor were not observed on beads treated only with hexane. Females walked over the control beads rapidly, spending an average of 1.1 sec. on each bead. Normally, they traversed the beads from the one mounted in a line until they reached the

TABLE 1. RESULTS OF PAIRED TESTS OF 20 *E. puttleri* FEMALES EXPOSED FOR 5 MIN TO HEXANE-TREATED (CONTROL) GLASS BEADS AND IMMEDIATELY THEREAFTER TO BEADS TREATED WITH HEXANE WASH OF COLORADO POTATO BEETLE EGGS (KAIROMONE)[a]

	Time (sec) spent on beads			Total No. of probing episodes
	Mean	$SE\bar{X}$	Range	
Kairomone	132.6	19.0	5.2–288.3[b]	21
Control	11.9	2.7	6.1–53.5	0

[a] Probing with the ovipositor occurred after drumming of beads with the antennae, events observed only on kairomone-treated beads.
[b] Time spent on kairomone-treated beads significantly different from time on control beads at $P < 0.05$ using the Wilcoxin Matched-Pairs Signed-Ranked Test.

last bead and exited to the screened base of the arena. While traversing the beads, females would occasionally tap the surface of the beads with the antennae held at an ca. 45° angle to the surface of the beads.

Females responding to kairomone-treated beads did not traverse the beads as rapidly, spending an average of 6.8 sec on each bead. The antennae were brought downward to about a 90° angle and vibrated rapidly, drumming on the surface of the bead with the distal portion of the flagellum as the parasite slowly turned and searched the bead. These behaviors we consider to be host-finding responses.

Antennal drumming always preceded probing of the egg with the ovipositor, but not all episodes of drumming were followed by probing. When probing occurred, females grasped the bead, extended the meso- and metathoracic legs, raised the abdomen, released the ovipositor, and probed the kairomone-treated surface of the bead with the distal tip of the ovipositor. After probing the bead for ca. 30–60 sec, the abdomen was lowered and the ovipositor was ensheathed. These behaviors we consider to be host-acceptance responses. After completion of the probing episode, females remained on the bead for several seconds, then moved to an adjacent bead and repeated the host-finding repertoire or left the beads.

Males were also exposed to kairomone-treated and control beads, but none of the 10 males tested displayed any response to kairomone.

The response of females to beads treated with kairomone for up to 8 hr prior to exposure showed that both antennal drumming and the probing with the ovipositor declined with the age of the kairomone (Figure 1). The drumming response was seen in all females tested at hours 1 and 2, with a gradual decline in females responding until hours 6 through 8, when four of 12 females responded. With the probing response, which was always preceded by drumming,

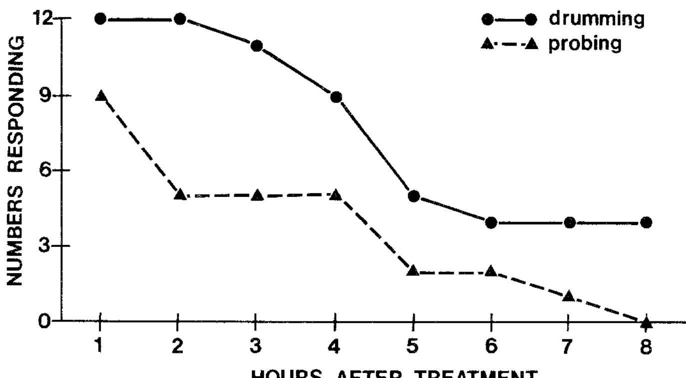

FIG. 1. Responses of female *E. puttleri* to beads treated with hexane wash of Colorado potato beetle eggs one to eight hours prior to exposure. Probing with the ovipositor was preceded by drumming on the bead with the antennal flagellum. Some females drummed, but did not probe with the ovipositor.

the highest numbers responding occurred 1 hr after beads were treated, followed by a drop in numbers responding. At hour 8, no probing with the ovipositor was noted; although four females drummed beads, the concentration of kairomone was apparently not sufficient to elicit the probing response. The reduction in attractiveness of the treated beads was not a function of "marking" of the beads with a substance to deter other females, for the test for marking showed that females spent a mean of 10.9 sec ($N = 45$ episodes, $SE\bar{X} = 0.91$) on beads not previously probed by females and 10.3 sec ($N = 53$ episodes, $SE\bar{X} = 0.66$) on beads previously probed by other females.

The data on responses of females to serial dilutions of kairomone (Figure 2) were similar to those in Figure 1. The first concentrations tested were 100%

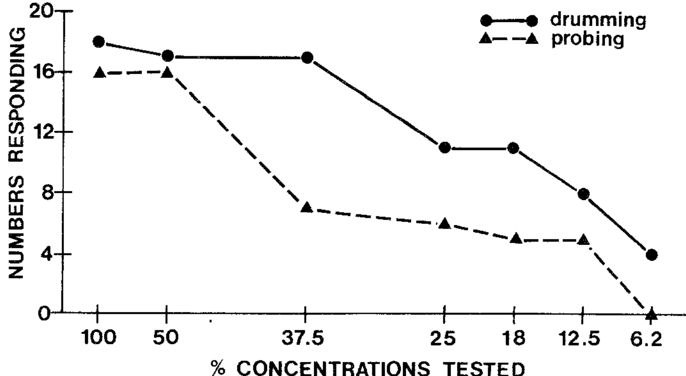

FIG. 2. Responses of female *E. puttleri* to serial dilutions of hexane wash of Colorado potato beetle eggs. The 100% concentration was the standard test concentration of 1000 eggs washed with 2.5 ml of *n*-hexane, with 10 μl applied to 25 glass beads.

(standard), 50%, and 25% of the standard concentration. The sharp reduction in activity at the 25% concentration prompted our testing of a 37.5% concentration. The data for the 37.5% concentration suggest that the greatest loss in activity occurs between 50 and 37.5% concentrations. The lowest dilution for probing beads with the ovipositor was 12.5%; although drumming was noted at 6.2% of the standard concentration, the level of activity of the kairomone was not sufficient to elicit a probing response with the ovipositor.

DISCUSSION

The loss in activity of kairomone in time, as shown in Figure 1, suggests that the kairomone degrades or is volatile. In some instances during bioassays, females on the cover slip above the treated beads extended their antennae downward and walked directly over the beads, but could not contact them with their antennae. A few dropped directly on the beads from the cover slip. We consider this a response to a volatile component of the hexane wash of eggs, rather than to visual cues, since we did not observe females exposed to untreated beads gaining access to the beads from the cover slip.

Our preliminary tests with the hexane wash of Colorado potato beetle eggs applied to filter paper showed weak arrestment responses of *E. puttleri* to the treated spots, little antennal drumming activity, and no attempts to probe with the ovipositor. Strand and Vinson (1983) showed that the parasite *Telenomus heliothidis* Ashmead (Hymenoptera: Scelionidae) required a rounded surface approximating the size and shape of the egg of the noctuid host, *Heliothis virescens* (F.) We also found that *E. puttleri* preferred a rounded surface. Unlike *T. heliothidis*, however, *E. puttleri* responded to 1 mm round glass beads which differ in shape and form from the ovoid egg of the Colorado potato beetle.

The host-finding and host-acceptance behaviors noted on kairomone-treated beads appeared to be the same as when *E. puttleri* were exposed to Colorado potato beetle eggs. The host-acceptance repertoire, however, normally resulted in the successful drilling of the egg with the ovipositor. After removal of the ovipositor, females commonly turned and fed on egg fluids at the wound. We observed no females turning to explore kairomone-treated beads after attempts to probe with the ovipositor.

The serious damage caused by the Colorado potato beetle, and current difficulties in controlling the pest due to high levels of resistance to an array of registered insecticides (Forgash, 1985), stresses the importance of the development of alternative strategies for control. The findings that *E. puttleri* readily parasitizes eggs of the Colorado potato beetle and has a restricted host range (Puttler and Long, 1983) make this insect an excellent candidate as a new, exotic parasite. The finding of a kairomone that elicits host-finding and host-acceptance behaviors in *E. puttleri* may have future application in field situa-

tions. This will, in large measure, depend on the identification and potential for synthesis of the kairomone.

We are interested, as well, in attempts to develop methods that will facilitate the mass rearing of this parasite, for it will be a necessary to release large numbers of parasites during each field season, since *E. puttleri* does not survive the winter in temperate regions. As noted by Strand and Vinson (1983), kairomones attractive to egg parasites constitute an important link in the efforts to develop acceptable artificial hosts for mass production of parasites and may also prove effective in stimulating parasites to attack previously unacceptable hosts which are more readily reared for mass production.

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COPULATION RELEASER PHEROMONE IN BODY SCALES OF FEMALE WHITEMARKED TUSSOCK MOTH, *Orgyia leucostigma* (LEPIDOPTERA: LYMANTRIIDAE): Identification and Behavioral Role

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Abstract—The copulatory behavior of the male whitemarked tussock moth, *Orgyia leucostigma,* was released by extracts of female body scales applied to rubber septum models baited with a female sex pheromone gland. The major compounds in the scale extracts were identified by GC-MS as a series of *n*-alkanes from C-21 to C-29. Of these, *n*-tricosane, *n*-tetracosane, *n*-pentacosane, and *n*-heptacosane, applied at 10 ng/septum, caused significantly more males to attempt copulation than hexane-treated controls. Mixtures of the *n*-alkanes, resembling the composition in the scale extracts, were no better than the two most active alkanes, *n*-tetracosane and *n*-pentacosane, alone. The releaser effect of the *n*-alkanes was dose dependent. EAG responses to the identified *n*-alkanes were small suggesting, along with the behavioral observations, that their perception occurred at very close range. Other factors releasing male copulatory behavior are discussed.

Key Words—Alkanes, copulation, cuticular hydrocarbons, sexual behavior, moth scales, releaser stimuli, whitemarked tussock moth, Lepidoptera, Lymantriidae, *Orgyia leucostigma.*

INTRODUCTION

In many moth species, including the whitemarked tussock moth (WMTM), *Orgyia leucostigma* (J.E. Smith), male copulatory behavior is released by contact

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with female scales (Grant 1981; Ono 1974, 1977, 1979, 1980, 1981; Sanders, 1979; Shimizu and Tamaki, 1980). This has been demonstrated by applying scales to surrogate female models treated with female sex pheromone and noting the increase in male copulatory response to these models over models without scales.

The copulation-releasing effect of female scales in most cases appears to be due to their physical characteristics, since extracting them with organic solvents had little or no consequence on their effectiveness as releasers (Ono, 1974; Sanders, 1979; Shimizu and Tamaki, 1980) whereas pulverizing them considerably reduced or eliminated their activity (Ono, 1979; Shimizu and Tamaki, 1980). By contrast, Grant (1981) found that while pulverizing scales of the WMTM reduced their activity, they still retained their ability to elicit a relatively high level of copulation. This result suggested that a chemical stimulus might also be involved in scale-induced copulatory behavior in the WMTM.

We investigated this possibility and in this report demonstrate that female WMTM scales provide a chemical stimulus which releases male copulatory behavior and identify the chemicals responsible.

METHODS AND MATERIALS

Insects. These were obtained from a laboratory culture reared on artificial diet (Grisdale, 1973, 1975). They were sexed as pupae and maintained in separate rooms. Adult males were held in continuous light and tested when two days old (Grant, 1975, 1981).

Bioassay. Female models consisted of sleeve type rubber septa (15 × 9 mm; A.H. Thomas Co.; Philadelphia, Pennsylvania), each baited with an excised female pheromone gland mounted on the small end (Figure 1). Glands were required because the sex pheromone has not been identified. The fringe of terminal abdominal scales protruding over the retracted gland was removed from the female with sticky tape before the glands were excised to avoid contaminating the gland or septum with scales.

The prepared septa were placed individually on filter paper disks lining the bottom of circular plastic arenas (15 cm diameter × 6 cm deep). A virgin male was introduced into each arena and observed for 5 min commencing when the sexually excited male contacted the septum. During the observation period, we recorded whether or not the male attempted copulation and, in some experiments, the duration of each encounter with the septum. A copulation attempt was defined as flexion of the male abdomen toward the model, followed by genital contact. A new septum and filter paper were used for each replicate to avoid possible contamination with extraneous scales. Experiments were carried out from 1000 to 1200 hr each day at ambient temperature and humidity.

Test Stimuli. The orientation of the models in the horizontal and vertical

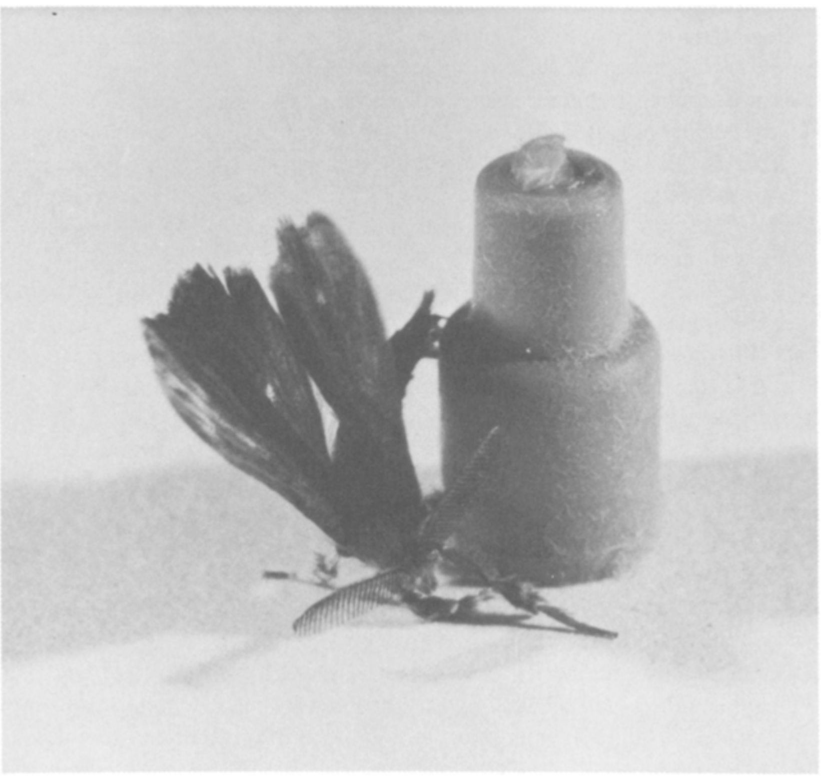

FIG. 1. Male *O. leucostigma* attempting to copulate with a vertical rubber septum model covered with female scales. Note the excised female pheromone gland sitting in the well at the small end of the rubber septum.

(standing on large end) positions was evaluated in the first series of experiments to determine which control model elicited the lowest frequency of copulatory response. The following stimuli were applied individually to the models to evaluate their effectiveness as releasers of male copulatory behavior: female body scales, pulverized female scales, extracts of female body scales, a fractionated whole-body wash of females, a female sex pheromone extract, individual alkanes identified in the scale extracts, and two mixtures, I and II, of these alkanes composed as follows: mixture I, which consisted only of odd chain alkanes, contained 5% *n*-heneicosane, 15% *n*-tricosane, 40% *n*-pentacosane, 20% *n*-heptacosane, and 20% *n*-nonacosane; mixture II, which contained most of the *n*-alkanes found in the scale extracts, consisted of 5% *n*-heneicosane, 10% *n*-tricosane, 15% *n*-tetracosane, 25% *n*-pentacosane, 10% *n*-hexacosane, 15% *n*-heptacosane and 20% *n*-nonacosane.

Untreated septa were coated with intact scales by rubbing them against live females or on the inside of a mortar containing scales removed with forceps from several females. It was estimated that less than 1 female equivalent of scales was applied in either case. Scales around the female genitalia that were likely to be contaminated with sex pheromone were avoided by removing them beforehand with sticky tape. Pulverized scales were produced by grinding with mortar and pestle and applied by rubbing the septa against the inside of the mortar to provide a covering of scale particles estimated to be equivalent to models with intact scales.

Scale extracts were prepared by removing scales from about 150 females and extracting these with 1 ml of hexane. They were applied, 4–5 female equivalents (FE) in 50 μl of hexane, to the outside surface of septa which were then allowed to stand in a fume hood for at least 40 min before testing. Waiting less time produced erratic results. The other test substances were also applied at least 40 min before evaluation. A whole-body extract was produced by immersing females in a minimum volume of hexane for 10 min to extract surface chemicals. It was then chromatographed on a small column to produce two fractions, one containing the alkanes and the other containing the remaining nonalkane material. The column was dry-packed in a Pasteur pipet (Carlson and Service, 1980) and consisted of 1 cm silica gel (J.T. Baker Chemical Co.) over 5 cm of 20% silver nitrate-impregnated silica (Hi-Flosil-Ag, 60/200 mesh; Applied Science Laboratories, Inc.). The extract (200 FE/100 μl) was eluted with 4 ml hexane (2 ml of which were absorbed by the dry column), 3 ml 5% ether–hexane, 3 ml 15% ether–hexane, and 3 ml 50% ether–hexane. The first 1 ml hexane fraction collected contained the alkanes; the second 1 ml contained only traces of alkanes and was discarded. The remaining fractions were combined to form the nonalkane fraction. The alkane and nonalkane fractions (4 FE each) were applied in 20 μl of solvent to separate septa. The female sex pheromone extract was obtained by surface rinsing pheromone glands from 50 virgin females with hexane (Grant, 1975) and applying 2 FE in 20 μl of hexane to the septa. The individual identified alkanes and the two alkane mixtures were applied in 20 μl of hexane. Controls consisted of septa treated with 20–50 μl of hexane and allowed to stand for 40 min before use.

Chemical Analysis of Scale Extracts. Two batches of scales from 120 and 150 females, respectively, were extracted with hexane and analyzed by splitless capillary gas chromatography (SCGC) on a Hewlett-Packard 5880A instrument fitted with a 30-m, 0.25-mm ID DB-1, fused silica cross-linked methyl silicone column (J&W Scientific Inc., Rancho Cordova, California). The column was operated at 80°C isothermal for 2 min, 10°C/min to 180°C, and then 2°C/min to 240°C, with a helium carrier gas flow of 1 ml/min throughout. The injector was maintained at 200°C and the flame ionization detector at 250°C.

Mass spectroscopic analysis (SCGC-MS) was performed with a 30-m, 0.32-mm ID DB-1 column and electron impact ionization. The column was

programmed at 80°C isothermal for 1 min and then 12°C/min to 220°C with a flow rate of about 2 ml/min.

The *n*-alkanes used as chemical standards and for bioassay were obtained from commercial sources (Applied Science Laboratories Inc., State College Pennsylvania; Analabs Inc., New Haven, Connecticut; Chemical Samples Corp., Columbus, Ohio). The compounds used in the behavioral tests were all 99% pure or better.

Electroantennograms (EAGs). EAG responses to *n*-alkane standards (1 μg at source) were obtained as described by Grant et al. (1972). Optimum responses were obtained from fresh (1-day-old) males.

RESULTS

Behavioral Observations. The main elements of the male response to a model treated with active material were similar to those reported earlier (Grant, 1981). The male usually initiated contact by means of his tarsi, and this was quickly followed by antennal contact. The male circled the septum, particularly a vertical one, touching it with his antennae and tarsi, and attempted to copulate while still on the substrate (Figure 1). If the copulation attempt was delayed, the head was kept in close contact with the septum, usually with the palps touching it. Even when models treated with active material failed to elicit copulatory behavior, they could often be distinguished from hexane-treated controls by the position of the male's head. With a vertical septum, males frequently climbed and circled around the top close to the pheromone gland and occasionally attempted to copulate from this position. Much more climbing, less antennal contact, little head contact, and little copulatory behavior were observed with control models.

Copulatory Response to Scales and Model Orientation. The addition of scales to either horizontal or vertical models significantly increased male copulatory attempts over the comparable scaleless models; moreover, the amount of time the males remained in contact with the model was significantly greater (Table 1). The addition of pulverized scales was less effective than intact scales, but their presence did elicit more copulatory responses than the controls, significantly so in the case of the vertical model.

The effect of model orientation on copulatory behavior was clear-cut (Table 1). Vertical models without scales elicited no copulatory responses and produced a significantly shorter contact time ($P < 0.05$, t test) than similar scaleless horizontal models. The horizontal septa elicited copulatory responses from 40% of the males, which was comparable to the 33% response elicited by scaleless horizontal plasticene models reported earlier (Grant, 1981). Because the vertical models produced a much lower control response, they were used in all subsequent tests to maximize the differences between treatments and controls.

TABLE 1. COMPARISON OF COPULATION RELEASING EFFECT OF HORIZONTAL AND VERTICAL RUBBER SEPTUM MODELS (+ FEMALE SEX PHEROMONE GLAND) TREATED WITH INTACT AND PULVERIZED FEMALE BODY SCALES OF *O. leucostigma*

Treatment	No. tests	Tests with at least 1 copulatory attempt (%)[a]	Duration contact with septum, $\bar{X} \pm$ SE (sec)[b]
Horizontal models			
Without scales	15	40 b	97 ± 14 b
With female scales	15	87 a	147 ± 20 a
With pulverized scales	15	53 b	89 ± 11 b
Vertical models			
Without scales	15	0 c	49 ± 05 b
With female scales	15	87 a	92 ± 11 a
With pulverized scales	15	33 b	72 ± 7 a

[a] Percents within the same group (horizontal or vertical model) followed by the same letter are not significantly different by G test, $P = 0.05$.
[b] Means within the same group (horizontal or vertical model) followed by the same letter are not significantly different by Student-Newman-Keuls multiple range test, $P = 0.05$.

Copulatory Response to Scale Extracts. Both of the scale extracts applied to vertical models elicited significantly more copulatory attempts than the hexane controls (Table 2). The sex pheromone gland extract, on the other hand, did not increase copulatory behavior, indicating that pheromone contamination

TABLE 2. EFFECTIVENESS OF VARIOUS FEMALE *O. leucostigma* EXTRACTS APPLIED TO VERTICAL RUBBER SEPTUM MODELS (+ FEMALE SEX PHEROMONE GLAND) IN RELEASING MALE COPULATORY BEHAVIOR

Treatment	No. tests	Tests with at least 1 copulatory attempt (%)[a]	Duration contact with septum $\bar{X} \pm$ SE (sec)[b]
Hexane (control)	30	3	89 ± 11
Scale extract 1 (5 FE)	13	46 *	101 ± 10
Scale extract 2 (4 FE)	15	33 *	90 ± 11
Whole-body extract			
Alkane fraction (4 FE)	15	40 *	123 ± 14
Nonalkane fraction (4 FE)	15	13	103 ± 13
Sex pheromone gland extract (2 FE)	15	7	74 ± 11

[a] Those percents marked with * are significantly different from control at $P = 0.05$, G test.
[b] None of the averages were significantly different from each other by Student-Newman-Keuls test, $P = 0.05$.

of the scales, if it occurred, was not the cause of the copulation attempts. Comparison of the alkane and nonalkane fraction of the whole-body extract showed that the alkane fraction significantly increased copulatory behavior compared to the control while the nonalkane fraction did not. In contrast to the previous experiment, there was no significant difference in the duration of contact caused by any of the treatments.

Compounds Identified in Scale Extracts. The two hexane extracts analyzed by SCGC were found to be very similar in composition. The major early eluting peaks showed retention times identical to those obtained from straight-chain hydrocarbons (C-19 to C-29). Mass spectral analysis of these mixtures confirmed the hydrocarbon nature of the peaks, exhibiting spectra identical to those obtained for corresponding straight-chain alkane standards. Additional later-eluting peaks were also present in the chromatograms, but these were not pursued. The linear hydrocarbon composition for two separate extracts as determined by SCGC is given in Table 3. The even-numbered hydrocarbons were consistently found in other WMTM scale extracts but not in solvent blanks or in extracts of other moth species analyzed at the same time.

Copulatory Response to Alkanes. Responses significantly greater than the control ($P = 0.05$, G test) were obtained with 10-ng doses of *n*-tricosane, *n*-tetracosane, *n*-pentacosane, and *n*-heptacosane (Figure 2), with the strongest responses elicited by *n*-tetracosane and *n*-pentacosane. Mixtures I and II, also evaluated at 10-ng doses, elicited copulatory responses from 47% of the males, the same as *n*-pentacosane or *n*-tetracosane alone (Figure 2), suggesting that the alkanes were not synergistic.

Effect of Concentration. Preliminary bioassays with whole-body extracts

TABLE 3. RELATIVE LINEAR HYDROCARBON COMPOSITION OF TWO FEMALE *O. leucostigma* SCALE EXTRACTS

Alkane	Extract A (%)	Extract B (%)
n-Nonadecane	—	1
n-Eicosane	1	2
n-Heneicosane	2.5	3
n-Docosane	7.5	5
n-Tricosane	12	7
n-Tetracosane	16.5	6
n-Pentacosane	25.5	25
n-Hexacosane	14.5	5
n-Heptacosane	11	16
n-Octacosane	2.5	1
n-Nonacosane	7	29

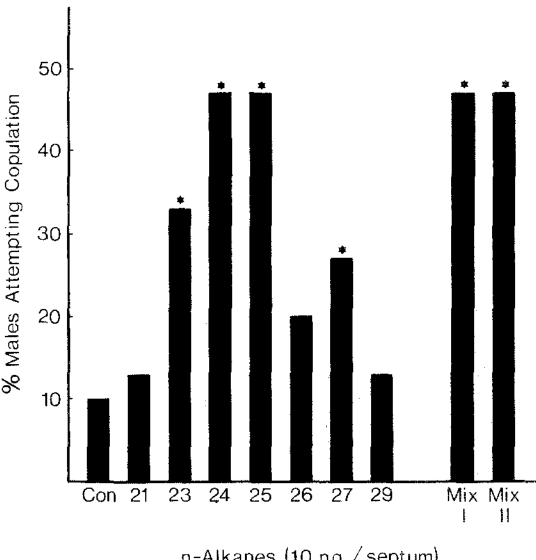

FIG. 2. Effectiveness of identified scale alkanes (10 ng/septum) and mixtures I and II of these alkanes (see text for mixture composition) in releasing *O. leucostigma* (N = 15) copulatory behavior. Controls (N = 30) were treated with 20 μl of hexane. Bars marked with * are significantly different from control at P = 0.05, G test.

and the *n*-alkanes indicated that the copulatory response was dose dependent. To assess this systematically, males were exposed to septa treated with *n*-pentacosane over a range of dosages from 0.1 ng to 10 μg. The maximum response was produced by 10 ng (Figure 3), which was the only dose that was significantly greater than the control (P = 0.05, G test), although substantial responses were elicited by 0.1- to 10-μg quantities. A similar dose–response curve was obtained with a 1 : 1 mixture of *n*-tricosane and *n*-pentacosane over the concentration range of 1 ng to 1 μg (not shown).

EAG Response. The responses to the *n*-alkanes were small, the maximum response being 0.4 mV (Table 4). The EAG response to the same quantity of (*Z*)-6-heneicosen-11-one, a female sex pheromone component (Slessor and Grant, unpublished data), was 2 mV. The most stimulating EAG compounds (tetracosane and tricosane) were also two of the behaviorally most active compounds, but *n*-pentacosane, also strongly active behaviorally, was one of the weakest EAG stimuli.

DISCUSSION

Hydrocarbons are frequently used as semiochemicals by insects (Howard and Blomquist, 1982). In the Lepidoptera, several moth species use saturated

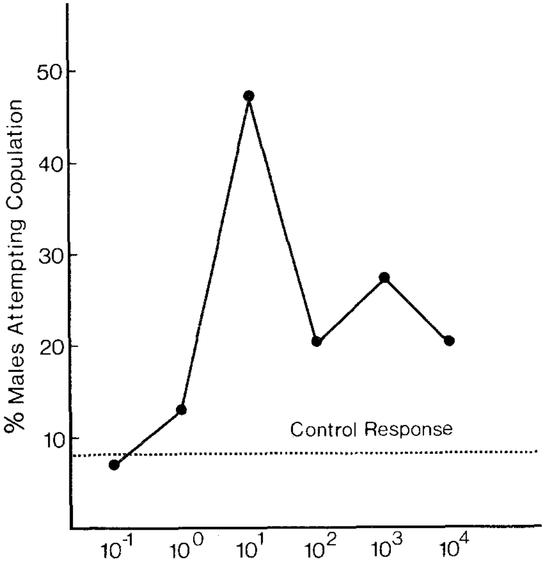

FIG. 3. Effect of concentration of *n*-pentacosane (applied to vertical septa) on percentage of male *O. leucostigma* (N = 15 for each dosage) attempting to copulate.

(Roelofs and Cardé, 1971) and unsaturated (Conner et al., 1980; Wong et al., 1984) hydrocarbons as sex attractants. In *Colias eurytheme,* a butterfly, 13-methylheptacosane found on male wings acts as a close-range "aphrodisiac," inducing acceptance behavior in the female, while two associated wing *n*-alkanes (*n*-heptacosane and *n*-nonacosane) appear to evoke female rejection behavior (Grula et al., 1980). Hymenopterous egg parasites use the alkanes found

TABLE 4. EAG RESPONSE OF *O. leucostigma* MALES TO IDENTIFIED *n*-ALKANES FOUND IN FEMALE SCALES[a]

Alkane	Ave. EAG response (mV)	N
n-Heneicosane	0.2	5
n-Tricosane	0.4	6
n-Tetracosane	0.4	3
n-Pentacosane	0.1	6
n-Heptacosane	0.2	5
n-Nonacosane	0.1	4

[a] All compounds tested at 1 μg at source.

in the scales of female *Heliothis zea* moths as kairomones to locate host material for oviposition (Jones et al., 1973).

We have demonstrated that at least four straight-chain alkanes present in the scales of female WMTM function as a copulation releaser pheromone. This is the first demonstration in moths of chemicals other than those originating from the sex pheromone gland (on the 8–9th intersegmental membrane) that are capable of doing so. The copulation releasing effect of these compounds, however, is dependent on the prior stimulation of males with female sex pheromone; that is, the sex pheromone acts as a primer for the releaser stimulus as observed with nonchemical releaser stimuli in other lepidopteran species (Shorey and Gaston, 1970; Haynes and Birch, 1984; Grant, in preparation). By contrast, the action of the copulation releaser pheromone, erectin, of the azuki bean weevil, *Callosobruchus chinensis,* appears to be independent of the sex pheromone (Tanaka et al., 1981), while in some Diptera the sex pheromone is nonvolatile and itself releases copulation when the male contacts the female (Howard and Blomquist, 1982). The chemicals releasing copulation in these species are also cuticular hydrocarbons, although they are somewhat larger and more complex than those of WMTM.

Many of the alkanes with an odd number of carbon chains found in the scales of female WMTM are also found in the scales or body surfaces of many other adult lepidopterans. They occur in male WMTM (unpublished data), both sexes of the cabbage looper, *Trichoplusia ni* (De Renobales and Blomquist, 1983), the corn earworm, *H. zea* (Jones et al., 1973), two species of *Colias* butterflies (Grula et al., 1980), and both sexes of several *Choristoneura* budworm species (unpublished data). The widespread occurrence of these hydrocarbons along with the similar physical characteristics of the scales probably accounts for the "universal" copulatory response (Ono, 1977) of male moths to male and female scales of other species (Grant, 1981; Ono, 1974, 1977, 1979, 1980, 1981; Sanders, 1979; Shimizu and Tamaki, 1980).

The apparent lack of specificity in stimuli which release copulation in these lepidopterans is not surprising because the factor controlling specificity is usually the female sex pheromone (Ono, 1980). Copulation-releasing stimuli such as the WMTM scale alkanes serve simply as sign stimuli indicating that the sex pheromone source is a suitable object for copulation. The system is not foolproof, however, because homocourtships often occur when two or more males arrive at a pheromone source at the same time. The chemical and physical similarity of male and female scales may also be responsible for this behavior.

The quantity (10 ng) of alkane required to elicit a copulatory response was surprisingly low. The EAG results suggest that males can perceive the scale alkanes with olfactory receptors on their antennae; however, the responses obtained with a relatively large stimulus dose (1 μg) were small, as was the case for female *Colias* butterflies stimulated with similar alkanes identified in male *Colias* wings (Grula et al., 1980). The low volatility of these alkanes could

account for the weak EAG responses, but the observation that WMTM males appear to make obligatory tarsal and antennal contact with the female or model before attempting copulation suggests that the alkanes are detected at very close range, probably by contact chemoreceptors. This could explain why *n*-pentacosane elicited a low EAG response. The perceptual mechanism by which the alkanes are detected requires further study.

Although we have demonstrated a role for the scale alkanes in releasing male WMTM copulatory behavior, other stimuli are also effective releasers and may not involve the scales at all. For example, the orientation of the model (vertical vs. horizontal) also affected the release of copulatory behavior, an observation repeated with the male spruce budworm, *C. fumiferana*, exposed to pheromone-treated septa (Grant, in preparation). Why male moths respond differently to horizontal and vertical models is not clear, but it could be related to differences in visual stimuli presented by the two models or to how the antennae or other appendages contact the model. For WMTM, vertical septa without scales may not provide an adequate tactile stimulus to release copulatory behavior.

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SYNTHESIS OF ENANTIOMERICALLY ENRICHED 2-HEPTANOL AND 3-OCTANOL BY MICROBIAL REDUCTASES OF *Curvularia falcata* AND *Mucor* SPECIES

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Abstract—Certain insects produce 2-heptanol or 3-octanol in various glandular secretions and recent studies have shown that the 3-octanol of two different genera of ants (*Crematogaster* and *Myrmica*) can be either the (*S*)-(+) or mainly the (*R*)-(−) enantiomer, respectively. Synthesis of each of these alcohols can be achieved in relatively high enantiomeric purity by certain microbial reductases. The corresponding ketone of each alcohol is reduced by *Curvularia falcata*, giving an alcohol which is about 90% the (*S*)-(+) enantiomer, and two *Mucor* species give as much as 80% the (*R*)−(−) enantiomer. The synthesis of certain chiral alcohols from their corresponding ketones by microbial reductases can offer a simple procedure for obtaining sufficient amounts of these substances for certain behavioral studies.

Key Words—Chiral alcohols, 2-heptanol, 3-octanol, microbial reductases, *Curvularia falcata*, *Mucor* species.

INTRODUCTION

The mandibular glands of three species of *Myrmica* ants contain 3-octanol as a component of their alarm pheromone blend, and this alcohol is >90% the (*R*)-(−) enantiomer (Attygalle et al., 1983). Bioassays of the pheromonal activity of the separate enantiomers of 3-octanol for three species of these ants indicated that they respond only to the (*R*)-(−) enantiomer; the (*S*)-(+) enantiomer is

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inactive (Cammaerts et al., 1985). The separate enantiomers of 2-octanol, which are commercially available, gave little or no response with these *Myrmica* ants. In contrast, two species of *Crematogaster* ants produce exclusively (*S*)-(+)-3-octanol in their mandibular glands (Brand, 1985), but no bioassays have been conducted with the separate enantiomers on these species.

The results of Cammaerts et al. (1985) illustrate the need for behavioral studies on the separate enantiomers of such simple alcohols as 3-octanol in insect species that produce them. However, the separate enantiomers generally are not available to the biologist wishing to bioassay them, and a chemist might not be persuaded easily to synthesize the enantiomers of such mundane alcohols. For their bioassays, Cammaerts et al. (1985) isolated (*S*)-(+)-3-octanol by preparative gas chromatography from oil of Japanese peppermint (*Mentha japonica*), and (*R*)-(−)-3-octanol from heads of worker ants of *Myrmica ruginodis*. However, in many cases, the preparation of microliter quantities of a compound from the insects themselves may not be feasible.

The two alcohols, 2-heptanol and 3-octanol, occur in various insect secretions (Blum, 1981). While the separate enantiomers of 2-heptanol are available from Norse Laboratories, Newbury Park, California 91320, the separate enantiomers of 3-octanol are not available commercially. The enantiomers of each of these simple alcohols could be produced by the action of an appropriate dehydrogenase on the corresponding ketone, a reaction obviously taking place in vivo. Reduction of ketones by microbial reductases, using whole cells, often with high yields of only one enantiomer, is quite common, and this procedure can be used to obtain appreciable amounts of product (MacLeod et al., 1964; Sariaslani and Rosazza, 1984, and references therein; Wong and Drueckhammer, 1985, and references therein).

We have synthesized each enantiomer of 2-heptanol and 3-octanol from the corresponding ketone in 80–90% purity with microbial reductases. The chirality of the alcohol obtained was determined by gas chromatography of the (*R*)-(+)-*trans*-chrysanthemoyl esters as described previously (Attygalle et al., 1983; Brand, 1985). *Curvularia falcata* gives approximately 90% (*S*)-(+)-2-heptanol and (*S*)-(+)-3-octanol, and two *Mucor* species produce 70–80% (*R*)-(−)-2-heptanol and (*R*)-(−)-3-octanol. These studies have been done using whole cells in their culture medium and the procedure can be scaled up to produce sufficient quantities of material for behavioral work.

METHODS AND MATERIALS

All fungal strains were obtained from the personal collection of J.P.N. Rosazza, University of Iowa. The successful strains used were *Curvularia falcata* QM-72 D, *Mucor recurvatus* UI-36, and *M. mucedo* 20094 P. The organisms were grown and maintained on soybean meal–glucose medium consisting of (grams per liter distilled water): glucose 20 g, yeast extract 5 g, soybean

meal 5 g, NaCl 5 g, and K_2HPO_4 5 g. The pH of the medium was adjusted to 7.0 before autoclaving.

Cultures were inoculated from an actively growing culture (5% inoculum), and grown with vigorous shaking in 100 ml medium in 250 ml Erlenmeyer flasks or 1 liter medium in Fernbach flasks. After 1 to 3 days of growth, the cultures were placed in an anaerobic chamber (Coy Mfg. Co.; gas phase: 85% N_2, 10% H_2, 5% CO_2) and allowed to become anaerobic for several hours before addition of either 2-heptanone or 3-octanone (200 μl for 100 ml medium or 2 ml for 1 liter medium). Preliminary studies showed that yields of the alcohol were higher with cultures kept anaerobic rather than aerobic. The period of exposure to either ketone varied from 6 hr to 8 days.

After exposure to either ketone, all the cultures were extracted twice with ether and the ether dried over anhydrous Na_2SO_4. *C. falcata* cultures turn black with time, and it is only when the organism is black that it is extracted easily with ether. Extraction of the earlier yellowish-brown stage results in an emulsion that is extremely difficult to break. *Mucor* cultures did not give any extraction problems.

Gas chromatographic determination of the ketone–alcohol ratio was done at 70°C using a Supelcowax 10 fused silica capillary column (15 m). Derivatization of the alcohols was done on the ketone–alcohol mixture with (*R*)-(+)-*trans*-chrysanthemic acid as described previously (Brand, 1985). Separation of the 2-heptanyl esters was achieved at 115°C and the 3-octanyl esters at 130°C on the Supelcowax 10 column. GC-MS of the standard 2-heptanyl and 3-octanyl esters and of the same esters from microbially produced alcohols was carried out on a 5% phenyl methyl silicone fused silica capillary column (25 m) at 140°C, interfaced to a Finnigan 1015 quadrupole mass spectrometer.

RESULTS

Gas chromatography of the ether extracts from *C. falcata* and *Mucor* cultures indicated that the added ketone and its corresponding alcohol were the only major volatile compounds in the extract. The formation of the (*R*)-(+)-*trans*-chrysanthemates was performed on the ketone–alcohol mixture without any further purification. Confirmation of the structures of the (*R*)-(+)-*trans*-chrysanthemoyl esters was obtained by comparison of the retention times with standards on the Supelcowax 10 column, and by GC-MS on the 5% phenyl methyl silicone column interfaced to a Finnigan 1015 quadrupole mass spectrometer. Equivalent mass spectra were obtained from the esters of standard 2-heptanol and 3-octanol and the microbially produced alcohols. The characteristic base peak at *m/e* 123 was obtained in all cases with a molecular ion at *m/e* 266 for the 2-heptanyl ester and at *m/e* 280 for the 3-octanyl ester.

2-Heptanone. *C. falcata* gave 2-heptanol that was 87–90% the (*S*)-(+) enantiomer, *M. recurvatus* gave about 76% the (*R*)-(−) enantiomer, and *M.*

mucedo gave 70–80% the (*R*)-(−) enantiomer. The recovery of the ketone plus alcohol as a percentage of the initial ketone added was as high as 70%.

3-Octanone. *C. falcata* gave 3-octanol that was 86–90% the (*S*)-(+) enantiomer, *M. recurvatus* gave about 76% the (*R*)-(−) enantiomer, and *M. mucedo* gave 75–80% the (*R*)-(−) enantiomer. The recovery of the ketone plus alcohol as a percentage of the initial ketone added was as high as 90%. In one experiment with 3 liters of *C. falcata* treated with 3-octanone and kept anaerobic for eight days, the ratio of ketone to alcohol recovered was 20:80, with the 3-octanol being 90% the (*S*)-(+) enantiomer. In another experiment, 3 liters of *M. mucedo*, kept anaerobic for 6 days, gave 10:90 ketone to alcohol but the 3-octanol was only 61% the (*R*)-(−) enantiomer. In each of these latter two experiments 0.3–0.4 ml of the alcohol was obtained.

DISCUSSION

Cammaerts et al. (1985) conducted satisfactory bioassays on *Myrmica* ants using 3-octanol that is approximately 90% the (*R*)-(−) enantiomer. This illustrates that certain behavioral experiments are possible with an alcohol that is highly enriched in only one enantiomer and that absolute purity of the enantiomer is not necessary for some studies. The (*S*)-(+)-2-heptanol and the (*S*)-(+)-3-octanol of about 90% purity produced by *C. falcata* and the (*R*)-(−) enantiomer of 75–80% purity produced by *M. mucedo* might be adequate for similar behavioral studies on some insects that produce these alcohols. However, the lack of activity of one enantiomer, as is the case with *Myrmica* (Cammaerts et al., 1985), may be an important criterion in the interpretation of bioassay data on alcohols of the above purity. An extensive literature documents the inhibitory effects of small percentages of unnatural enantiomers on the response of certain insects, and a cautionary note is offered on this point when bioassaying enantiomers that are only highly enriched and are not absolutely pure.

Neither the *C. falcata* nor the *Mucor* species reduced all the ketone to the alcohol. The best conversions obtained were a 20:80 ratio of 3-octanone to 3-octanol with *C. falcata* after anaerobic exposure to the ketone for eight days, and a 10:90 ratio with *M. mucedo* after six days. Sih and Chen (1984) have pointed out that a major complication in using intact cells for the reduction of ketones is that the process may be only partially enantioselective. They conclude that this usually arises from the combined action of competing enzymes of opposite chirality in intact cells. A ratio of 9:1 of the *S*:*R* enantiomers was obtained with *C. falcata* whether the exposure time was 6 hr or eight days, and in contrast, the *M. mucedo* usually gave about 80% the (*R*)-(−) enantiomer with an 18-hr exposure, but only 61% after six days. We have not attempted to optimize conditions for these organisms and do not know whether competing enzymes are present. Certain additional microorganisms tested, e.g., *C. pallescens* ATCC 12018 and bakers' yeast, did not produce any alcohol under the

usual conditions employed. However, it seems certain that suitable organisms and conditions could be found that would give better than 90% of either enantiomer in good yield.

As only one enantiomer of the alcohol was a major product, we wondered whether these organisms would selectively oxidize the same enantiomer if given the racemic alcohol under aerobic conditions. Our attempts to do this failed completely, and the reduction of the ketone seems irreversible with these organisms. This irreversibility of reduction has been mentioned by MacLeod et al. (1964) and by Sih and Chen (1984) and is understandable under anaerobic fermentative conditions. We have no explanation as to why our organisms failed to reoxidize any alcohol under aerobic conditions. In previous experiments we have found a fungus that would oxidize racemic *trans*-verbenol to verbenone quantitatively under aerobic conditions (Brand et al., 1976). A different approach employing the enantioselective hydrolysis of certain racemic acyclic alcohol acetates by microbial esterases, to yield chiral alcohols such as (*S*)-(+)-3-octanol, has been described by Oritani and Yamashita (1980).

As the reduction of the ketone is incomplete, solvent extracts of the culture medium contain variable amounts of the added ketone. Separation of the alcohol from the ketone could be achieved by adsorption column chromatography and preparative gas chromatography.

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IMPORTANCE OF LICHEN SECONDARY PRODUCTS IN FOOD CHOICE OF TWO ORIBATID MITES (ACARI) IN AN ALPINE MEADOW ECOSYSTEM

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Abstract—In an alpine meadow ecosystem in the Swiss National Park, the lichens *Cetraria islandica* and *Cladonia symphycarpa* occur in small vegetation patches dominated by *Carex firma* (Cyperaceae) and *Sesleria coerulea* (Gramineae). Laboratory food-choice experiments with two oribatid mites *Fuscozetes setosus* and *Carabodes intermedius* show that the thallus structure and secondary products of *C. islandica* are repellent factors against herbivorous mites. In contrast, secondary products of *C. symphycarpa* may be an attractant for the mites.

Key Words—Lichen, herbivores, secondary products, oribatid mites, Acari, food choice, *Cetraria islandica*, *Cladonia symphycarpa*, *Fuscozetes setosus*, *Carabodes intermedius*.

INTRODUCTION

The aim of this study was to investigate the influence of lichen secondary products on the food choice of two mite species, *Fuscozetes setosus* (C.L. Koch, 1841) and *Carabodes intermedius* Willmann, 1951, in an alpine meadow ecosystem in the Swiss National Park.

This meadow, 2550 m above sea level [vegetation type *Caricetum firmae* (Kerner) Br.-Bl.1926] has been studied since 1976 by a team of ecologists. A description of the study site is given by Matthey et al. (1981).

In this habitat, lichen phytomass accounts for only 1.1–5.5% of total phytomass (Galland, personal communication). The most abundant lichen species are *Cetraria islandica* (Iceland moss) and *Cladonia symphycarpa*. The first spe-

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cies is associated with *Carex firma* (Cyperaceae) and *Sesleria coerulea* (Gramineae) living in small vegetation patches without soil contact, while the second species grows on the edge of the vegetation cluster on disturbed humous subsoil.

About 50 species of oribatid mites occur in the habitat. Of these, 14 fed regularly on lichens, particularly on *C. symphycarpa*. In contrast, *C. islandica* was strictly avoided by all mite species. Two mite species, *F. setosus* and *C. intermedius*, the most active feeders on thalli of *C. symphycarpa* were chosen for food choice experiments. They were also the mite species most easily maintained in the laboratory. Both species belong to the panphytophagous feeding group (after Luxton, 1972). Whereas *F. setosus* is a dominant species that occurs regularly distributed in all vegetation types (Rohrer and Reutimann, 1984), *C. intermedius* was found predominantly associated within the small lichen microhabitats.

METHODS AND MATERIALS

The secondary products of both lichen species were identified by thin-layer chromatography (Culberson and Ammann, 1979). In *Cladonia symphycarpa*, the depside atranorin occurs in the cortex, the depsidone norstictic acid, as well as an unidentified substance occurring in the medulla. In the medulla of *Cetraria islandica* protolichenesterinic acid, an aliphatic lichen acid, as well as the depsidone fumarprotocetraric acid (a bitter substance) have been found (Culberson, 1969; Culberson et al., 1977).

The secondary products of freshly collected thalli of *C. symphycarpa* and *C. islandica* were extracted in acetone up to four times for a period of approx. 20 min each time. Thalli subjected to extraction constituted the substrate types B and G for food choice experiments (Figure 1). Other extracted thalli were then impregnated with the soluble secondary products of the respective species (repeated submersion of the thalli in acetone and desiccation in a thermal cupboard) (substrate types C and F).

The above-mentioned soluble secondary products were also applied on cellulose-free filter paper (diameter 10 mm, Balston Ltd) (substrate types D and E). Traces of secondary products were still detected in thalli B and G after three extractions. Lichenological spot tests (color reaction on lichen after application of an indicator solution) showed, however, typical discoloration of the inoculated lichen material on thalli C and F. Untreated desiccated thalli were used as controls (A and H).

The following eight substrate types were used in dual combinations for food-choice experiments with the mites: A, *C. islandica*, thalli, freshly collected, dried (control); B, *C. islandica*, thalli, with most secondary products

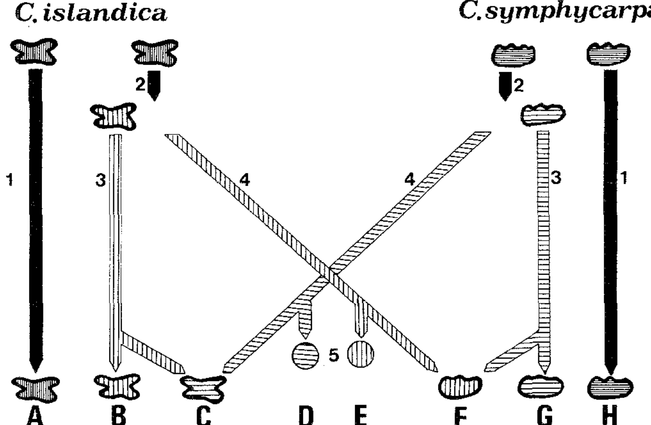

FIG. 1. Diagram of treatments: (1) no treatment, (2) acetone extractions of secondary products, (3) thalli subjected to acetone extractions, (4) acetone-soluble secondary products for impregnations, (5) disks of filter paper with secondary products dissolved in acetone. For further explanation, see text.

removed by acetone extractions; C, *C. islandica*, thalli, treated as in B, but with the secondary products of *C. symphycarpa*, applied in acetone solution; D, *C. islandica*, acetone-extracted secondary products on glass filter paper; E, *C. symphycarpa*, acetone-extracted secondary products on glass filter paper; F, *C. symphycarpa*, thalli, treated as in E, but with the secondary products of *C. islandica* applied in acetone solution; G, *C. symphycarpa*, thalli, with most secondary products removed by acetone extractions; and H, *C. symphycarpa*, thalli, freshly collected, dried (control).

Feeding experiments were conducted in covered glass dishes, 48 mm in diameter, containing substrate made of a mixture of plaster of Paris and charcoal (15 : 1) and with a relative humidity of 90–99%. In each dish, two of the eight food types (A–H, see above), were offered. Five adults of each species were used in each experiment. Seven pairwise combinations were chosen for the experiments (see Table 1 and Figure 1). The animals were kept at 20°C and observed once a day for a period of 6–19 days. Preferences in each pairwise combination were tested for with chi-square tests for goodness of fit (Wonnacott and Wonnacott, 1977). The null hypothesis (no preference) was that mites would be distributed equally between the two substrates. Observations of distribution on substrates were made once daily. Preferences were scored by summing the individual daily observations for the 6–19 days of each experiment. Feeding observations, feeding marks on the substrates, or fecal pellets were recorded as well.

Table 1. Results of Food-Choice Experiments with 7 Binary Combinations offered to *Fuscozetes setosus* and *Carabodes intermedius*[a]

	F. setosus						*C. intermedius*					
	1	2	3	4	5	6	1	2	3	4	5	6
A-H	7	0:11	H	H	H	<0.05	13	0:27	H	H	H	<0.001
D-E	6	0:9	E			<0.05	9	0:0				
A-B	6	0:15	B		B	<0.01	6	0:24	B		B	<0.001
C-H	17	0:26	H		H	<0.001	16	1:27	H		H	<0.001
C-G	12	9:20	G			<0.01	17	6:31	G		G	<0.001
G-H	6	4:5	H			<0.5	19	15:31	H	H	H	<0.0001
B-G	9	2:20	G	G	B, G	<0.01	13	2:9	G	G	B, G	<0.05

[a] (1) number of controls, (2) number of individuals on the two substrates, (3) preferred substrate, (4) substrates on which feeding was directly observed, (5) substrates on which feeding marks and fecal pellets were observed, (6) values of P (chi-square tests).

RESULTS

The individuals of both species were repeatedly observed to eat thallus tissue of *Cladonia*. They strictly refuse untreated *Cetraria* (A and H, Table 1). Both species prefer to feed on untreated *Cladonia* even in the presence of *Cetraria* impregnated with *Cladonia* substances (C and H). This indicates a preference for *Cladonia* as substrate.

Carabodes significantly prefer untreated *Cladonia* to *Cladonia* without lichen substances (G and H). *Cetraria* with a reduced concentration of lichen substances are only eaten by *Fuscozetes* (A and B). No feeding attempts were noticed on the glass filter paper. When lichen products were nearly completely removed, lichen thalli of both species were eaten by both *Fuscozetes* and *Carabodes* (B and G).

Examination of gut contents show that both species digest only hyphae of lichen fungi and excrete lichen algae undigested. In laboratory experiments, unicellular green algae (unidentified) are only eaten by *Carabodes* (Reutimann, 1985).

DISCUSSION

Carabodes clearly avoids lichen substances on filter paper, where *Fuscozetes* reacts positively to the *Cladonia* lichen substances (atranorin, norstictic acid, and an unidentified substance) (D and E). Secondary products from *Cladonia* are an important factor leading to acceptance of *Cladonia* by *Fuscozetes*. They seem to have only a limited antiherbivore function against the two mite species (cf.Rundel, 1978). The distal sensory organs of the mite would perceive these compounds in the case of direct contact with the substrate by means of the chemosensory setae on the distal segments of legs I and II as well as the palpi (Krantz, 1978), but the compounds seem to lack a decisive stimulating quality in eliciting feeding behavior.

Lichen substances of *Cetraria* (protolichenesterinic acid, fumarprotocetraric acid) have, on the other hand, a certain repellent effect on these two mite species. The presence of appreciable concentrations of fumarprotocetraric acid (Culberson et al. 1977) might be responsible for this. Various authors report that reindeer avoid *Cladonia* species with fumarprotocetraric acid, whereas they eat species not containing it (Rundel, 1978). In contrast to *Cetraria islandica*, *Cladonia symphycarpa* does not contain these substances (Culberson, 1970; Culberson et al., 1977).

The different reactions of the two mite species to lichens may be seen in the context of other differences in feeding behavior. While both species are classified as panphytophagous feeders (after Luxton, 1972), *Fuscozetes* also captures living (and dead) animal prey (zoophagous and necrophagous feeding

subtype, after Luxton 1972; Reutimann, 1985). It is a relatively active species, unlike *Carabodes*, which seems to be a relatively inactive, predominantly fungivorous species. Patterns of spatial distribution in the habitat showed that *Carabodes* is closely associated with lichens (Reutimann, 1985). *Cladonia* thalli in alpine meadows often show traces of feeding of the type made by these mites.

In addition, *Carabodes* proved to be very constant in its substrate preferences for lichen in laboratory tests with different organic materials (Reutimann, 1985). Lichen-mite associations have been found repeatedly by various authors (e.g, Bellido, 1975; Andre, 1975; Gjelstrup and Søchting, 1979; see Seyd and Seaward, 1984, for a comprehensive view).

A main consequence of the acetone treatment is the abrupt death of the fungal and algal cells in the lichen thalli caused by lysis of the plasmalemmas. In addition, the traces of lichen substances remaining in the thalli B, C, F, and G show that the method should be improved upon for further experimental work. The efficiency of extraction could perhaps be increased with other solvents.

Relatively few studies have documented the ecological importance of lichen products in relation to herbivorous animals. A few lichen products have antibiotic effects. Lichens with certain lichen products (bitter constituents, protocetraric acid, fumarprotocetraric acid) are avoided by a few lichen-eating invertebrates (Gerson and Seaward, 1977). It is not exactly known in individual cases if secondary products ensure effective protection against consumption. Gerson and Seaward (1977) argued that various secondary products do not greatly influence the consumption of lichens.

Lawrey (1983) suspects that secondary products may be only one of several factors affecting food preferences of lichen herbivores. The quality of food may be diminished by a lower concentration of essential nutrients. In this connection the nature of the algal symbiont may be of importance. Lichens with the nitrogen-fixing bluegreen algae may have higher nitrogen contents. The symbiont in the case of the lichens examined in this study is a green alga. Our experiments show definitely that thallus structure is another important factor in mite feeding preferences. The relative importance of secondary products and other factors influencing food preferences may vary among lichen species. These species may be expected to differ in secondary product concentrations. Species with high growth rates, for example, may be able to compensate for herbivore damage, and there may be less selection for antiherbivore defense in these species.

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INTRANIDAL WORKER REACTIONS TO VOLATILE COMPOUNDS IDENTIFIED FROM CEPHALIC SECRETIONS IN THE STINGLESS BEE, *Scaptotrigona postica* (HYMENOPTERA, MELIPONINAE)

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Abstract—From pentane extracts of worker heads of the stingless bee (*Scaptotrigona postica*), 70 volatile compounds were identified by combined gas chromatographic–mass spectroscopic analyses. A bioassay was developed to evaluate intranidal reactions of workers to synthetic volatiles. Thirty-six of the cephalic volatiles were tested. Thirteen types of behavioral reactions were recorded in a semiquantitative manner. The test was run in the brood nest where mainly young nurse bees are present and also in the storage area of the nest with old foragers traffic. The results obtained were compared and discussed in order to understand the chemical communication system of this species. Especially in the dark interior of the nest, which in nature is found in hollow tree cavities, chemical messages obviously play a particularly important role in the communication systems of the bees.

Key Words—Cephalic volatiles, stingless bee workers, intranidal reactions, *Scaptotrigona postica*, Meliponinae, Apidae, Hymenoptera.

INTRODUCTION

The enormous behavioral diversity in the more than 300 species of stingless bees (Hymenoptera: Apidae, Meliponinae) seems to depend on elaborate com-

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munication systems. Ritualized movements and sound production, as evident in the dance language (von Frisch, 1967) of honeybees (Apinae), seem to be of minor importance in stingless bees (Kerr and Esch, 1965; Gould et al., 1985). They widely use chemical information (Kerr, 1960; Kerr et al., 1963). Scent marks placed by scout bees guide recruited foragers to food sources (Lindauer and Kerr, 1958; Kerr et al., 1981). Massive colony defense is released by alarm pheromones (Blum, 1981) or kairomones (Wittmann, 1985). Drone mating assemblies are probably stabilized by a male-specific aggregation pheromone (Engels and Engels, 1984).

We hypothesize that in stingless bees an expressive chemical communication system is essential for their highly evolved social organization. Nothing is known about pheromones involved in reproduction (Sakagami, 1982; Wille, 1983) and only little about chemocommunication in the dark interior of nest cavities (Keeping et al., 1982). Therefore, we developed a bioassay to be employed within the nest.

Recently we reported a considerable age- and function-dependent variation in the numerous cephalic volatiles found in workers of *Scaptotrigona postica* (Francke et al., 1983); the list of identified compounds is completed here. In order to decode the information transmitted by volatiles, we only tested in this first study worker reactions to single synthetic compounds. The objective was to clarify whether all identified constituents of the cephalic volatiles would release a specific behavioral response. The expected results will enable us to design biotests with various mixtures of volatiles.

METHODS AND MATERIALS

Bees. Free flying *Scaptotrigona postica*[4] (Latreille) colonies of normal size, as found in natural nests, were kept in indoor observation hives in the bee laboratory of the Department of Genetics on the Ribeirão Preto Campus, Sao Paulo State, Brazil.

The nest boxes constructed for the bioassay (Figure 1) are subdivided into a brood nest and a storage part, connected only by two narrow tunnels to avoid rapid odor exchange. The nest entrance leads to the storage area. The brood nest consists of horizontal combs built from bottom to top. New vertical cells are added on the edge of the combs. Following the provisioning of a completed cell with larval food by the nurse bees, and ovipositioning by the queen, the cell is sealed. The brood nest is normally surrounded by several thin layers of cerumen. The uppermost part of this involucrum was always removed in order

[4] According to the subdivision of the *Scaptotrigona postica* superspecies as proposed by Moure (1942), our bees belong to *Sc. depilis.*

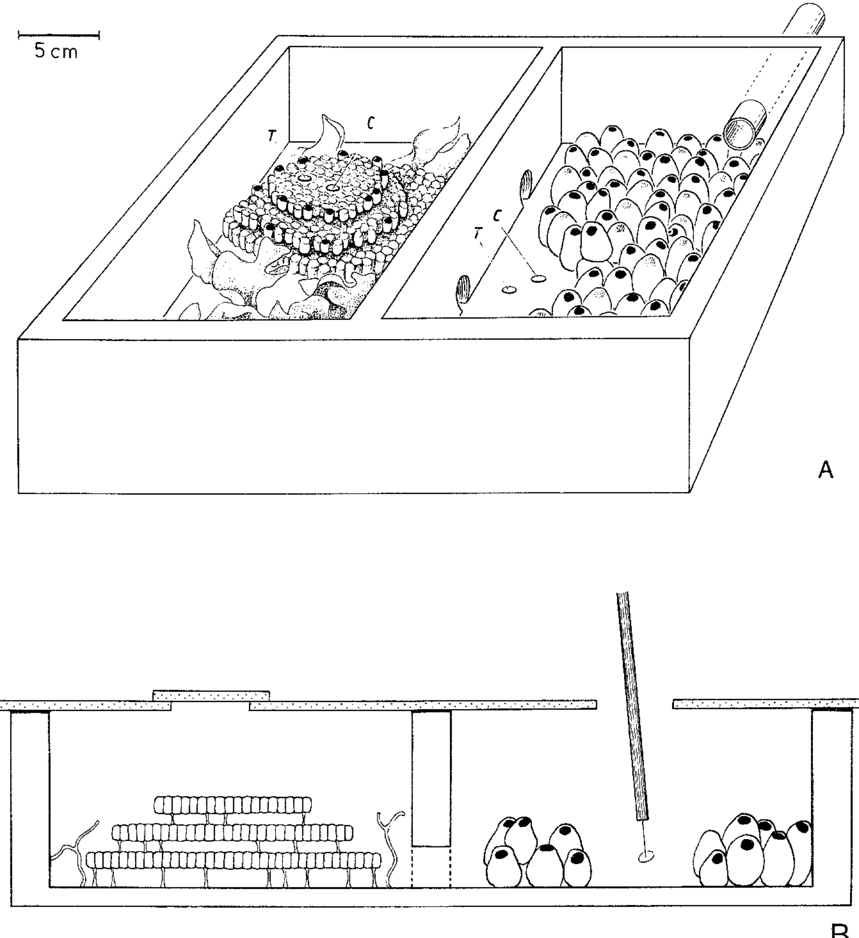

FIG. 1. Experimental wooden nest box used in the volatile bioassay with free flying stingless bee (*Scaptotrigona postica*) colonies. (A) Overview, (B) Cross-section. In order to have two test compartments, the nest box was separated into the brood nest (left) and the storage area (right) connected by two tunnels. The entrance tube led to the storage area with honey and pollen pots which in part are closed. The nest box was covered with a glass plate. Through perforations, normally closed by small pieces of glass, the wax test disks could be manipulated by a stickpin. The test disks were placed on the bottom of the box in a corner free of pots or on the uppermost brood comb. Bees are not shown in this drawing. T = beeswax test disk impregnated with pentane-dissolved synthetic volatiles, C = control disk, impregnated with pure pentane or blank.

to use the upper comb as a test platform. Tests were never run simultaneously in the brood nest and storage area.

Identification of Compounds. Pentane extracts of the heads of old adult worker bees were analyzed using a gas chromatographic–mass spectroscopic coupling system (Varian MAT 311 A) as previously described (Francke et al., 1983). Up to now 70 volatile compounds have been identified, and their relative proportions have been determined. The total amount of volatile material per individual was found to range between 150 and 250 μg. However, the absolute amount of compounds secreted by the bees to produce a distinct odor signal is as yet unknown. The purity of test compounds was higher than 98%; the chiral compounds were racemic. Single components were used as 5% pentane solutions.

Bioassay. Thirty-six of the identified compounds were bioassayed (Table 1). Small disks of beeswax (8 mm diameter) were punched out of comb foundation. Test solutions were applied on the wax disks in aliquots of 1, 2, or 5 μl. According to the actual concentrations of the respective compounds in natural secretions (Table 1), the applied volumes of the test solutions represent 1–500 bee equivalents. Due to the lack of "natural solvents or fixatives" such as high-boiling hydrocarbons and lipids present in the natural secretion, the synthetic compounds evaporated more rapidly during the test runs. The impregnated test disk was immediately transferred into the colony by spitting it on top of a fine pin to avoid tool contamination. The bottom of the storage area or the uppermost brood comb were used as a test compartment. The glass lid of the observation box was perforated to facilitate handling of the wax disk. A blank disk was always placed a few centimeters away from the test disk (Figure 1). After termination of the observation period, the test disk was removed and discarded in order to avoid habituation to odors. Tests were carried out on three different colonies placed in the same observation laboratory. The study site has a wet tropical climate. The tests were usually run in the morning and afternoon and only under favorable weather conditions for normal foraging. Ambient temperatures were between 25 and 35°C. Testing was carried out from October 1983 to November 1984, excluding the dry months June, July, and August, and during March and April 1985.

Bioassay Records. The reactions of the worker bees in the tests were highly specific. General and special behavioral responses could be distinguished (Figure 2). The course of the reactions was recorded every 30 sec over a period of 3–5 min. The number of bees exhibiting any type of reaction towards the test disk was noted and compared with the bees' behavior around the control disk. Our purpose here was to guarantee that specific volatile-released behavior was recorded and not circumstantial excitement. Depending on the type and intensity of the reactions observed, a break of at least 5 min was made between consecutive tests. All compounds were tested on different days, at least two to

TABLE 1. VOLATILE SUBSTANCES IDENTIFIED FROM PENTANE EXTRACTS OF OLD WORKER HEADS (*Scaptotrigona postica*)[a]

Substance class (No. of compounds)	Compound	%	Tested
Esters (31)	(Z)-7-Hexadecenyl butanoate	16.70	x
	(Z)-5-Tetradecenyl butanoate	9.80	x
	Hexyl hexanoate	1.90	
	Tetradecyl butanoate	1.60	x
	2-Pentadecyl butanoate	1.40	
	γ-Decalacton	1.40	x
	δ-Decalacton	1.40	x
	Hexyl butanoate	0.80	
	Hexyl (*E*)-2-hexenoate	0.80	x
	2-Heptyl butanoate	0.50	
	2-Heptyl hexanoate	0.40	x
	3-Methylbutyl butanoate	0.40	
	2-Heptyl (*E*)-2-hexenoate	0.40	x
	Benzyl hexanoate	0.40	
	3-Methylbutyl (*E*)-2-hexenoate	0.40	x
	3-Methylbutyl hexanoate	0.30	x
	2-Tridecyl butanoate	0.30	
	Dodecyl butanoate	0.30	x
	Hexyl benzoate	0.30	
	3-Methylbutyl acetate	0.30	
	Hexyl acetate	0.30	
	(Z)-5-Tetradecenyl acetate	0.20	
	2-Methylpropyl butanoate	0.15	
	Ethyl hexanoate	0.15	
	3-Methylbutyl 3-methylbutanoate	0.15	
	2-Hexyl hexanoate	0.15	x
	Heptyl butanoate	0.15	
	2-Pentyl hexanoate	0.05	x
	Hexyl butanoate	0.05	
	Hexyl 3-methylbutanoate	0.05	x
	Hexyl pentanoate	0.05	x
		41.25	15
Alcohols (17)	2-Heptanol	8.60	x
	(Z)-7-Hexadecen-1-ol	6.60	
	(Z)-5-Tetradecen-1-ol	4.10	
	2-Tridecanol	2.70	x
	2-Pentadecanol	2.70	x
	1-Tetradecanol	1.10	
	2-Phenylethanol	0.80	x
	1-Hexanol	0.70	x
	2-Nonanol	0.70	x
	2-Undecanol	0.50	x
	1-Dodecanol	0.50	
	3-Pentanol	0.30	

TABLE 1. (*Continued*)

Substance class (No. of compounds)	Compound	%	Tested
	2-Pentanol	0.30	
	1-Heptanol	0.30	
	3-Methyl-1-butanol	0.20	x
	2-Hexanol	0.20	x
	2-Octanol	0.10	x
		30.40	10
Carbonyl compounds (10)	2-Tridecanone	11.70	x
	2-Pentadecanone	5.80	x
	Benzaldehyde	1.90	x
	2-Heptanone	1.10	x
	2-Undecanone	0.80	x
	2-Nonanone	0.40	x
	2-Pentanone	0.30	
	(Z)-6-Undecen-2-one	0.10	x
	(Z)-6-Tridecen-2-one	0.10	x
	(Z)-8-Tridecen-2-one	0.10	x
		22.30	9
Carboxylic acids (5)	Geranoic acid	2.10	
	Nerolic acid	2.10	
	Butanoic acid	0.40	x
	Pentanoic acid	0.40	
	Hexanoic acid	0.40	x
		5.40	2
Hydrocarbons (7)	Tridecane	0.15	
	Pentadecane	0.15	
	Dodecane	0.10	
	Tetradecane	0.10	
	Undecane	0.05	
	p-Xylene	0.05	
	m-Xylene	0.05	
		0.65	0
Total compounds (70)		100.00	36

[a]The compounds of the different classes are listed according to their relative proportions found in the natural extract. Only substances contributing at least 0.05% were listed. Of the 70 cephalic volatiles, 36 have been used in the bioassay.

three times, in changing random sequences. If alarm was released, a long break was organized so that normal behavior could be reestablished.

Biotest Evaluation. Our semiquantitative records were scaled according to the following classes of reaction intensity: 0 = no reaction; 1 = specific reaction seen in 1–5 bees at the same time or during the first 3 min; 2 = the same

FIG. 2. Intranidal reactions of stingless bee workers (*Scaptotrigona postica*) to pure synthetic volatiles. Semiquantitative records, heights of bars (cf. bottom right) represent means of reaction intensity (1–5) shown in 2–5 test series. Specific reactions released by the various compounds may be compared by reading the table horizontally; vertical reading shows the pattern of different reactions evoked by a single compound. Solid and open bars represent the response obtained in the storage area and the brood nest, respectively. As can be seen from the chart, all single volatiles released a typical behavior.

for 6–10 bees; 3 = 11–15 bees; 4 = 16–25 bees; 5 = 26 bees or more, mostly all bees of the area reacted, i.e., several hundred bees.

RESULTS

Analysis of Cephalic Volatiles in Old Worker Bees. As an increase with age in *Scaptotrigona postica* worker cephalic volatile concentrations was found in most of the compounds (Francke et al., 1983), we used head extracts from old guarding and foraging bees in the present analyses. We could now complete our previous list to 70 compounds (Table 1). Within this bouquet, there is a predominant number (31 identified compounds) and total amount (more than 40% of all volatiles) of esters, (Z)-7-hexadecenyl butanoate alone contributing 16.7%. The 17 alcohols represent about 30%, the 10 carbonyl compounds about 20%, the 5 carboxylic acids about 5%, and 7 hydrocarbons less than 1% of the extract mixture. In this complex multicomponent blend, the seven most abundant compounds (2-tridecanone, 2-pentadecanone, (Z)-7-hexadecenyl butanoate, (Z)-5-tetradecenyl butanoate, the two corresponding secondary alcohols, and 2-heptanol) make up more than 60% of the bouquet. Trace compounds contributing less than 0.05% were not analyzed and are neglected here. About half the identified compounds (36 of the total of 70, Table 1) have been tested. The results are summarized in Figure 2.

General Observations. Stingless bees, especially of this species, immediately remove small intruders or particles found within the nest. Large objects which cannot be bitten into fragments are plastered with cerumen. Pieces of paper, foam rubber, isopore, or wood as well as dead bees—if placed in the nest—always released local alarm, intensive handling or plastering and, therefore, were unsuitable carriers of test volatile compounds. As cerumen is a somewhat changing mixture and normally attractive in itself for all workers, we could not use this material. We found that melted honeybee wax is indifferent and a suitable material for exposing pentane solutions of volatile test compounds.

Reaction Types. We were surprised to find that all the 36 individual compounds tested evoked different behavioral responses. As pointed out above, we tried to avoid any habituation or even conditioning. In the test colonies containing thousands of bees, most of the workers probably reacted only once or a few times to a test disk.

The behavioral responses observed were highly stereotypic and can be easily classified into three general and ten specific reactions (Figure 2). Except for some antennating, pure pentane never released any specific response. As in preliminary tests, other hydrocarbons only released weak or unspecific reactions; at a later stage this class of compounds (Table 1) was not tested extensively.

Overall Reactions. Of the different classes of compounds tested (Table 1), the alcohols released the most diverse and intensive reactions, followed by the esters and the carbonyl compounds. The two carboxylic acids also evoked strong reactions (Figure 2). There was no uniform response to compounds belonging to the same chemical class. Alarm can be taken as an example for this, representing the typical social behavior of colonial bees. In our intranidal bioassay situation, intense alarm was released by six alcohols, two esters, one carbonyl compound, and one carboxylic acid. The response recorded normally included (especially in the brood nest) several other reactions like antennating, cleaning the antennae, fanning, and often also the plastering of the test disk with cerumen particles. But only one alarming compound, 2-heptanol, additionally released the mandibulating of the test disk. This reaction, on the other hand, was also seen after exposing nonalarming attractive odors, and mainly in the storage test area (Figure 2).

General Reactions

1. Attractive. Many workers approached the test disk. Most of the alcohols, several esters and carbonyl compounds, but not the two carboxylic acids tested, were attractive to the bees.

2. Repellent. Bees avoided contact with the test disk. If they got close to it, they immediately retreated. Butanoic and also hexanoic acid were found to release a pronounced repellence both in the brood and storage area of the nest. Besides this, one alcohol, 2-phenylethanol, and one carbonyl compound, (Z)-8-tridecen-2-one, were also strongly repellent, but only in the storage area of the nest.

3. Alarm. Many bees became nervous, ran away, hid, or sought an intruder to attack it. Two alcohols, 2-octanol and 2-heptanol, the ketone 2-nonanone, and butanoic acid released a "big alarm." Several other alcohols and two esters also caused alarm, but to a minor extent.

Specific Reactions

4. Antennate. Most of the compounds tested here caused the bees to antennate more or less intensively. This reaction was more pronounced in the brood nest.

5. Clean antennae. Nearly all antennating was immediately followed by a cleaning of the antennae with a corresponding intensity. Remarkable cleaning activities were released by the two carboxylic acids and the alcohol 2-phenylethanol both in the storage and brood nest.

6. Lick test disk. Approximately half the compounds tested, except for the carboxylic acids, were licked from the test disk. But only a few bees always exhibited this reaction.

7. Fan. The fanning behavior was correlated with alarm in most of the tests and was particularly observed in the alcohols and the carboxylic acids.

None of the esters and only one carbonyl compound, 2-nonanone, released a strong fanning reaction.

8. Bite test disk. This reaction was one of looking aggressive and was especially observed when 2-heptanol impregnated disks were exposed in the brood nest. 2-Phenylethanol and some of the esters released medium attacks. Many other compounds also released disk biting reactions, but only in a few bees.

9. Wing beat. This very characteristic response was directed towards other bees and was observed exclusively in the brood nest. This behavior was released by many compounds, but was especially pronounced when testing the alcohols hexanol, 2-octanol, 2-nonanol, the ester 2-heptyl (*E*)-2-hexenoate, and benzaldehyde.

10. Attack. Many different volatiles evoked aggressive worker interactions. The attacking of other bees was pronounced in the brood nest, where the esters hexyl 3-methylbutanoate and 2-heptyl hexanoate, apart from benzaldehyde, were the most effective aggression releasers.

11. Trophallaxis. A few food-exchange reactions were recorded in many tests, especially with the alcohols. Relatively frequent trophallaxis was noted when testing the ester hexyl pentanoate.

12. Attach cerumen on test disk. This reaction was rare, but also very characteristic. It was released by five of the alcohols, especially by hexanol and 2-heptanol, and a few times also by two esters and two carbonyl compounds. Never more than a few bees deposited pieces of cerumen on the test disk, and there was some correlation with weak alarm reactions.

13. Sit on test disk. This rare, but also highly specific, behavior was released by several compounds of all classes and was mainly observed in the brood nest. The same worker bee was often standing on the wax disk and defending it. If the disk was abandoned, sometimes another worker immediately occupied it.

Reactions to Chemically Related Compounds. Of several possible examples, the reactions released by 2-heptanol and derived esters and carbonyl compounds will be compared here (Figure 2). 2-Heptanol was highly attractive and alarming and also caused intensive fanning and mandibulating of the test disk, especially in the brood nest. 2-Heptyl hexanoate provoked similar reactions, but to a lesser extent and was more pronounced in the storage area. Apart from this, some wing beating and, in particular, attacks on other bees were recorded in the brood nest. 2-Heptyl (*E*)-2-hexenoate was extremely attractive both in the brood and storage area, but only caused minimal alarm in the latter part of the nest. 2-Heptanone clearly released a weaker response. The compound was only slightly attractive in the storage nest. No alarm was recorded at all. The pattern of other specific reactions was a mixture of the responses recorded while testing the alcohol and the esters.

Reaction Patterns. None of the 36 compounds tested only evoked a single specific reaction (Figure 2). The minimal response was obtained with 2-hexyl hexanoate which released four types of reactions, the maximal with 2-phenylethanol and 2-nonanone, both resulting in 12 of the 13 different reactions. The average number of different reactions released by one compound was 9.8 in the alcohols, 8.9 in the carbonyls, 8.2 in the esters, and 5.5 in the carboxylic acids.

There was considerable variation in the type and intensity of reactions recorded in the brood and in the storage test areas. Some compounds evoked exactly the same, others extremely different responses in both parts of the nest. Generally speaking, the bees in the brood nest reacted more strongly than those in the storage area (Figure 2). For instance 2-tridecanol released only two weak reactions in the storage part, but nine of medium intensity in the brood nest.

DISCUSSION

Chemistry of Cephalic Volatiles of Scaptotrigona postica *worker bees.* The overall pattern in the volatile cephalic secretion of *Sc. postica* resembles that of other stingless bees, especially of the tribe Trigonini (Blum et al., 1970; Luby et al., 1973; Blum, 1981; Duffield et al., 1984; Johnson et al., 1985), but also of the Meliponini (Smith and Roubik, 1983). With only a few exceptions (3-methyl-1-butanol and its derivatives, the aromatic compounds, and the terpenoid acids), the identified oxygen-containing compounds obviously originate from the acetate pool and form typical rows of bis homologs. Two principal groups of compounds may be distinguished: one represents methylketones and some of their corresponding chiral reduction products, the methylcarbinols. The second comprises primary alcohols, acids, and the respective esters.

Methylketones and methylcarbinols are particularly widespread among Hymenoptera (Blum, 1981). They also occur as plant volatiles and are probably formed from β-ketoacyl precursors. A particular species may produce them in distinct qualitative and quantitative proportions, and they certainly play a role as intra- and interspecific chemical messengers. It is possible that enantiomeric composition in chiral alcohols and double-bond positions in unsaturated ketones contribute to the specificity of the bouquet. Enantiomeric composition of chiral pheromones often plays an essential role in the optimal activity of a signal. In *Sc. postica* the 2-alkanols could be shown to be approx. 95:5 mixtures of *S*- and *R*-enantiomers (König and Francke, unpublished data). Preliminary tests with a synthetic mixture of enantiomers revealed that the "natural" 2-heptanol evokes a particularly strong alarm and, in contrast to pure (*R*)-(−)-2-heptanol, induces the attaching of cerumen to the test disk. 2-Heptanol was also identified as a major cephalic volatile in other stingless bees of the genera *Trigona* (Kerr et al., 1981) and *Melipona* (Smith and Roubik, 1983), but nothing is known about enantiomeric differences in these cases.

The unsaturated methylketones of *Sc. postica* carry the double bond at even-numbered positions, while the unsaturated esters and primary alcohols show uneven-numbered double-bond positions. Desaturase systems of insects usually insert double bonds at uneven positions in the acyl precursors of respective compounds (Roelofs and Bjostad, 1984). Since during the formation of methylketones by β-oxidation–decarboxylation reactions, the initial C-1 is lost, the double bonds appear to "move" one position towards the new functional end. Because of their stereotyped occurrence and less variable chemical structures, saturated unbranched methylketones and methylcarbinols seem to be less suitable for generating a volatile signal which a conspecific receiver may unambiguously distinguish from the "background noise" caused by the environmental odors.

In contrast, esters form a particularly versatile class of compounds. They may vary in chain length on both sides of the functional group; the insertion of double bonds provides additional information (Francke et al., 1984). (*Z*)-9- and (*Z*)-11-tetradecenol as well as (*Z*)-11-hexadecenol, together with the corresponding acetates, are widespread sex pheromones of Lepidoptera. Respective compounds from *Sc. postica* carry the double bonds in positions 5 and 7 and, thus, are more closely related to certain ant volatiles which show the identical distribution of double bonds (Francke et al., 1985).

It should be mentioned that volatile constituents from individuals of colonies kept in Brazil and Germany were qualitatively identical. Relative proportions were also found to be the same. However, the total amount of volatiles in bees collected in Brazil was higher.

Wax Disk Setup as Behavioral Bioassay to Check Communicative Functions of Volatiles in Bees. Previously there has been no bioassay to test behavioral responses of stingless bees to volatiles within the nest (Hummel and Miller, 1984). In the only intranidal tests yet described (Keeping et al., 1982), dichloromethane head extracts were used. The extracts were placed on a 18-mm-diameter filter paper disk which was located on a piece of paper in the nest container. Reactions were recorded within the surface of the paper. Wing fanning, antennal contacts, walks onto the test disk, and repellence were observed, out of which contacts and repulsions were quantified. In our tests, *Sc. postica* workers attacked all test papers placed in the nest, but they tolerated beeswax. Control wax disks did not interfere with normal behavior at all. As such disks could easily be manipulated in different parts of the nest, our bioassay could be run in the brood nest and in the storage area where distinct age groups of workers are present. Keeping et al. (1982) found *Trigona gribodoi* nurse bees to be totally oblivious to the pheromones exposed. In contrast to this, *Sc. postica* nurse age workers showed pronounced reactions to synthetic volatiles (Figure 2).

In most of the few other papers dealing with the reactions of stingless bees to volatile chemicals, tests were run at the nest entrance. The test samples were

exposed on small rods (Weaver et al., 1975), pieces of filter paper (Johnson et al., 1985), or a cotton wool ball (Kerr et al., 1981). The few behavioral responses obtained in such situations were mainly those of alarm together with attack or repellence, and much circumstantial excitement was observed. Artificial trails made up of filter papers treated with 2-heptanol—the main compound in cephalic extracts of *Trigona spinipes*—were attractive to foraging workers. Therefore, 2-heptanol was considered to be the major trail pheromone constituent in this species (Kerr et al., 1981).

Our intranidal wax disk setup seems to have some advantages when compared with techniques used until now in bioassays of volatiles of stingless bees. The behavioral responses observed could be reproduced sufficiently, and the specific responses consisted of typical sets of a varying number of distinct reactions (Figure 2).

Cephalic Secretion of Scaptotrigona postica*: A "chemical language"?* Today it is generally accepted that in odor communication, insects use mixtures instead of single compounds, and pattern recognition plays an essential role (Schneider, 1980). Stingless bees seem to use a particularly complicated "chemical language." The complex multicomponent mixture secreted by *Sc. postica* is an excellent prerequisite for the formation of unique polyfunctional messages. Depending on the differences in the thermodynamic stability of various components and differences in their vapor pressure, the composition of a released signal will change in a well-defined way and thus inform the receiver about the age of the message and the distance to its origin. Since the composition of the cephalic secretion varies characteristically with age, an individual bee should also be able to recognize the age of its "interlocutor" and its social function within the colony.

As shown in Figure 2, the response to a specific and pure volatile was not one single specific reaction, but a set of reactions. This is true for all 36 synthetic compounds tested and could be interpreted in such a way that the message represents a "sentence." Such a "language" might use classes of compounds for general instructions: for instance, alcohols as alarm releasers, esters as attractants, and carboxylic acids as repellents. But the diversity of test responses recorded here do not fit into such a simple scheme. The comparison of test results revealed that all 36 compounds evoked specific response patterns (Figure 2). For example, the pronounced repellent effects of four compounds within the storage area included not only intensive antennating and cleaning the antennae, but also fanning. Besides this, 2-phenyl-ethanol, (*Z*)-8-tridecen-2-one, and hexanoic acid released some trophallaxis, but butanoic acid did not. The mandibulation of the test disk was seen only in the experiments with the repellent alcohol and carbonyl compounds, but not in the two carboxylic acids. It would seem that the complete information given by the four repellent substances tested here varied to a considerable extent.

Bioassay Reactions Compared with Behavioral Repertoire. Differences in

the responses obtained by the tests in the brood nest and the storage area probably depend on the differences in age and function of the worker bees involved (Engels et al., 1980; Sakagami, 1982). Worker polyethism in stingless bees is pronounced, and young house bees mainly build combs and attend the brood, whereas old field bees forage. We previously described corresponding age-specific variations in the cephalic volatiles in *Scaptotrigona postica* (Francke et al., 1983). Correlated differences in worker responsiveness to pheromonal signals can be expected and have to be tested in more detail.

As young bees contain fewer cephalic volatiles and smaller amounts (Keeping et al., 1982; Francke et al., 1983), it is possible that their use of the chemical communication channel is more restricted and exclusively concerned with intranidal communication. Nothing is known about this, but as the nest interior of all stingless bees is completely dark, only tactile, acoustic, or olfactory stimuli can be used there for interindividual messages. In such an enclosed area with a low level of "background noise," only a simple bouquet is needed for chemical communication.

Old bees defend the nest, carry out garbage, and forage and, therefore, need alarm systems, chemical recruitment, scent marking of the nest entrance as well as of forage trails and routes to filial nests, etc. (Kerr, 1960; Kerr et al., 1963). This broad polyethism (Simões and Bego, 1979) is, of course, related to different ecological situations and probably requires a corresponding complexity of chemocommunication, especially in old worker interactions. Besides this, worker–queen interactions have to be considered. Within the nest, the complicated oviposition behavior of stingless bees (Sakagami and Zucchi, 1963) will probably involve pheromonal messages. Nothing is yet known about these interesting questions. This is also the case for mating and swarming behavior (Imperatriz-Fonseca, 1977) and male aggregations (Engels and Engels, 1984). Consequently, future biotests must include queen and drone reactions. In addition to the test of single compounds, work is in progress to test bioassay mixtures of identified compounds in naturally occurring proportions. Enantiomeric composition of chiral compounds must be determined and tests with respective optically active samples should be carried out.

Various reactions can be reinforced by the release of pheromones by the reacting bees. Under natural conditions, the response to a chemical signal will often include the emittance of a second one. "Chemical dialogues" are probably important elements of behavioral sequences. Of course, any analysis of such complicated relationships requires fundamental knowledge not only of the behavior (Simões and Bego, 1979; Engels et al., 1980), but also of the complete life history of a given species. Although *Scaptotrigona postica* is one of the best known stingless bees (Sakagami, 1982; Engels and Engels, 1984), our knowledge does not yet suffice, especially as this species is considered to have highly evolved communication systems (Lindauer and Kerr, 1958; Kerr et al., 1963).

The data presented here concerning the spectrum of cephalic volatiles and odor-specific reactions in stingless bee workers should be regarded as initial steps. Further work is in progress to investigate the role that chemical messengers play in the sociobiology and ecology of these fascinating tropical insects.

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SEX PHEROMONES OF THE SEA LAMPREY (*Petromyzon marinus*): Steroid Studies

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Abstract—Pheromone-containing and pheromone-devoid samples of male sea lamprey (*Petromyzon marinus*) urine were analyzed for the concentrations of nine steroids [dehydroepiandrosterone (DHEA), testosterone (T), dihydrotestosterone (DHT), progesterone (P), androstenedione (A), estrone (E_1), estradiol (E_2), corticosterone (B), and cortisol (F)] by radioimmunoassay (RIA). Samples analyzed included native urine that had been enzymatically hydrolyzed with mixed β-glucuronidase/sulfatase. Values of the analyses were used to prepare solutions of the individual steroids for bioassay at concentrations which bracketed the urinary concentrations. Results show that only testosterone elicited a preference response in spawning-run female sea lampreys, and in concentrations three to four orders of magnitude greater than those found in active, unhydrolyzed male urine. The possibility that testosterone acts as a pheromone in this species is discussed.

Key Words—Sea lamprey, *Petromyzon marinus*, pheromone, urine, steroids, radioimmunoassay, RIA, behavior.

INTRODUCTION

Intraspecific chemical signals (pheromones) have been implicated in a variety of behavioral processes in fish, including schooling, parent–young interactions, homing, pair formation, and spawning (Solomon, 1977; Liley, 1982). Preferences of spawning-run landlocked sea lampreys (*Petromyzon marinus*) for substances released by sexually mature conspecifics of the opposite sex indicate that pheromones may play a role in the reproductive behavior of this species (Teeter, 1980). Pheromone release in sea lampreys coincides with the appearance of secondary sex characteristics. At this time, male sea lampreys release in their urine a pheromone that is attractive to female conspecifics (Teeter, un-

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published observation). The possibility that this pheromone could be steroidal in nature led us to assay both pheromone-containing (behaviorally active) and pheromone-devoid (behaviorally inactive) male urine for its content of several metabolically important steroids; the values obtained from this analysis were then used to establish concentrations for examining the effects of these compounds on the preference behavior of female sea lampreys.

METHODS AND MATERIALS

Spawning-run sea lampreys (*Petromyzon marinus*) were captured in tributaries of Lake Huron and held in large raceways supplied with lake water at the Hammond Bay Biological Station, Millersburg, Michigan. Urine from both sexually immature and sexually mature male sea lampreys (approximately 400 animals per collection, four collections per day) was expressed by applying gentle abdominal pressure and allowing urine to spray into a Teflon trough. During the collection, the animals were held well away from the trough to ensure that no water, skin mucus, or other material contaminated the urine. Once in the trough, the urine was collected by pipet, pooled in daily batches, and frozen for subsequent bioassay and analysis. The results of previous experiments (Teeter, 1980) showed that the presence of a sex pheromone in male urine (obtained as previously described or by catheterization) was associated with the presence of secondary sex characteristics; milt did not, however, elicit preference responses in spawning-run females. Consequently, urine was collected from "sexually immature" males (those lacking secondary sex characteristics) and from "sexually mature" males (those which displayed secondary sex characteristics). Urine from spermiated males (that which contained a noticeable quantity of milt) was kept in a separate pool and was not used in this study. The urine was separated into pools on the basis of positive or negative preference assays, as described below.

Bioassays. Pooled male urine and steroid solutions were assayed as previously described (see Lisowski et al., 1986, for details of apparatus and procedures) for pheromone content (behavioral activity) using spawning-run female sea lampreys as test subjects in a two-choice preference test tank. As an example of a representative test, females were given a choice between a compartment containing lake water and a compartment containing lake water with 13 μl/liter male urine. Data were analyzed using the Wilcoxon matched-pairs, signed-ranks test (Brown and Hollander, 1977). Presence of a pheromone was indicated if females spent a significantly longer time in the stimulus arm of the apparatus than in the lake water arm. Gender of all animals used in the study was confirmed by autopsy.

Steroid Extraction and Separation. Ten-milliliter aliquots of male sea lamprey urine were used for the radioimmunoassays (RIAs). For samples that were

to be hydrolyzed, a mixture of 15,000 Fishman units of *Helix pomatia* β-glucuronidase/aryl sulfatase (Sigma Chemical Co., St. Louis, Missouri) in 0.8 ml water and 1.2 ml of 1.2 M acetate buffer (pH 4.8) was added; the hydrolysis was allowed to proceed at 37°C for 2 hr. At the end of the hydrolysis period, the mixture was extracted twice with 20-ml portions of acid-washed diethyl ether. Unhydrolyzed samples were also extracted twice with 20-ml portions of diethyl ether. These extracts were dried under a gentle stream of nitrogen in a warm (50°C) sand bath, redissolved in 10 ml of acid-washed diethyl ether, washed with 0.5 ml of 0.5% $NaHCO_3$, and dried again. Each extract was dissolved in 0.1 ml of a mixture of (all freshly distilled) chloroform–*n*-heptane–methanol–water, 500:500:75:3 (Zamecnik et al., 1977) and fractionated on a 2.5-g (8-ml) Sephadex LH-20 column, eluting with the same mixture. Elution patterns and recovery values of tritiated steroids run on these columns are given in Table 1. The glass columns (8 mm ID, acid-washed) and polyethylene fittings (QSH Practicolumns, Isolab Co., Akron, Ohio) were treated for 1 hr with 1% trichloromethylsilane (Aldrich Chemical Co., Milwaukee, Wisconsin) in chloroform prior to packing. Material eluted from the columns was dried and taken up in 1.0 ml steroid radioimmunoassay buffer (0.1 M sodium phosphate in saline, pH 6.9, containing 0.1% gelatin and 0.01% thimerosal).

Steroid RIA. All determinations were done on precoded samples whose identity was known only to an investigator not directly involved in the analytical procedures. Aliquots of the samples in steroid RIA buffer were analyzed in specific RIA systems (Table 2). The following antibodies and tritiated steroids were used: (1) rabbit anti-progesterone-11α-hemisuccinyl-bovine serum albu-

Table 1. ELUTION PATTERNS AND RECOVERY VALUES OF STANDARD (PREPURIFIED) STEROIDS[a]

Fraction no. (and ml eluted)	Steroid eluted		Recovery (%)
	Trivial name	Systematic name	
1. (0–2.5)			
2. (2.5–5.5)	Progesterone (P)	4-Pregnen-3,20-dione	98
	Androstenedione (A)	4-Androsten-3,17-dione	96
3. (5.5–9.0)	DHEA	5-Androsten-3β,-ol-17-one	91
	Testosterone (T)	4-Androsten-17β-ol-3-one	94
	DHT	5-Androstan-17β-ol-3-one	87
4. (9.0–14.0)	Corticosterone (B)	4-Pregnen-11β,21-diol-3,20-dione	96
	Estrone (E_1)	1,3,5(10)-Estratrien-3-ol-17-one	86
5. (14.0–24.0)			
6. (24.0–33.0)	Cortisol (F)	4-Pregnen-11β,17α,21-triol-3,20-dione	72
	Estradiol (E_2)	1,3,5(10)-Estratrien-3,17β-diol	94

[a]Steroids eluted according to Zamecnik et al. (1977) from 2.5 g Sephadex LH-20 columns.

Table 2. CROSS-REACTIVITY VALUES[a] OF COELUTED STEROIDS IN RIA SYSTEMS USED DURING STUDY

Fraction	Steroid tested	Coeluted steriod's RIA system	Cross-reactivity value
2	Progesterone	Androstenedione	0.01
	Androstenedione	Progesterone	0.01
3	DHEA	Testosterone	0.01
	DHEA	DHT	1.46
	Testosterone	DHT	19.71
	Testosterone	DHEA	0.01
	DHT	Testosterone	29.33
	DHT	DHEA	0.01
4	Estrone	Corticosterone	0.01
	Corticosterone	Estrone	0.01
6	Cortisol	Estradiol	0.01
	Estradiol	Cortisol	0.01

[a]Percent cross-reactivity determined at 50% binding of tested antibody.

min (BSA) and [1,2,6,7-^{3}H]progesterone, 89 Ci/mmol; (2) rabbit anti-androstenedione-7α-carboxyethylthioether-BSA and [1,2,6,7-^{3}H]-androstenedione, 114 Ci/mmol; (3) rabbit anti-testosterone-7α-carboxymethylthioether-BSA and [1,2,6,7,16,17-^{3}H]testosterone, 150 Ci/mmol; (4) rabbit anti-5α-dihydrotestosterone-1α-carboxyethylthioether-BSA and [1,2,4,5,6,7,16,17-^{3}H]-5α-dihydrotestosterone, 190 Ci/mmol; (5) rabbit anti-estrone-6-(*O*-carboxymethyl)oxime-thyroglobulin and [2,4,6,7-^{3}H]estrone, 85 Ci/mmol; (6) rabbit anti-estradiol-17β-6-(*O*-carboxymethyl)oxime-BSA and [2,4,6,7,16,17-^{3}H]-17β-estradiol, 152 Ci/mmol; (7) rabbit anti-cortisol-21-hemisuccinyl-thyroglobulin and [1,2,6,7-^{3}H]hydrocortisone, 93 Ci/mmol; (8) rabbit anti-dehydroepiandrosterone-15α-carboxymethylthioether-BSA and [1,2,6,7-^{3}H]dehydroepiandrosterone, 79 Ci/mmol; and (9) rabbit anti-corticosterone-21-hemisuccinyl-thyroglobulin and [1,2,6,7-^{3}H]corticosterone, 89 Ci/mmol. All the antibodies were produced by Miles-Yeda, Rehovoth, Israel, except anti-dehydroepiandrosterone (anti-DHEA, Sigma Chemical Co.) and anti-estradiol, which was a gift of Dr. D. T. Armstrong, London, Ontario, Canada; the tritiated steroids were purchased either from New England Nuclear, Boston, Massachusetts, or Amersham Corp., Arlington Heights, Illinois, and were purified before use (on the Sephadex LH-20 columns). Assays were performed as previously described (Katz et al., 1982).

Separation of bound from free steroids in the RIA tubes was effected by dextran-coated charcoal methodology. Centrifuged supernatants were counted

for radioactivity in ACS II aqueous scintillation solution (Amersham) using a Packard Instruments liquid scintillation spectrometer, model 2425, and were corrected for quenching. Data reduction was performed on a Hewlett-Packard 9815A/9871A desktop computer using log–logit transformations. Extraction and recovery procedures were validated using standard steroid mixtures as previously described (Katz et al., 1982).

Behavioral Activity Assays of Urinary Steriods. As little as 0.5 ml (6.5 μl/liter of water) of pooled urine from sexually mature male sea lampreys has been shown to elicit preference responses in females in our two-choice tanks (Teeter, 1980). We arbitrarily use 1 ml of urine per test (13 μl/liter) when determining whether or not a pool of urine contains the pheromone. Consequently, samples of the steroids were tested in the preference tank at the concentrations at which they were determined to be present in 1 ml of behaviorally active male urine by RIA ("active urine–unhydrolyzed," Table 3). Solutions of the steroids were prepared in ethanol and the appropriate amount of material delivered by microliter syringe to the stimulus side of the test tank. An equivalent amount of solvent was added to the blank side of the tank as a control in each experiment.

RESULTS AND DISCUSSION

Urine of sexually immature and sexually mature male sea lampreys was analyzed by RIA for the content of nine steroid hormones (dehydroepiandrosterone, testosterone, dihydrotestosterone, progesterone, androstenedione, estrone, estradiol, corticosterone, and cortisol). To measure the degree of steroid conjugation, both active and inactive urine were hydrolyzed with a sulfatase–

TABLE 3. STERIOD CONTENT OF NATIVE AND HYDROLYZED MALE SEA LAMPREY URINE, EITHER PHEROMONE-CONTAINING (ACTIVE) OR PHEROMONE-DEVOID (INACTIVE)[a]

	Active urine		Inactive urine	
Steroid	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed
DHEA	0	2.05 ± 0.43	10 ± 0.05	1.11 ± 1.41
T	1.28 ± 0.08	1.48 ± 0.04	0.42 ± 0.03	0.56 ± 0.04
DHT	0.15 ± 0.01	0.35 ± 0.01	0.18 ± 0.07	0.26 ± 0.01
P	0.38 ± 0.01	1.08 ± 0.05	0.67 ± 0.02	0.60 ± 0.02
A	1.02 ± 0.04	1.34 ± 0.19	1.13 ± 0.01	1.16 ± 0.06
E_2	0.44 ± 0.06	6.91 ± 0.86	1.64 ± 0.07	3.89 ± 0.20
E_1	0.84 ± 0.06	2.09 ± 0.09	0.60 ± 0.04	2.28 ± 0.10
B	0	19.42 ± 1.92	4.82 ± 0.27	15.30 ± 0.92
F	6.58 ± 0.61	9.65 ± 0.33	4.87 ± 0.50	5.21 ± 0.49

[a]All concentrations are ng/ml except DHEA, which are pg/ml.

TABLE 4. BEHAVIORAL PREFERENCE RESPONSE VALUES FOR SEVERAL MALE SEA LAMPREY URINARY STEROIDS, TESTED INDIVIDUALLY, AS MEASURED IN TWO-CHOICE TEST TANK (1 ml STIMULUS SOLUTION PER TEST)

Steroid	Stim. soln. concentration (ng/ml)	No. of animals	Mean % time (±SE) on stimulus side	Attractive?[a]
DHEA	100[b]	24	56.9 ± 6.2	N
	10[b]	23	53.4 ± 6.4	N
	1[b]	24	56.7 ± 6.1	N
	0.1[b]	24	52.9 ± 5.5	N
DHT	0.2	24	42.1 ± 4.9	N
P	0.4	24	57.1 ± 6.3	N
A	10	24	44.0 ± 6.5	N
	1	24	51.7 ± 5.3	N
	0.1	24	53.9 ± 5.3	N
E_1	10	24	57.9 ± 5.0	N
	1	24	52.4 ± 6.6	N
	0.1	24	55.4 ± 5.8	N
	0.01	24	53.8 ± 5.5	N
B	100	18	57.7 ± 6.0	N
	10	24	42.4 ± 5.4	N
	1	30	52.7 ± 4.4	N
F	100	24	53.0 ± 5.7	N
	10	24	49.9 ± 6.0	N
E_2	0.5	24	45.8 ± 5.8	N
Urine control (1 ml[c])		24	63.7 ± 5.0	Y

[a] $P < 0.05$, Wilcoxon matched-pairs signed-rank test, two-tailed; Y = yes, N = no.
[b] pg/ml.
[c] 1 ml of bioactive male urine used as a control.

glucuronidase enzyme mixture prior to RIA analysis. Numerical values for these analyses are presented in Table 3 and graphed in Figure 1.

Using results obtained with inactive, unhydrolyzed urine as baseline values, and comparing them with steroid concentrations found in active urine, one may note that testosterone increases threefold, and cortisol increases by 1.5 times; progesterone decreases twofold, and estradiol shows a fourfold decrease, while DHEA and corticosterone decrease to zero. DHT, androstenedione, and estrone concentrations remain roughly the same. Results for hydrolyzed and unhydrolyzed progesterone suggest the presence of a steroid capable of forming a conjugate and capable of cross-reacting with progesterone antibody. One likely candidate for this contaminant is deoxycorticosterone, which is known to cross-

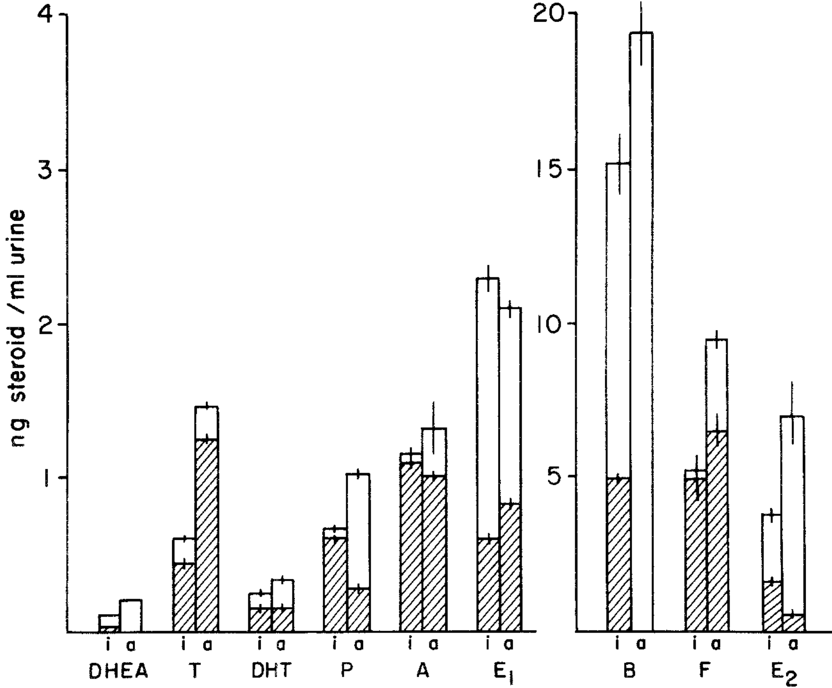

FIG. 1. Steroid content of biologically active, pheromone-containing (a) and biologically inactive, pheromone-devoid (i) male spawning-run sea lamprey urine. Shaded bars show unhydrolyzed values, clear bars represent hydrolyzed values.

react with the antibody and which coelutes with progesterone from the Sephadex LH-20 column.

Using enzyme-hydrolyzed urine as a measure of total steroid content, it can be seen that in all cases but that of estrone, active urine contains a greater quantity of the steroids measured than does inactive urine. The estrone content in hydrolyzed, active urine is not significantly lower than its unhydrolyzed counterpart.

It is known that production and/or release of male pheromone in landlocked sea lampreys corresponds with the onset of sexual maturity and concomitant development of secondary sexual characteristics (Teeter, 1980). It seemed reasonable that sea lampreys might have developed a pheromone communication system using steroid hormones whose concentrations might surge during this period of development. The possibility of chemical communication using an externalized hormone as a signal is generally recognized (Liley, 1982); however, as of the present, this has not been reliably demonstrated for any aquatic species. With the exception of testosterone (see below), the results of our bioassays with unconjugated urinary steroids were uniformly negative. In no case

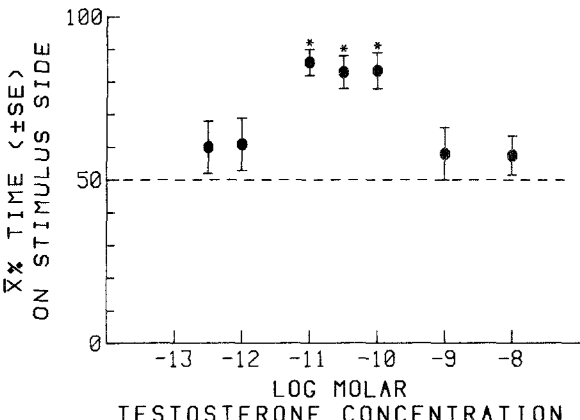

FIG. 2. Plot of log molar testosterone concentration vs. mean percent time that female spawning-run sea lampreys spend on the stimulus side of a two-choice behavioral preference tank. An asterisk denotes a significant preference for testosterone ($P < 0.05$, Wilcoxon matched-pairs, signed-tank test, two-tailed).

did any of the assayed steroids, when presented at concentrations which would be found in 1 ml of male urine (concentrations known to elicit female preference when using bioactive male urine), elicit preference in female lampreys (Table 4). Testosterone was assayed over a range of concentrations, and the results are presented in Figure 2. Bioactive male sea lamprey urine contains approximately 5×10^{-9} M unconjugated testosterone (1.5 ng/ml). When 1 ml of bioactive urine is added to the stimulus side of the behavioral test tank, the testosterone concentration that results from dilution into 78 liters of water is ca. 6.6×10^{-14} M. The behavioral response to pure testosterone occurs at concentrations between 1×10^{-11} M and 1×10^{-10} M (Figure 2). Thus, bioactive male urine may contain a substance other than testosterone which elicits a preference response in female sea lampreys. As the concentration of testosterone was increased above 1×10^{-9} M, the preference effect disappeared (Figure 2). It is clear from these results that females show a preference for water in which testosterone is present. The testosterone concentration necessary to evoke this response is approximately three to four orders of magnitude greater than the testosterone concentration of native, bioactive male lamprey urine that is observed to evoke a preference response in females.

It is possible that the preference shown by spawning-run female sea lampreys for testosterone is of no biological significance. It is also possible that testosterone, or a closely related structural derivative, functions as a sex pheromone in sea lamprey when present at the appropriate concentration. In this case, the testosterone concentrations necessary to elicit a response in females in the bioassay apparatus are representative of the amounts that the female must

sense in the open stream before preference behavior is elicited. This would argue for the release of pheromone by one or more males in close proximity to the female, perhaps taking place over the animals' spawning nest. At close range, dilution of the pheromone would be minimal, and it is possible that the critical concentration range of 10^{-11}–10^{-10} M might be reached. Even if this is the case and testosterone is functioning as a short-range attractant, then our general bioassay results indicate that there is a second substance exerting its attractive effect on females at much lower concentrations. This is evidenced by the fact that 0.5–1.0 ml of bioactive male urine, when placed in the bioassay device, will elicit a behavioral preference response in female lamprey. The concentrations that obtain in this situation are on the order of 6–13 μl of urine per liter of water. This amounts to a 1 : 78,000 dilution of the urine and, for testosterone in male urine, the result is that the concentration is much lower than the effective preference-producing concentration. Thus, the presence of another pheromone is implicated. Experiments directed to isolating and identifying this material are currently being undertaken in our laboratory.

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IDENTIFICATION OF TRAIL PHEROMONE OF LARVA OF EASTERN TENT CATERPILLAR *Malacosoma americanum* (LEPIDOPTERA: LASIOCAMPIDAE)

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Abstract—Previous studies have shown that larvae of the eastern tent caterpillar (*Malacosoma americanum* F.) mark trails, leading from their tent to feeding sites on host trees, with a pheromone secreted from the posterior tip of the abdominal sternum. 5β-Cholestane-3,24-dione (**1**) has been identified as an active component of the trail. The larvae have a threshold sensitivity to the pheromone of 10^{-11} g/mm of trail. Several related compounds elicit the trail-following response. Two other species of tent caterpillars also responded positively to the pheromone in preliminary laboratory tests.

Key Words—Eastern tent caterpillar, *Malacosoma americanum,* Lepidoptera, Lasiocampidae, larva, trail pheromone, recruitment, 5β-cholestane-3,24-dione.

INTRODUCTION

The presocial eastern tent caterpillar (*Malacosoma americanum*) lives in colonies containing 50–300 or more individuals. Foraging larvae (caterpillars) mark trails with a secretion from the sternum at the posterior tip of the abdomen (Fitzgerald and Edgerly, 1982). Foragers mark exploratory trails as they move from their tent in search of food. Successful foragers over-mark these trails as they follow them back to the tent after feeding. Over-marked trails are analogs

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of the recruitment trails of the eusocial insects and serve to lead tentmates to patches of young leaves, the preferred larval food (Fitzgerald and Peterson, 1983). Although trail marking by communal and solitary caterpillars has been reported for a number of other species, including economically important defoliators such as the gypsy moth (*Lymantria dispar*) and the large white butterfly (*Pieris brassicae*) (McManus and Smith, 1972; Long, 1955), no larval trail pheromone has been previously isolated or characterized. We report results of a study to identify and synthesize the trail pheromone of the larva of the eastern tent caterpillar, and to assess its activity.

METHODS AND MATERIALS

Bioassay. Eastern tent caterpillar egg masses were collected from several locations in central New York State and held at 2°C until needed. Colonies were hatched at room temperature and fed young leaves of black cherry (*Prunus serotina*). Second-instar larvae were used for the bioassay. Five-microliter quantities of chemical fractions dissolved in hexane were deposited in 2-cm-long lines on paper cards. A caterpillar was placed at one end of a line, and a positive response to the fraction was recorded when the caterpillar traveled to the end of the line, turned, and followed the line back to the starting point. Following the identification and synthesis of the pheromone, additional bioassays were performed with whole colonies foraging in host trees under both simulated and actual field conditions.

Chemical Isolation and Identification. Proton magnetic resonance ($[^1H]$NMR) spectra and carbon magnetic resonance spectra ($[^{13}C]$NMR) were obtained on a Varian FT-80A instrument, and mass spectra on a Shimadzu QP1000 GC-MS instrument, 25 m × 0.2 mm BPI:SGE, 1 min at 250°C, 10°/min to 290°C, splitless, or on a Finnigan 4000, direct inlet instrument. High-resolution mass spectra were obtained on an AEI MS30 instrument. Flash chromatography was performed on silica according to Still et al. (1978) using hexane–ethyl acetate (4:1) for elution. High-pressure liquid chromatography (HPLC) separations were achieved with a Spectra-Physics SP8700 system with a Waters differential refractometer R401 detector and a 25 × 0.4 cm Merck Hibar Lichrosorb RP-18 (5 μm) column using acetonitrile (1 ml/min) as solvent.

Hexane (14 liters) was percolated through 35,000 terminal segments from eastern tent caterpillar larvae to afford a hexane extract that was active in the trail-following bioassay, and the solution was concentrated to a green gum by distillation through a 25-cm Vigreux column.

Purification of 450 mg of the gum in hexane by flash chromatography followed by reverse-phase HPLC gave an active fraction (ca. 0.1 mg) that showed a single peak with the RI detector. The mass spectrum (Shimadzu GC-MS) of this compound suggested a cholestanedione type of structure: M^+ 400(2),

315(2), 314(2), 297(4), 271(9), 231(2), 215(1), 191(2), 147(7), 135(6), 122(7), 121(8), 119(12), 109(12), 107(17), 105(13), 95(21), 93(20), 91(15), 81(34), 79(24), 71(29), 67(25), 57(14), 55(49), 43(100). Peaks in the mass spectrum at 315 and 314 place a cholestanedione side chain carbonyl group at C-24 (315 = M-C_5H_9O, β-cleavage, and 314 = M$-C_5H_{10}O$, McLafferty rearrangement). A peak at 231 (corresponding to a loss of $C_{11}H_{20}O$) is a residue common in steroids, with oxygenation in ring A, after loss of the side chain and ring D with H transfer. This compound was identified as 5β-cholestane-3,24-dione (**1**) after comparison of its GC-MS spectrum with that of **1** prepared from lithocholic acid. Coinjection of the isolated compound and synthesized **1** on capillary GC in addition to identical retention times in sequential experiments on HPLC and GC confirmed its identity. Although the mass spectrum of the 5α epimer (**2**) is similar to that of **1**, these compounds are readily separated on HPLC and capillary GC [reverse phase HPLC, acetonitrile, 1 ml/min, R_t (5β) = 0.8 R_t (5α); capillary GC 25 m BPI, 280°, R_t (5β) = 0.88 R_t (5α)].

Synthesis. 5β-Stigmastan-3-one (**5**) was prepared from β-sitosterol (**9**) by Jones oxidation followed by catalytic hydrogenation (Augustine, 1972a). 5-α-Stigmastan-3-one (**6**) was prepared from β-sitosterol (**9**) by catalytic hydrogenation followed by Jones oxidation (Augustine, 1972b) (see Figure 1).

For 5β-cholestane-3,24-dione (**1**), 3α-hydroxy-5β-cholestan-24-one was prepared from lithocholic acid according to the method of Ochi et al. (1979), then oxidized with the Jones reagent to afford 5β-cholestane-3,24-dione (**1**) as an oil that solidifed on standing but resisted recrystalization. Mass spectrum (Shimadzu GC-MS) M^+ 400(5), 315(3), 314(5), 297(5), 271(10), 231(4), 215(2), 191(2), 147(7), 135(4), 122(7), 121(10), 119(7), 109(10), 107(17), 105(11), 95(10), 93(22), 91(12), 81(27), 79(21), 71(27), 67(27), 57(9), 55(48), 43(100); M^+ calculated for $C_{27}H_{44}O_2$ 400.3341, found 400.3336; [^{13}C]NMR ($CDCl_3$) δ12.18(C-18), 18.42(C-26, C-27), 18.58(C-21), 21.31(C-11), 22.76(C-19), 24.27(C-15), 25.88(C-6), 26.74(C-7), 28.27(C-16), 29.90(C-23), 34.99(C-10), 35.46(C-20), 35.65(C-8), 37.14(C-2), 37.29(C-1, C-22), 40.18(C-12), 40.88(C-9, C-25), 42.45(C-4), 42.87(C-13), 44.43(C-5), 56.23(C-17), 56.55(C-14), 213.25(C-3), 215.32(C-24); [^{1}H]NMR ($CDCl_3$) δ0.68 (s, 3, C-18 Me), 1.02 (s, 3, C-19 Me), 1.08 (d, J = 7 Hz, 6, C-26 and C-27 Me's).

For cholest-4-ene-3,24-dione (**10**), one equivalent of bromine (1 M in acetic acid) was added dropwise at room temperature to 5β-cholestane-3,24-dione (**1**) (130 mg) in acetic acid (2 ml). The mixture was stirred for 5 min, diluted with water, filtered, and the product was taken up in ethyl acetate, dried and evaporated to yield 4-bromo-5β-cholestane-3,24-dione as a solid. The crude product in *N,N*-dimethylacetamide (1 ml) was added to a refluxing mixture of calcium carbonate (150 mg) and *N,N*-dimethylacetamide (5 ml). After heating under reflux for 30 min, the mixture was cooled, diluted with water, and extracted with ether. The ether extracts were washed with dilute HCl, then water, dried, and evaporated. Purification by preparative TLC on silica gel afforded cholest-

(1) (2)

(3) (4)

(5) (6)

(7) (8)

(9) (10)

FIG. 1.

4-ene-3,24-dione (**10**) (100 mg) mp 116–118° (MeOH). Mass spectrum (Finnigan) M^+ 398(28), 383(3), 355(5), 313(19), 269(12), 229(17), 124(38), 107(25), 95(27), 93(28), 91(29), 81(28), 79(32), 71(39), 67(28), 55(51), 43(100); [^{1}H]NMR ($CDCl_3$) δ0.71 (s, 3, C-18 Me), 1.08 (d, J = 7 Hz, 6, C-26 and C-27 Me's). 1.17 (s, 3, C-19 Me), 5.65 (bs, 1, C-4H).

For 5α-cholestane-3,24-dione (**2**), cholest-4-ene-3,24-dione (**10**) (100 mg) was hydrogenated (Combe et al., 1967) at 20°C under 1 atm of hydrogen in propan-2-ol (10 ml) with Pd–$CaCO_3$ catalyst (100 mg) to give a mixture of 5β- and 5α-cholestane-3,24-dione 3 : 2 (with Pd-C catalyst the ratio of 5β to 5α = 2 : 1). The products were separated on HPLC (acetonitrile solvent, 1 ml/min) to give pure 5α-cholestane-3,24-dione, mp 125–127° (MeOH). Mass spectrum (Shimadzu) M^+ 400(6), 385(3), 315(9), 314(7), 297(2), 278(9), 271(9), 217(7), 215(5), 209(3), 191(2), 149(9), 147(6), 135(12), 123(15), 121(16), 119(11),

109(17), 107(17), 105(16), 95(23), 93(23), 91(18), 81(30), 79(28), 71(32), 69(17), 67(25), 57(5), 55(50), 43(100); M^+ calculated for $C_{27}H_{44}O_2$ 400.3341, found 400.3346; [^{13}C]NMR ($CDCl_3$) δ11.46(C-19), 12.09(C-18), 18.31 and 18.35(C-26 and C-27), 18.47(C-21), 21.46(C-11), 24.21(C-15), 28.13(C-16), 28.98(C-6), 29.83(C-23), 31.72(C-7), 35.40(C-10 and C-20), 35.67(C-8), 37.23(C-22), 38.19(C-2), 38.59(C-1), 39.91(C-12), 40.85(C-25), 42.66(C-13), 44.73(C-4), 46.72(C-5), 53.82(C-9), 56.06(C-17), 56.29(C-14), 212.05(C-3), 215.35(C-24); [^{1}H]NMR ($CDCl_3$) δ0.67 (s, 3, 18 Me), 1.00 (s, 3, 19 Me), 1.08 (d, J = 7 Hz, 6, 26 and 27 Me's).

RESULTS AND DISCUSSION

The response of caterpillars to 5β-cholestane-3,24-dione (**1**) and to similar compounds laid out in narrow trails on paper cards is shown in Table 1. The threshold sensitivity of the caterpillars to 5β-cholestane-3,24-dione lies within the range of values previously reported for the trail pheromones of ants (Tumlinson et al., 1972; Jaffe and Howse, 1979; Morgan, 1984).

Caterpillars deposit the pheromone on the surface of the substrate and perceive the nonvolatile compound by contact chemoreception. Since the compounds we bioassayed were dissolved in hexane and applied to absorbent paper cards, the detectable surface residue was probably less than it would have been if an equivalent quantity had been deposited directly by the caterpillar. Indeed, tests of 5β-cholestane-3,24-dione on a nonabsorptive substrate showed threshold activity an order of magnitude lower than on paper. Behavioral threshold concentrations may, therefore, be lower than indicated by this bioassay procedure.

Since these preliminary bioassays were done only on readily available analogs of the pheromone, structure–activity correlations cannot yet be made. Most remarkable, however, is the observation that 5β-cholestan-3-one (**3**) elicits a response at a 10-fold lower concentration than does the pheromone itself (Table 1); thus, the carbonyl oxygen at C-24 is not essential. In two pairings (**1** and **2**, **5** and **6**) the β configuration seems essential.

Although extensive testing of the biological activity of synthetic 5β-cholestane-3,24-dione remains to be done under both laboratory and field conditions, preliminary tests with laboratory colonies maitained under simulated natural conditions showed that the compound at a dose of 10^{-8} to 10^{-10} g/mm is competitive with authentic exploratory trails. In addition, when the compound was used to lay trails to previously unexploited sections of colonized trees in the field, caterpillars concentrated their foraging activity on the treated branches during their next foraging bout. Further testing is needed, however, to determine if additional factors are involved in the recruitment response. Two other species of tent caterpillar, *M. disstria* and *M. neustria* also responded positively to the pheromone in preliminary laboratory tests.

TABLE 1. NUMBER OF SECOND-INSTAR EASTERN TENT CATERPILLARS SHOWING POSITIVE RESPONSE TO SYNTHESIZED TRIAL PHEROMONE AND TO RELATED COMPOUNDS ($N = 6$ TRIALS/DILUTION)

	Dilution (10^{-x})g/mm trial							
Compound tested	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}
5β-cholestane-3,24-dione (1)	6	6	6	6	6	4	0	0
5α-cholestane-3,24-dione (2)	5	3	0	0	0	0	0	0
5β-cholestan-3-one[a] (3)	6	6	6	6	6	4	2	0
5α-androstan-3-one[a] (4)	0	0	0	0	0	0	0	0
5β-stigmastan-3-one (5)	6	6	6	2	0	0	0	0
5α-stigmastan-3-one (6)	0	0	0	0	0	0	0	0
5-cholesten-3-one[a] (7)	6	5	3	1	0	0	0	0
5β-cholestane[a] (8)	6	5	3	0	0	0	0	0

[a] Sigma Chemical Company, St. Louis, Missouri.

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ISOLATION, CHARACTERIZATION AND ACTIVITY OF PHYTOTOXIC COMPOUNDS FROM QUACKGRASS [*Agropyron repens* (L.) *Beauv.*][1]

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Abstract—Previous experiments showed that legumes grown in the presence of living or herbicidally treated quackgrass residues or extracts exhibited reduced seedling root and shoot growth and decreased nodulation and nitrogen fixation. Aqueous extracts of quackgrass shoots were most inhibitory to plant growth. Upon sequential partitioning of an aqueous extract of quackgrass shoots, the ether extract possessed the most activity and caused 50% reductions in radicle elongation of eight crop and weed species at concentrations of less than 240 μg/ml (small-seeded species) and 1000 μg/ml (large-seeded species). Snapbeans (*Phaseolus vulgaris* L. "Bush Blue Lake") grown aseptically in agar containing an ether extract at 100 and 200 μg/ml exhibited severe root browning, lack of root hair formation, and a two- to three-fold reduction in root and shoot dry weights. The ether extract of quackgrass shoots had no inhibitory effect on the growth of four *Rhizobium* species in Petri dishes or two species in broth culture. Inhibitors present in the ether extract may influence the legume *Rhizobium* symbiosis indirectly by reducing legume root growth and root hair formation. The ether extract of quackgrass shoots was separated using high-pressure liquid, thin-layer, and liquid column chromatography in an attempt to isolate and identify the inhibitors responsible for the inhibition of seedling growth. Two closely related flavonoid inhibitors were isolated from the ether extract. One was identified as 5,7,4'-trihydroxy-3',5'-dimethoxyflavone (tricin). Both flavonoids caused 50% inhibition of radicle elongation in cress (*Lepidium sativum* L. "Burpee curly") seeds at concentrations of less than 125 μg/ml. Both flavonoids were found in ether extracts of quackgrass shoots and rhizomes, but the largest amounts of both compounds occurred in quackgrass shoots collected from the field.

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Key Words—*Agropyron repens* L. Beauv., allelopathy, radicle elongation, flavonoid, flavone, tricin, shoot, rhizome.

INTRODUCTION

Quackgrass is a highly aggressive perennial plant that reproduces mainly by rhizomes (Holm et al., 1977). It is a widespread weed problem in grain, forage, vegetable, and fruit crops grown in the cooler zones of the Northern Hemisphere (Holm et al., 1977; Wyse, 1980).

Previous research in both the field and the greenhouse has demonstrated the detrimental interference of quackgrass on the growth of higher plants. However, the literature concerning quackgrass interference does not clearly differentiate the relative roles of allelopathy and competition in the total interference (Rice, 1984). In the field, quackgrass has been reported as an aggressive competitor for light, water, and nutrients (Kommedahl et al., 1970; Ohman and Kommedahl, 1964). Others have reported considerable allelopathic activity by quackgrass due to the direct production of inhibitors by quackgrass shoots or rhizomes (LeFevre and Clagett, 1960, Oswald, 1948; Phlak, 1967; Welbank, 1960), inhibitors such as acetic and butyric acid (Patrick et al., 1963) which arise from the decay of materials in the soil (Lynch and Penn, 1980; Ohman and Kommedahl, 1964; Toai and Linscott, 1979; Welbank, 1963), or the interaction of decaying quackgrass with pathogenic soil microbes (Penn and Lynch, 1982).

In the field and greenhouse, Weston and Putnam (1985) reported reduced root and shoot growth, nodulation, and nitrogen fixation by legumes grown in the presence of living and herbicidally treated quackgrass. Legumes grown in the presence of quackgrass displayed chlorotic leaves and necrotic roots. In the laboratory, extracts of quackgrass shoots were particularly inhibitory to seed germination, radicle elongation and root growth of legumes. Root systems were stunted, necrotic, and lacked root hairs when grown in the presence of shoot or rhizome extracts, indicating that quackgrass indirectly inhibits the legume-*Rhizobium* symbiosis by inhibiting root hair formation rather than directly inhibiting *Rhizobium* growth (Weston and Putnam, 1986).

The production and release of toxic compounds by quackgrass have been documented in the laboratory. Dried quackgrass rhizomes and leaves were reported by both Toai and Linscott (1979) and Ohman and Kommedahl (1964) to contain water-soluble inhibitors which reduce seedling growth. Two inhibitors from quackgrass rhizomes have been tentatively characterized by Gabor and Veatch (1981) and LeFevre and Clagett (1960); one is suggested to be a glycoside. However, the structures of these inhibitors have never been fully elucidated. Characterization of inhibitors present in quackgrass shoots, the portion of the plant most toxic to seedling growth (Weston and Putnam, 1986), has not been previously reported.

To further explore the possible allelopathic potential of quackgrass shoots under well-controlled conditions, we attempted to: (1) assess the effect of partitioned quackgrass shoot extracts on seed germination, root development, and the legume-*rhizobium* symbiosis, (2) isolate and identify the inhibitors) present in quackgrass shoots responsible for the reduction of seedling root growth, and (3) quantify the amount of inhibitor(s) in aqueous extracts of both field and greenhouse-grown rhizome and shoot tissue.

METHODS AND MATERIALS

Procedure for Collection of Quackgrass Material. Quackgrass was collected from the Horticultural Research Farm, East Lansing, Michigan. The site had a soil classified as a Marlette sandy loam (Glossoboric Hapludalf, fine-loamy, mixed, mesic) and supported a community dominated by quackgrass that had grown undisturbed for approximately 15 years. On June 10, 1984, field-grown quackgrass shoots (25–30 cm in height) were collected and placed in a drying oven at 35–40°C for four days. Rhizomes were also carefully removed from field soil at this time, washed thoroughly with tap and distilled water, and placed in a drying oven under similar conditions.

Sods (0.3 × 0.3 m) were collected from this field site and placed in square wooden flats, 15 cm in depth. Flats were placed under metal halide lighting in the greenhouse with photosynthetic photon flux density (PPFD) of 425 μmol/m^2/sec measured at the top of the canopy. The greenhouse was maintained with a 14-hr- light–10-hr-dark photoperiod at 28 and 22°C. Flats were fertilized once weekly with 600 ml soluble 20-20-20 fertilizer at a rate of 3.9 g/liter of water. When quackgrass shoots reached a height of 20–25 cm, they were repeatedly harvested with shears by trimming 3–4 cm above the soil surface. After 60 days, rhizomes were carefully removed from the flats and washed thoroughly with tap and distilled water. Rhizomes and shoot material were then placed in a drying oven at 35–40°C for four days. The entire process of collecting material from the greenhouse was repeated six times between 1983 and 1985 to gather sufficient material for experimental use.

All field-grown and greenhouse-grown plant material was ground separately in a Wiley mill (mesh screen size 1 mm). Powdered material was stored in tightly closed glass containers until used for extraction and quantification of quackgrass phytotoxins.

Procedure for Extraction of Quackgrass Shoot Material. Shoot tissue (100 g) was extracted for 24 hr with 3 liters of distilled water on a shaker in a 4°C coldroom (Figure 1). The mixture was filtered through four layers of cheesecloth and centrifuged for 20 min at 16,000*g* to remove particulate material. Nine liters of chilled acetone were slowly added to the aqueous extract. The mixture was stirred at slow speed on a stir plate for 24 hr in a 4°C coldroom. Proteinaceous material precipitated out of solution in the presence of acetone and was

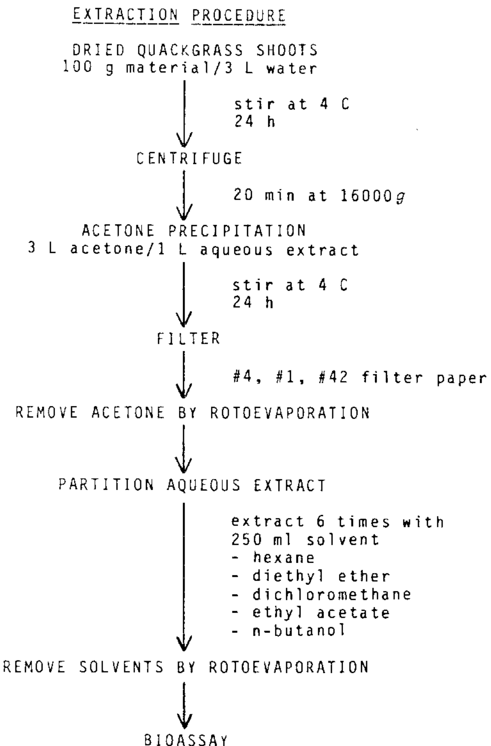

FIG. 1. Flow diagram of the procedure for extraction and partitioning of dried quackgrass shoots.

removed by vacuum filtration through Whatman No. 4, 1, and 42 filter paper and discarded. (This procedure prevents dense emulsions from forming during partitioning). Acetone was removed from the extract by rotary evaporation at 35°C. The clear, aqueous extract was sequentially partitioned with the following solvent series ranging from least polar to most polar: hexane, diethyl ether, dichloromethane, ethyl acetate, and *n*-butanol. Each liter of the aqueous shoot extract was sequentially partitioned six times with 250 ml of each of the five solvents. Solvent was removed from each fraction by rotary evaporation at 35°C. The entire process of extraction and partitioning was repeated five times to gather enough material for experimentation.

Each fraction from the liquid–liquid extractions was redissolved in methanol to form solutions at a concentration of 0.5 mg/ml. One milliliter of each fraction was placed in separate glass Petri dishes (60 × 15 mm), which were lined with filter paper (Whatman No. 1). Methanol was allowed to evaporate from each dish, and 1.5 ml distilled water was added to form a concentration of 0.33 mg material/ml. In addition to the fractions obtained from the liquid–

liquid extractions, the following materials were also tested for toxicity: a distilled water control (applied to the filter paper after evaporating 1.0 ml of methanol), crude aqueous extract of quackgrass shoots, and the aqueous extract of quackgrass shoots remaining after partitioning. Twenty seeds of "Burpee curly" cress were then added to each dish. Radicle elongation of curly cress was consistently used as a bioassay throughout our experiments because of its availability, uniformity, and sensitivity. Three replicates of each treatment were arranged in a completely randomized design and placed in a 26°C growth chamber with relative humidity near saturation. Radicle lengths of seeds were measured at 72 hr. Data were subjected to analysis of variance and means separated by least significant difference (.05).

Influence of Ether Extracts on Radicle Growth. Ether extracts were redissolved in methanol to form a dilution series ranging in concentration from 1600 μg/ml to 25 μg/ml. One milliliter of each treatment solution was applied separately to glass Petri dishes. After the addition of 1.5 ml distilled water per dish, concentrations ranged from 1066.7 μg/ml to 16.7 μg/ml. A distilled water control was also included, and 20 seeds were arranged per dish. Seed indicators used were: "Burpee curly" cress, lettuce (*Lactuca sativa* L. "Ithaca"), alfalfa (*Medicago sativa* L. "Vernal"), redroot pigweed (*Amaranthus retroflexus* L.), soybean [*Glycine max* (L.) Merr. "Corsoy 79"], navybean (*Phaseolus vulgaris* L. "Seafarer"), barnyardgrass [*Echinochloa crus galli* (L.) Beauv.] and smooth crabgrass [*Digitaria ischaemum* (Schreb. ex Schweig.) Schreb. ex Muhl.]. Two replicates of each treatment were arranged in a completely randomized design and placed in a 26°C growth chamber with relative humidity near saturation. Radicle lengths of all species were measured at 72 hr, and data were analyzed as described previously. Standard errors were calculated for each mean.

Influence of Ether Extracts on Aseptically Cultured Snapbeans. Fahraeus medium (125 ml of 1% agar) was poured into 250-ml Ehrlenmeyer flasks. This medium is well suited for culture of legumes in sterile agar culture (Dazzo, 1982), excluding microorganisms which may alter the toxicity of plant compounds. Snapbeans were selected for this bioassay because they can be easily cultured in agar media as opposed to other legume crops. Flasks were closed with styrofoam stoppers and autoclaved at 121°C for 25 min. "Bush Blue Lake" snapbean seeds were placed in a 0.5% solution of calcium hypochlorite for 4.5 min for surface sterilization and then rinsed for 1 min with sterile distilled water. Seeds were placed on moist sterile filter paper in glass Petri dishes in a 27°C growth chamber for four days in the dark.

When seedling radicles were approximately 3 cm in length, the Fahraeus agar in the flasks was remelted by autoclaving at 121°C for 10 min (agar was made beforehand for convenience). Treatment solutions were then added and mixed before the agar solidified; these consisted of ether extracts of quackgrass shoots dissolved in water at two concentrations (12.5 mg/ml and 6.25 mg/ml). To one third of the flasks, 2.0 ml (25 mg) of filter-sterilized extract solution

was added to give a final concentration of 200 μg ether extract/ml agar medium. To another third of the flasks, 2.0 ml (12.5 mg) of filter-sterilized extract solution was added to give a final concentration of 100 μg/ml agar medium. In the controls, 2.5 ml distilled water were added to the agar. The pH of the agar mixtures ranged from 5.2 to 5.4. Pregerminated seeds were planted in the treated agar at a 1.5 cm depth, one seed per flask. Seeds were inoculated with 0.1 ml of *R. phaseoli* in the exponential phase of growth in yeast extract mannitol broth. Flasks were placed in a lighted growth chamber at 27°C day and 23°C night temperatures. A 16-hr photoperiod under fluorescent and incandescent bulbs was maintained with an average PPFD of 175 μmol/m^2/sec at the top of the flasks.

The treatments in the experiment were replicated five times (one flask each) and arranged in a completely randomized design. The experiment was repeated, also using five replicates. Only the plants remaining sterile were harvested after 21 days, when beans reached the second to third trifoliate leaf stage. Root and shoot dry weights were recorded. Data from the two experiments were combined and analyzed using analysis of variance and mean separation by least significant difference (0.05) test.

Isolation and Characterization of Toxins from Ether Extracts of Quackgrass Shoots. The ether extract was further separated in an attempt to isolate and identify the compounds in quackgrass shoots responsible for inhibition of seedling root growth. When the dried ether fraction (240.0 mg) was redissolved in dichloromethane (40 mg/ml), a gold crystalline precipitate was formed. The precipitate was collected in a fritted glass filter and washed with 100 ml hexane followed by 100 ml diethyl ether. The precipitate was dried, weighed (11.3 mg), and bioassayed. The standard bioassay used throughout these separation procedures was the inhibition of the radicle elongation of "Burpee curly" cress measured at 72 hr. Since the precipitate possessed considerable inhibitory activity (I_{50} = 149.0 μg/ml) further separation was conducted using silica gel thin-layer chromatography (TLC) plates (Merck, 0.25 mm, F-254). Plates were run in 8:1 chloroform–methanol. Visualization under shortwave ultraviolet (UV) light at 254 nm and exposure to 5% vanillin in sulfuric acid showed three distinct bands (R_f = 0.20, 0.35, and 0.40). The bands appeared bright yellow, and band 3 (R_f = 0.40 appeared to make up the majority of the mixture.

Band 3 (R_f = 0.40) was scraped off the plate and the scrapings were eluted with 4:1 chloroform–methanol through a fritted glass filter. Band 3 was bioassayed and also subjected to spectral analyses. Band 1 (R_f = 0.20) was also isolated from the TLC plate, but it was in small amounts and was contaminated by some of band 3. Therefore, band 1 was not bioassayed but was subjected to spectral analysis. All spectral analyses were performed at the ARCO Plant Cell Research Institute. UV spectra were obtained with a Perkin Elmer Lambda 5 UV/VIS spectrophometer, while chemical ionization mass spectroscopy (CI-MS) was performed with a VG 7070E direct probe inlet using isobutane or

ammonia as the ionizing gas. Nuclear magnetic resonance spectroscopy (NMR) was performed in deuterated methanol or chloroform on a Varian XL 300 MHz instrument. A dose-response curve was constructed to determine the concentration of this material (compound 1) required for 50% inhibition of cress radicle elongation (I_{50}).

The ether fraction was also separated using liquid column chromatography (LC). This crude material (500 mg) was loaded onto a silica gel (60g Baker, 200–250 mesh) flash column. Pressure for the column was provided with a laboratory air line at a rate of 40 ml solvent/min and eluted with 500 ml 65:35 dichloromethane–ethyl acetate, followed by 500 ml of ethyl acetate and 500 ml methanol. Fractions (25 ml) were collected, examined by TLC (silica gel-eluted with 65:35 dichloromethane–ethyl acetate) and combined to provide 11 distinct fractions. The 11 fractions were bioassayed separately for inhibitory activity using 250 μg material/dish, or 166.7 μg/ml. Activity was concentrated in fractions 6–8, and the material from these fractions was combined.

This material (55.5 mg) was loaded on a silica gel TLC plate and run in 10:1 chloroform–methanol. Seven distinct bands were scraped off the plate after visualization under UV (254 nm) light. Plate scrapings were eluted with 4:1 chloroform–methanol through a fritted glass filter, and the eluent from each zone was collected, dried, weighed, and bioassayed at 250 μg material/dish or 166.7 μg/ml. The majority of the activity occurred in zones 2 (R_f = 0.10–0.21) and 3 (R_f = 0.21–0.30). This material was combined (15.0 mg) and further separated by high-pressure liquid chromatography (HPLC) using a Waters, μBondapak C_{18}, 8-mm × 10-cm radial compression column. The UV detector (Waters Lambda-max model 481) measured absorbance at 380 nm. The solvent system used was 75:25 methanol–water at a flow rate of 1.5 ml/min. The mixture was resolved into two peaks and two fractions were collected after repeated injections.

The two fractions were bioassayed on an equal weight basis at 200 μg material/Petri dish or 133.3 μg/ml after the removal of the solvents by rotary evaporation. Strong inhibitory activity was associated only with fraction 1, which appeared as one large, distinct, needle-shaped peak in a variety of HPLC solvent systems. The material was dried, weighed (6.5 mg), and a small amount was subjected to analysis by TLC (silica gel in 10:1 chloroform–methanol). Visualization by UV (254 nm) light showed one distinct band at R_f = 0.21. Exposure to 5% vanillin in sulfuric acid showed the same band (R_f = 0.21) which turned a pale pink. The material was subjected to spectral analysis by UV, MS, and NMR (as described previously). A dose–response curve was constructed to determine the concentration of this material (compound 2) required for 50% inhibition of cress seed radicle elongation (I_{50}).

HPLC Quantification of Toxins from Various Quackgrass Tissues. An attempt was made to extract and quantify the amounts of compounds 1 and 2 (characterized in the previous section) in rhizomes and shoots grown in two

different environments. Treatments consisted of 100 g each of field- or greenhouse-grown rhizome or shoot tissue, which was extracted separately for 24 hr as described previously. The four extracts were sequentially partitioned with the same solvent series, and the ether extract of each tissue treatment was retained and weighed. Each extract (40 mg) was loaded separately onto a silica gel TLC plate and run in 10:1 chloroform–methanol. Bands tentatively identified as compounds 1 and 2 were observed under UV (254 nm) light in all tissue treatments. Plate scraping was used to separate the mixture before further evaluation by HPLC. Three zones were scraped off each plate (R_f = 0.14–0.34, R_f = 0.34–0.45, and R_f = 0.45–0.55) which contained variable amounts of compounds 1 and 2. All scrapings were eluted in 4:1 chloroform–methanol, and the eluent was filtered and dried. The HPLC system described previously (75:25 methanol–water) was used to separate and quantitate the amount of compounds 1 and 2 in each fraction. Standard curves of each purified compound were generated and used to estimate the total amount of compound 1 and 2 in each tissue extract.

RESULTS AND DISCUSSION

Extraction of Quackgrass Shoot Material. Aqueous extraction of quackgrass shoot material was performed at 4°C to prevent the breakdown of secondary products by microorganisms. Greenhouse-grown tissue was used because of its uniformity of size, its availability, and because it was exposed to more uniform growing conditions throughout the year. The greatest amount of material (mg) was removed from the aqueous shoot extract through partitioning with *n*-butanol (Table 1). However, the most inhibitory activity occurred in the hexane, ether, and ethyl acetate fractions, as measured by the inhibition of cress radicle elongation at 72 hr. Repeated partitioning with a solvent series was successful in removing considerable toxicity from the crude aqueous extract of shoots. Since the greatest toxicity and a comparatively larger weight of material were concentrated in the ether fraction, the crude ether extract was used in further separations and bioassays in an attempt to isolate quackgrass inhibitors responsible for the reduction in seedling growth.

Influence of Ether Extracts on Radicle Growth. The ether extract of quackgrass shoots also caused significant decreases in radicle length of eight crop and weed species after 72 hr of growth (Figure 2). Radicle length was a more sensitive indicator of inhibition than seed germination or shoot length (data not presented). The crude ether extract was more inhibitory to smaller-seeded alfalfa, cress, redroot pigweed, and lettuce (mean I_{50} value, 170 μg/ml) than to larger-seeded soybeans and navybeans (mean I_{50} value, 1000 μg/ml). The smaller-seeded monocots (barnyardgrass and smooth crabgrass) were also inhibited by ether extracts (mean I_{50} value, 242 μg/ml). These experiments in-

TABLE 1. QUANTITY OF QUACKGRASS SHOOT EXTRACTS PARTITIONED FROM 100 g DRIED SHOOT MATERIAL AND THEIR INFLUENCE ON RADICLE LENGTH OF CURLY CRESS AT 72 HR AT CONCENTRATION OF 0.33 mg/ml

Treatment	Quantity extracted (mg)	Radicle length (mm)[a]
Distilled water control		21.2
Crude Aqueous		13.9
Hexane	14.9	1.0
Diethyl ether	65.4	0.0
Dichloromethane	34.5	6.1
Ethyl acetate	31.4	1.6
Butanol	2759.7	11.9
Remaining Aqueous	13416.7	18.9
F value	—	**
LSD (0.05)	—	5.1

[a]5% level of probability, **1% level of probability, NS = no significant difference.

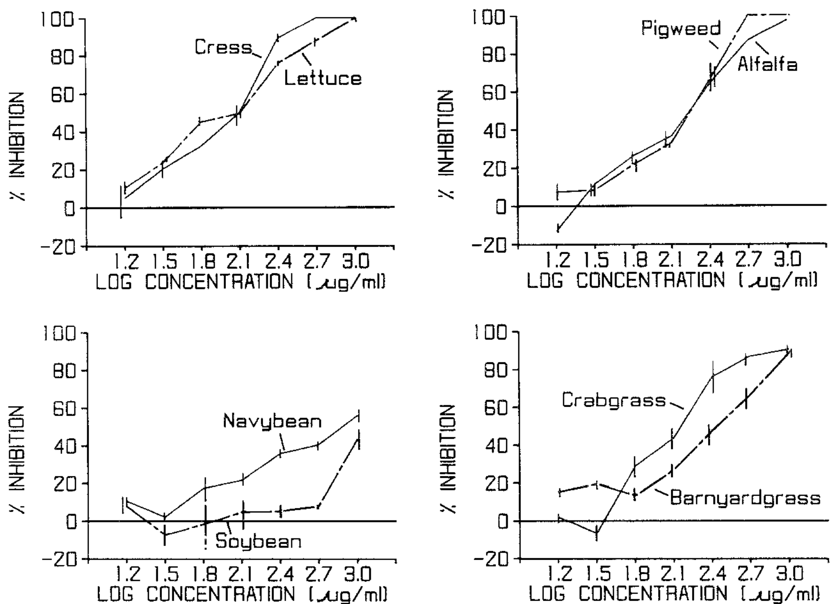

FIG. 2. Inhibition of seedling radicle elongation of eight indicator species by ether extracts of quackgrass shoots after 72 hr of growth. Bars represent ± one standard error unit.

dicate that ether extracts of quackgrass shoots are inhibitory to the radicle growth of a variety of crop and weed species when present at relatively low rates.

Influence of Ether Extracts on Aseptically Cultured Snapbeans. Snapbeans grown with 100 or 200 μg/ml ether extract of quackgrass shoots exhibited severe brown discoloration of the root system, swelling of the roots, and a lack of root hair formation. Leaves appeared green but were smaller and cupped. Similar concentrations had also reduced radicle elongation of most seed indicator species (previous section). In comparison, the controls produced a white fibrous root system with a healthy appearance of roots and shoots. A two- to threefold decrease in both root and shoot dry weights occurred in the presence of ether extract as compared to the control (Table 2). These experiments indicate that quackgrass inhibitors are effectively removed from extracts of shoots by ether. Ether-soluble quackgrass inhibitors may reduce nodulation and nitrogen fixation in legumes (Weston and Putnam, 1985) as a result of the reduction in legume root growth and root hair formation, thereby eliminating suitable sites for *Rhizobium* infection.

In previous experiments, aqueous extracts of quackgrass shoots or rhizomes had no effect on the growth of *Rhizobium* bacteria (Weston and Putnam, 1986). The ether fractions from quackgrass shoot extracts at 4.0 or 0.4 mg/ml also had no effect on the growth of four species of *Rhizobium* in Petri dishes (data not presented). When the ether extract was incorporated into yeast extract mannitol or Bergersen's III broths at a concentration of 200 μg/ml, no inhibition of either *R. phaseoli* or *R. japonicum* growth occurred as compared to a control of distilled water (data not presented). Apparently the quackgrass inhibitors which reduce seedling growth do not play a direct role in inhibiting the growth of several common species of *Rhizobium* that participate in the legume-*Rhizobium* symbiosis.

Isolation and Characterization of Phytotoxins from Ether Extracts of

TABLE 2. INFLUENCE OF STERILE ETHER EXTRACT OF QUACKGRASS SHOOTS ON DRY WEIGHT OF ASEPTICALLY GROWN SNAPBEANS

Treatment	Shoot weight (mg)[a]	Root weight (mg)[a]
Distilled water control	222	78
Ether extract (100 μg/ml)	91	28
Ether extract (200 μg/ml)	71	26
F value	**[b]	**
LSD (0.05)	40	20

[a]Measurements were taken 21 days after planting and are the means of eight plants per treatment.
[b]5% level of probability, **1% level of probability, NS = no significant difference.

ISOLATION OF ACTIVE COMPOUNDS FROM ETHER FRACTION

COMPOUND 1

ETHER FRACTION
↓
DISSOLVE IN DICHLOROMETHANE
↓ (1 ml/40 mg)
COLLECT PRECIPITATE
↓ Wash with 100 ml hexane and diethyl ether
BIOASSAY
↓
LOAD ON SILICA TLC PLATES
↓ Run in 8:1 chloroform:methanol
SCRAPE ZONE 3 (rf = 0.40)
↓
BIOASSAY
↓
UV, IR, NMR, MS
↓
TRICIN

COMPOUND 2

ETHER FRACTION
↓
LIQUID COLUMN CHROMATOGRAPHY
↓ Silica gel, Run in: 1. 65:35 DCM:EtOAc 2. EtOAc 3. MeOH
COLLECT AND BIOASSAY 11 FRACTIONS
↓ TLC
COMBINE FRACTIONS 6-8
↓
LOAD ON SILICA TLC PLATE
↓ Run in 10:1 chloroform:methanol
SCRAPE ZONE 2
↓
BIOASSAY
↓
HPLC
↓ Run in 75:25 methanol:water
COLLECT PEAK 1
↓
BIOASSAY
↓
UV, IR, NMR, MS
↓
COMPOUND 2

FIG. 3. Flow diagram of the separation procedures used for purification of the ether extract of quackgrass shoots.

Quackgrass Shoots. Two toxic compounds were isolated from the ether extract of greenhouse-grown quackgrass material (Figure 3). It was necessary to extract relatively large amounts of dried quackgrass shoot material (500 g) to obtain enough purified product for the spectroscopic analyses. Since the ether extract was a complex mixture, the isolation of both compounds required the use of a number of chromatographic separation techniques.

Compound 1 was isolated from a gold crystalline material which precipitated when the ether fraction was dissolved in dichloromethane. This gold crystalline material was shown by TLC and HPLC to be three closely eluting compounds. Of these, compound 1 ($R_f = 0.40$) was eventually identified as 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone (tricin), while the running component, compound 3 ($R_f = 0.20$), which was contaminated with a small amount of tricin, was deduced to be 5,7,3′-trihydroxy-4′,5′-dimethoxyflavone.

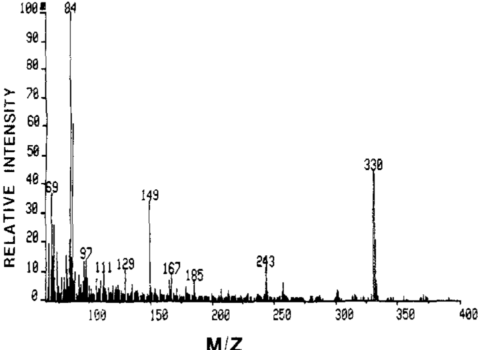

FIG. 4. Chemical ionization mass spectrum (isobutane) of 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone (tricin).

Compound 1 is yellow gold in color and soluble in alcohols. Examination of the EI mass spectrum this compound showed a low intensity ion at *m/z* 330, which indicated a probable molecular ion of 330. Under chemical ionization condition (Figure 4) intense *m/z* 331 and 330 ions showed these to be the $M+H^+$ and M^+ ions, respectively.

Analysis of the [^{1}H]NMR spectrum (Figure 5) of compound 1 in deuterated methanol disclosed information suggesting it to be a substituted methoxyflavone. In this spectrum we may discount certain signals for several impurities associated with the methods used. The singlet at δ 1.27 represents a coeluate from TLC adsorbents, an artifact usually observed when small quantities of material are purified by preparative TLC. Other singlets at δ 3.30, 4.88, and 7.90 correspond to undeuterated methanol, partially deuterated water, and chloroform from the separation procedure, respectively. For compound 1, the six-

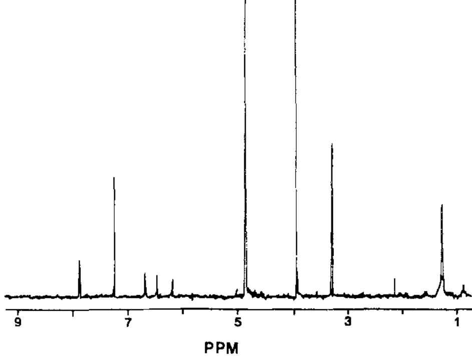

FIG. 5. [^{1}H]Nuclear magnetic resonance spectrum (300 MHz) of 5,7,4′-trihydroxy 3′,5′-dimethoxyflavone (tricin) dissolved in deuterated methanol.

A) $R=R_{II}=Me$, $R_I=H$
5, 7, 4'-Trihydroxy - 3'-5'-dimethoxyflavone
(Tricin)

B) $R=R_I=R_{II}=Me$; 5,7-dihydroxy-3',4',5'-
trimethoxyflavone

C) $R=H$, $R_I=R_{II}=Me$; 5,7,3'-trihydroxy-4',5'-
dimethoxyflavone

FIG. 6. The molecular structure of 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone (tricin), **A**; 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone, **B**; and 5,7,3′-trimethoxy-4′,5′-dimethoxyflavone, **C**.

proton singlet at δ 3.95 is assigned to two equivalent methoxyl groups which are therefore placed at the 3′ and 5′ positions. The other protons of compound 1 appeared at δ 6.22 (1H, doublet, $J = 2$ Hz, H-6), 6.49 (1H, doublet, $J = 2$ Hz, H-8), 6.68 (1H, singlet, H-3) and 7.28 (2H, singlet, H-2′ and H-6′). The correctness of these assignments was shown by synthesis of 5,7-dihydroxy-3′4,′5′-trimethoxyflavone, **B** (Figure 6), the 4′-*O*-methyl derivative of tricin, from 2′,4′,6′-trihydroxyacetophenone and 3,4,5-trimethoxybenzoyl chloride by the method of Wheeler (1963), and by comparison (Table 3) of the spectrum (Figure 7) of this synthetic compound with that of tricin. From these spectra it is clear that compound 1 with two equivalent methoxyl group at C-3′ and C-5′ lacks the 4′-methoxyl group which appears at δ 3.85 in the spectrum of the synthetic compound. The presence of the 4′-methoxyl in this synthetic 4′-methoxytricin is observed to have small, −0.05 and +0.03 ppm, but distinguishing effects on the chemical shifts of the proton H-3 and the equivalent protons H-2′ and H-6′. The 3′- and 5′-methoxyl groups in both compound 1 (tricin) and the synthetic derivative were equivalent, appearing as singlets (6H) at δ 3.95 and 3.94, respectively.

The structure **A** (Figure 6) for compound 1 (tricin) was further confirmed by examining its UV spectra. Its spectrum under alkali conditions showed the usual bathochromic shift expected of phenolic compounds. In addition, the pattern of the UV spectrum in the presence of aluminum chloride was identical to that of tricin. In 1983, Voiron (1983) published a paper on the identification of methoxyflavones with different patterns of OH and OCH_3 substitution using UV spectral differentiation. The UV spectrum is observed in methanol and also in

TABLE 3. [¹H]NMR ASSIGNMENTS (δ VALUES) OF CHEMICAL SHIFTS FOR TRICIN AND RELATED COMPOUNDS

Protons	Tricin (**A**) (R_f 0.40)	Mixture **A**:**C** (1:3)	Synthetic product (**B**)	Isomer **C**[a] (R_f 0.20)
H-3	6.68	6.68 6.74	6.73	6.74
H-6	6.22	6.22	6.22	6.22
H-8	6.49	6.49	6.49	6.49
H-2' H-6'	7.28	7.25 7.28	7.25	7.25
3'-OMe 5'-OMe 4'-OMe	3.94	3.94 3.93 3.94	3.95 3.85	3.93 3.84

[a]Signals assigned by subtracting peaks for tricin.

the presence of methanol plus aluminum chloride, a spectral shift reagent. The majority of flavonoids may be easily identified by their purple fluorescence in UV light and by their spectra in methanol, since they exhibit an absorption maxima in the long UV range (band I) above 326 nm, and generally the ratio of the absorption of band I to band II is greater than 0.7. Compound 1 exhibits a UV maxima at 268 and 350 nm in methanol and, when in the presence of aluminum chloride, exhibits a bathochromic shift to 277 and 393 nm (Figure 8) (Voiron, 1983). This, along with other spectral information, is characteristic only of tricin (5,7,4'-trihydroxy-3',5'-dimethoxyflavone).

Another golden yellow compound ($R_f = 0.20$) which we assigned structure **C** (Figure 6) was also isolated together with tricin. However, because of the small quantities available and the presence of tricin as an impurity, no bioassays

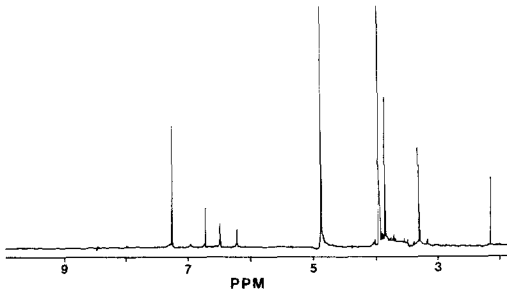

FIG. 7. [¹H]Nuclear magnetic resonance spectrum of synthetic 5,7-dihydroxy-3',4',5'-trimethoxyflavone dissolved in deuterated methanol.

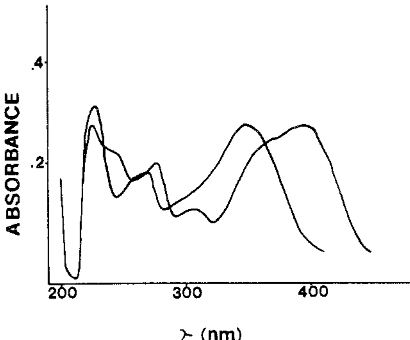

FIG. 8. Ultraviolet absorbance spectrum of 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone (tricin) in methanol and its corresponding bathochromic shift when placed in methanol plus aluminum chloride.

were conducted. The UV spectrum was similar to that of tricin but the method reported by Voiron using aluminum chloride was not effective because of the mixture. However, the [^{1}H]NMR spectrum of the mixture supported the TLC analysis and indicated that the mixture consisted of tricin, **A**, and 5,7,3′-trihydroxy-4′,5′-dimethoxyflavone. The chemical shift assignments for this compound could be deduced by subtracting the peaks for tricin. The values obtained (Table 3) were remarkably consistent with the presence of a 4′-methoxyl group as indicated by two nonequivalent methoxyl groups at δ 3.93 and 3.84 for the methoxyl groups at C-5′ and C-4′, respectively. The singlet for H-2′ and H-6′ appears at δ 7.25, which is identical to the position of H-2′ and H-6′ in the synthetic 4′-methoxyl compound (**B**) and distinctly different from the value (δ 7.28) in tricin. This deduction from the [^{1}H]NMR spectra was supported by MS measurements. An intense ion ($M+H^+$) (100%) at *m/z* 331 in the chemical ionization spectrum of the mixture revealed the molecular species involved and that the molecular weight was identical to that of tricin. This fact, when considered in conjunction with the similarity with [^{1}H]NMR spectra, indicates that the compound ($R_f = 0.20$) is 5,7,3′-trihydroxy-4′,5′-dimethoxyflavone, **C**. This compound has recently been found in extracts of *Poa huecu* Par. (Graminae) by Pomilio and Rofi (1985).

Compound 2 is also golden yellow in color, soluble in alcohols and fluoresces purple under UV (254 nm) light. We believe that compound 2 is also a flavonoid, closely related in structure to tricin. From the HPLC and TLC data obtained, we believed that we had purified the mixture to only one component. The mass spectrum (data not presented) also shows the fragmentation pattern of an oxygen-containing structure. The last discernable fragment occurred at *m/z* 300, (10%) and was not expectedly intense. The true molecular ion, therefore, remains uncertain.

The complexity in the [^{1}H]NMR spectrum indicates the presence of im-

purities (data not presented). However, analytical HPLC using a variety of solvent systems did not resolve compound 2 into anything other than one needle-shaped peak. This, along with the NMR spectrum, suggests that the impurity may be one or more very closely related flavonoids. The NMR spectrum resembles that of compound 1 and the synthesized flavonoid. However, the region from δ 6.7–7.2 is not clearly resolved. The NMR data strongly suggest that compound 2 is also a methoxyflavone, but the presence of impurities prevented the discovery of the substitution pattern upon the A and B rings.

Flavonoids are secondary phenolic compounds that are closely related to both the coumarins and the substituted cinnamic acids. The structural attribute which appears to confer biological activity upon these compounds is substitution upon the benzene moiety. Many possible roles for flavonoids in the physiology of higher plants have been postulated. In particular, flavonoids may play a role as screening pigments for the protection of plant tissues from UV light (Harborne, 1965; McClure, 1977; Robinson, 1980).

The resinous oils of many plants are particularly rich in flavonoid aglycones. Flavonoids are excreted by the root systems of various plants, and some have shown considerable allelopathic activity. For example, in wheat roots, striking inhibition of ion uptake occurs in the presence of various flavones, flavonols, and isoflavones. Various flavonoids also inhibit photophosphorylation in plant mitochondria at extremely low concentrations (McClure, 1977).

Quackgrass extracts contain at least two related flavonoid compounds (tricin and compound 2) which inhibit cress seedling germination and radicle elongation (Table 4). The I_{50} value for tricin upon cress seed radicle elongation is 123.3 μg/ml, while compound 2 has an I_{50} of 59.3 μg/ml. These compounds are both inhibitory to radicle elongation in cress seeds at relatively low concentrations in comparison to several other alleged allelochemicals (Rice, 1984). Neither compound was previously screened for phytotoxic activity. The flavone tricin has also been isolated from the leaves of several *Triticum* species. It appears to be restricted in distribution to a few monocotyledonous plants (Har-

TABLE 4. INFLUENCE OF TRICIN AND COMPOUND 2 ON INHIBITION OF RADICLE ELONGATION OF CURLY CRESS AT 72 hr.

	I_{50} value (μg/ml)[a]
Tricin	123.3
Compound 2	59.3

[a] I_{50} values represent the point at which 50% inhibition of radicle elongation of curly cress occurred. A dose-response curve was calculated on the basis of mean elongation of 40 seeds at five concentrations of the chemicals.

TABLE 5. QUANTITY OF ETHER EXTRACT, TRICIN AND COMPOUND 2 OBTAINED FROM PARTITIONING AND HPLC QUANTIFICATION OF DIFFERENT SOURCES OF QUACKGRASS MATERIAL.

Quackgrass Source	Ether extract	Tricin	Compound 2
		(mg/100 g)	
Greenhouse shoot	66	10	6
Field shoot	208	30	36
Greenhouse rhizome	62	1	8
Field rhizome	375	5	9

borne, 1965). The ether extract of quackgrass shoots not only contains tricin and compound 2, but also appears to contain a number of other closely related flavonoids. These may possess varying degrees of biological activity depending upon the substitution pattern upon the flavonoid rings (McClure, 1977). Previously, Gabor and Veatch (1981) isolated a golden inhibitor, soluble in alcohol, which was extracted from quackgrass rhizomes and characterized as a glycoside. It is possible that the aglycone portion of that molecule was also a flavonoid.

HPLC Quantification of Phytotoxins from Various Quackgrass Tissues. Quackgrass shoots and rhizomes collected from the field produced three to five times more dried ether extracts per 100 g of dried tissue than did shoots or rhizomes grown in the greenhouse (Table 5). The amounts of the two phytotoxic compounds (tricin and compound 2) in field- and greenhouse-grown shoots or rhizomes were also compared by TLC and HPLC quantification (Table 5). Quackgrass shoots grown under field conditions yielded the greatest amounts of both tricin and compound 2 (29.8 and 35.5 mg/100 g dried tissue, respectively) when compared to other tissues. Rhizomes grown in both environments also contained tricin and compound 2, but in generally smaller quantities than those which occurred in the shoots. Rhizomes and shoots grown in the field also yielded higher levels of tricin and compound 2 than did those grown in the greenhouse. This is not surprising, since it is known that plant tissues exposed to greater environmental stress generally produce higher levels of flavonoids (McClure, 1977). The figures shown represent an estimate of the quantities present in the ether extracts and may or may not reflect relative amounts of the two inhibitors present in the whole plant before extraction. However, partitioning with ether did remove the majority of flavonoid compounds from the aqueous extracts (as observed by TLC and the cress bioassay).

These experiments clearly show that quackgrass shoots and rhizomes possess at least two inhibitors that are flavonoid compounds. These flavonoid com-

pounds reduce radicle elongation and root growth. They are present in larger proportions in the ether extracts of quackgrass shoots as compared to extracts of rhizomes. Extracts of quackgrass shoots are also more toxic to seedling root growth in a number of plant species than are extracts of rhizomes (Weston and Putnam, 1986). Tricin and compound 2, along with other related compounds, may be released from plant tissues by exudation or degradation and may play a role in the alleged allelopathic activity of living and dead quackgrass. This observed allelopathic activity includes reductions in root and shoot growth of crop and weed species and inhibition of legume nodulation and nitrogen fixation in the presence of quackgrass or its extracts in the field, greenhouse and laboratory (Weston and Putnam, 1985, 1986). The specific mode of action of the flavonoid compounds, their rate of release from quackgrass tissue, and their role in the soil rhizosphere should be further investigated.

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MICROSOMAL OXIDATION OF ALLELOCHEMICALS IN GENERALIST (*Spodoptera frugiperda*) AND SEMISPECIALIST (*Anticarsia gemmatalis*) INSECT

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Abstract—Midgut microsomes prepared from larvae of the fall armyworm [*Spodoptera frugiperda* (J.E Smith)], a generalist insect, and the velvetbean caterpillar (*Anticarsia gemmatalis* Hubner), a semispecialist, were used to study their oxidative activity toward a variety of allelochemicals. Allelochemicals such as terpenoids, alkaloids, indoles, glucosinolates, flavonoids, coumarins, cardenolides, phenylpropenes, and a ketohydrocarbon were all metabolized by the microsomal cytochrome P-450 monooxygenases in both species. Fall armyworm microsomes oxidized monoterpenes more favorably than other types of terpenes, indicating a preference for these compounds. In all instances, the oxidative metabolism of these allelochemicals can be induced by dietary allelochemicals such as indole 3-carbinol, indole 3-acetonitrile, menthol, flavone, or peppermint oil ranging from 1.3- to 9.5-fold. In the case of certain triterpenes, tetraterpene, alkaloid, coumarin, and cardenolides, metabolic activity can only be observed after induction. The monooxygenase activities toward these allelochemicals were generally higher in the generalist than in the semispecialist. These findings provide strong evidence that microsomal monooxygenases play an important role in the detoxification of plant toxins and hence host-plant selections in herbivorous insects.

Key Words—Allelochemical metabolism, microsomal oxidation, fall armyworm, velvetbean caterpillar, *Spodoptera frugiperda*, *Anticarsia gemmatalis*, Lepidoptera, Noctuidae, enzyme induction.

INTRODUCTION

The microsomal cytochrome P-450 monooxygenase [also known as mixed-function oxidase (MFO)] system of insects plays an important role in the oxi-

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dative metabolism of various xenobiotics including pesticides (Hodgson, 1985). The versatility of this system is mainly due to its broad substrate specificity, capable of oxidizing various functional groups of lipophilic organic molecules. This system is believed to play a major role in insect herbivory on the basis of the following indirect evidence. First, aldrin epoxidase activity in polyphagous larvae is higher than in monophagous larvae, oligophagous ones being intermediate (Krieger et al., 1971). Second, MFO activity in larvae is low in the nonfeeding stage and high in the actively feeding stage (Krieger and Wilkinson, 1969; Ahmad, 1983). Finally, allelochemicals induce MFO activity which is presumed to enhance the detoxification of allelochemicals (Brattsen et al., 1977, 1984; Yu et al., 1979; Yu, 1983).

However, unlike pesticides, little is known about the microsomal metabolism of allelochemicals in insects. Most of the work has been done in mammals because of the toxicological and pharmacological importance of many allelochemicals in humans and domestic animals. Because of insufficient metabolic data in insects (Dowd et al., 1983), the MFO theory has recently been challenged by Dowd et al. (1983) and Gould (1984). These authors also argue that although allelochemicals induce MFO activity, the inducers have never been shown to stimulate the MFO which detoxifies the inducers.

In the present study, I investigated microsomal oxidations of an array of structurally different allelochemicals in a generalist and a semispecialist insect. The role of enzyme induction in the detoxification of allelochemicals was also examined in these insects.

METHODS AND MATERIALS

Insects. Larvae of the fall armyworm [*Spodoptera frugiperda* (J.E. Smith)], a generalist insect, and the velvetbean caterpillar (*Anticarsia gemmatalis* Hubner), a semispecialist insect, were reared on an artificial diet and maintained in environmental chambers at 25°C with a 16:8 light–dark photoperiod as described previously (Yu, 1982).

Chemicals. The allelochemicals used in this study and their sources were (−)-menthol, β-myrcene, (+)-camphor, geraniol, farnesol, santonin, phytol, gibberellic acid, stigmasterol, sitosterol, squalene, cholesterol, ergosterol, β-carotene, xanthotoxin, rotenone, flavone, nicotine, atropine, strychnine, colchicine, morin, cytisine, indole 3-carbinol, indole 3-acetaldehyde, indole 3-acetonitrile, indole 3-lactic acid, myricetin, estragole, umbelliferone, scopoletin, digitoxin, and eugenol (Sigma Chemical Company, St. Louis, Missouri); (+)-α-pinene, (−)-α-pinene, (−)-β-pinene, (+)-limonene, *l*-menthone, (+)-pulegone, *d*-carvone, (+)-camphene, (−)-camphene, nerolidol, safrole, *trans*-anethole, caffeine, monocrotaline, sinigrin, coumarin, digitoxigenin and digoxigenin (Aldrich Chemical Co., P.O. Box 355, Milwaukee, Wisconsin); myr-

isticin (Saber Laboratories, Inc., Box 232, Morton Grove, Illinois); 2-phenylethyl isothiocyanate, 2-tridecanone, and isosafrole (Fluka Chemical Corp., 255 Oser Avenue, Hauppauge, New York). All other chemicals were of analytical quality and purchased from commercial suppliers.

Treatment of Insects. When the toxicity of allelochemicals was studied, groups of 10 first-instar fall armyworm larvae were individually fed artificial diets containing the allelochemicals and maintained in 1-oz plastic cups. Larval weights were taken 13 days after feeding began. Adult emergence was also recorded at the end of the experiments.

When allelochemicals were used as inducers of microsomal monooxygenases, groups of newly molted sixth-instar larvae were collected from the culture and fed artificial diets containing the allelochemicals for two days. Controls were fed the artificial diet only. At the end of the experiments, larvae were removed from their respective diets and used for enzyme assays. No mortality was observed due to the treatments.

Preparation of Microsomes for Metabolic Studies. To prepare microsomes for the present study, midguts were dissected from 2-day-old sixth-instar larvae and their gut contents removed. They were then washed in 1.15% KCl and homogenized in 25 ml of ice-cold 0.1 M sodium phosphate buffer, pH 7.5, in a motor-driven tissue grinder for 30 sec. The crude homogenate was filtered through cheesecloth, and the filtered homogenate was centrifuged at $10{,}000 g_{max}$ for 15 min in a Beckman L5-50E ultracentrifuge. The pellet (cell debris, nuclei, and mitochrondria) was discarded, and the supernatant was recentrifuged at $105{,}000 g_{max}$ for 1 hr. The microsomal pellet was suspended in ice-cold 0.1 M sodium phosphate buffer, pH 7.5, to make a final concentration of 1 mg protein/ml and was used immediately. The above procedures were conducted at 0–4°C.

Microsomal Oxidation of Allelochemicals. The in vitro oxidative metabolism of allelochemicals was studied using either the NADPH-dependent substrate disappearance method or allelochemical-dependent NADPH oxidation. The former method was used to study the 10 compounds listed in Table 1. The 5-ml incubation mixture contained 1–2 mg of microsomal protein; 0.1 M sodium phosphate buffer, pH 7.5; an NADPH-generating system consisting of 1.8 μmol of NADP, 18 μmol glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase; and 10–50 μg of substrate (allelochemical) in 0.1 ml of methyl Cellosolve. Incubation mixture which contained no NADPH-generating system was used as blank. Duplicate incubations were conducted in a water bath with shaking at 30°C in an atmosphere of air for 1 hr. The unreacted parent compound was extracted with 10 ml of chloroform (for xanthotoxin, indole 3-acetonitrile and flavone) or ethyl acetate [for myristicin, safrole, rotenone, nicotine, (+)-pulegone, *trans*-anethole and estragole] by shaking in the water bath for 1 hr. The extracts were then analyzed by either high-pressure liquid chromatography (HPLC) or gas chromatography (GC).

TABLE 1. EFFECT OF ALLELOCHEMICALS IN LARVAL DIET ON DEVELOPMENT OF FALL ARMYWORM

	% of control	
Treatment (0.1% in diet)[a]	Larval weight after 13 days	Adult emergence
Control	100	100
Xanthotoxin	1	0
Rotenone	20	20
Flavone	20	100
Nicotine	0	0
Myristicin	1	10
Safrole	0	0
(+)-Pulegone	0	0
trans-Anethole	0	0
Estragole	0	0
Indole 3-acetonitrile	32	95

[a] First-instar larvae were fed meridic diets containing the allelochemicals.

A Beckman Series 340 high-performance liquid chromatograph equipped with a UV detector was used to analyze the allelochemicals with the exception of nicotine. Two types of columns were used. For normal-phase column (25 cm × 4.6 mm ID, Ultrasphere-Si, Altex), 5% isopropyl alcohol in hexane was used as the solvent system. This column was employed to analyze xanthotoxin, indole 3-acetonitrile, safrole, flavone, and rotenone. For reverse-phase column (25 cm × 4.6 mm ID, Ultrasphere-ODS, Altex), 85% methanol was used as the solvent system. This column was employed to analyze myristicin, (+)-pulegone, *trans*-anethole, and estragole. A flow rate of 1 ml/min was used for both columns.

A Varian model 3740 gas chromatograph equipped with a thermionic specific detector was used to analyze nicotine. The column used was 1.83 m × 2 mm ID glass column packed with 2% OV-101 on 80–100 mesh Ultra-Bond 20 M. The operating conditions were: column, 185°C; injection port, 200°C; detector, 250°C; nitrogen carrier gas, 30 ml/min; air, 175 ml/min; and hydrogen, 4.5 ml/min.

In tests with blank systems (−NADPH), 95–100% of added allelochemicals [xanthotoxin, indole 3-acetonitrile, myristicin, safrole, flavone, rotenone, (+)-pulegone, *trans*-anethole] was recovered by the extraction procedures. When boiled microsomes were used as the tissue source, there was no difference in the recovery rate, indicating that these allelochemicals were not metabolized by microsomes other than MFO under the assay conditions employed. However, a poor recovery (49%) for nicotine was encountered in the blank system. Apparently, nicotine was degraded by microsomes other than MFO since 70%

of added nicotine was recovered using boiled microsomes. Estragole showed a recovery rate of 87% in the blank system; however, the loss was not found to be enzymatic.

For some allelochemicals, especially those which cannot be detected by GC or HPLC, the allelochemical-dependent NADPH oxidation method was used to study their oxidative metabolism. The 4.6-ml mixture which contained 0.5–1 mg microsomal protein, 0.1 M sodium phosphate buffer, pH 7.5, was first incubated for 3 min at 30°C and then 0.1 mM NADPH in 0.4 ml of the same buffer was added and mixed. After the addition of 50 μM substrate (allelochemical) in 5 μl of methyl Cellosolve to 2.5 ml of the mixture, the rate of NADPH oxidation was recorded at 340 nm against the same reaction mixture in the absence of substrate in a Beckman model 5260 UV/VIS spectrophotometer. This system cancels endogenous NADPH oxidation automatically because both reference and sample cuvettes contain microsomes and NADPH.

RESULTS

Toxicity of Allelochemicals to Fall Armyworm Larvae. Ten compounds which were selected for bioassays against fall armyworm larvae included coumarin (xanthotoxin), flavonoids (rotenone, flavone), alkaloid (nicotine), phenylpropenes (myristicin, safrole, *trans*-anethole, estragole), terpenoid [(+)-pulegone] and indole (indole 3-acetonitrile). As shown in Table 1, these allelochemicals were all toxic to the fall armyworm when first-instar larvae were fed artificial diets containing 0.1% of the compounds. In the case of nicotine, safrole, *trans*-anethole, estragole, and (+)-pulegone, none of the test larvae progressed to sixth instar. However, flavone and indole 3-acetonitrile had no marked effect on adult emergence.

Microsomal Oxidation of Allelochemicals. The validity of the NADPH-dependent allelochemical disappearance method is shown in Figure 1 using xanthotoxin as a model substrate. When NADPH was added in the incubation mixture, the level of xanthotoxin decreased 56% as compared with the control (−NADPH). Two metabolites were produced from the metabolism but were not identified. Addition of piperonyl butoxide (PB) (10^{-4} M) or CO (both are known inhibitors of microsomal monooxygenases) caused an 89% reduction in the metabolic activity in each case. The results suggest that xanthotoxin is oxidized by the microsomal cytochrome P-450-dependent monooxygenases in fall armyworm larvae.

Using the NADPH-dependent method described above, we showed that the allelochemicals in Table 2 were all metabolized by a MFO system from fall armyworm larvae. The specific activity for the MFO system ranged from 0.86 to 34.34 nmol/hr/mg protein, estragole being the best substrate for the enzyme among those studied. The metabolism of each compound was inhibited by pi-

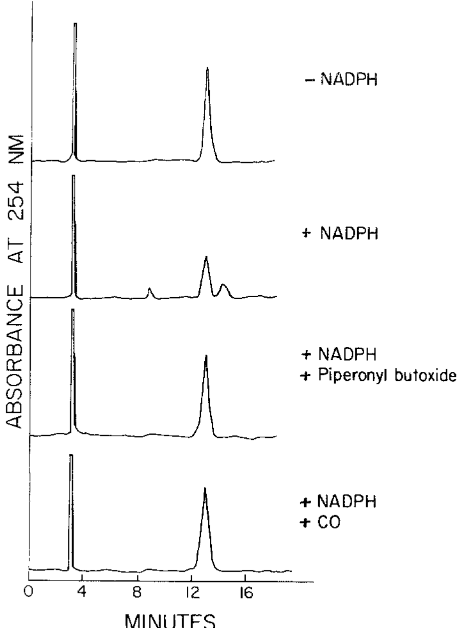

FIG. 1. Liquid chromatograms showing metabolism of xanthotoxin by fall armyworm microsomes under different conditions. Newly molted sixth-instar larvae were fed a diet containing indole 3-carbinol (0.2%) for two days prior to preparation of microsomes. See text for details (incubations were conducted for 15 min).

TABLE 2. OXIDATIVE METABOLISM OF ALLELOCHEMICALS BY FALL ARMYWORM MICROSOMES

	Allelochemical metabolized (nmol/hr/mg protein)[a]	
Enzyme substrate	Control	PB (10^{-4} M) (% of control)
Xanthotoxin	6.33 ± 0.35	25
Indole 3-acetonitrile	2.27 ± 0.28	31
Myristicin	2.74 ± 0.10	14
Safrole	3.93 ± 1.06	10
Flavone	1.11 ± 0.56	25
Rotenone	31.35 ± 1.65	36
Nicotine	0.86 ± 0.16	12
(+)-Pulegone	10.35 ± 1.59	7
trans-Anethole	14.39 ± 0.98	45
Estragole	34.34 ± 2.55	24

[a] Newly molted sixth-instar larvae were fed artificial diets containing 0.2% of the compounds for two days prior to enzyme assays. The NADPH-dependent substrate disappearance method was used to measure oxidative metabolism. Mean ± SE of two experiments, each assayed in duplicate.

peronyl butoxide, again indicating the involvement of cytochrome P-450. Data in Table 3 shows that the oxidative metabolism can be enhanced by feeding the larvae with certain allelochemicals such as indole 3-carbinol, indole 3-acetonitrile, menthol, flavone, or peppermint oil, all of which are inducers of various MFO activities in fall armyworms (Yu, 1982, 1983, 1984; Yu and Ing, 1984). The induction ranged from 1.6- to 4.1-fold depending on the allelochemical used as substrate.

Further studies of allelochemical metabolism were made by the allelochemical-dependent NADPH oxidation method. The results are summarized in Tables 4, 5, and 6. It is seen that addition of PB and CO greatly decreased the enzyme activity, indicating the involvement of cytochrome P-450. Data in Table 4 show that fall armyworm microsomes generally oxidize monoterpenes more favorably than other types of terpenes, indicating a preference for these compounds. In addition to terpenoids, other plant toxins such as alkaloids, indoles, glucosinolates, coumarins, cardenolides, ketohydrocarbon, and phenylpropenes were also metabolized by the MFO system (Table 5). In all instances, the oxidative metabolism of these allelochemicals can be induced by dietary indole 3-carbinol ranging from 1.3- to 9.5-fold in this insect. It is interesting to note that in the case of certain triterpenes, tetraterpene, alkaloid, coumarin, and cardenoilides, metabolic activity can only be observed after induction.

Among those studied, santonin, gibberellic acid, cytisine, colchicine, morin, indole 3-lactic acid, myricetin, umbelliferone, and digitoxigenin were not metabolized by the MFO system from fall armyworm larvae. Lack of suitable functional groups, steric/conformation factors, and/or polarity may explain their failure as substrates for these membrane-bound enzymes.

The microsomal monooxygenase system from velvetbean caterpillar larvae (noninduced) also metabolized various allelochemicals. From Table 6, it can be seen that the MFO activities toward these allelochemicals were generally lower in the velvetbean caterpillar than in the fall armyworm with the exception of *d*-carvone, geraniol, nerolidol, farnesol, and cholesterol for which the reverse was true.

DISCUSSION

The results of the present study clearly demonstrate that plant allelochemicals with diverse chemical structures including terpenoids, alkaloids, indoles, glucosinolates, flavonoids, coumarins, cardenolides, phenylpropenes, and ketohydrocarbon are all metabolized by the microsomal monooxygenase system prepared from larvae of the fall armyworm and velvetbean caterpillar. Although attempts were not made to identify the oxidative metabolite(s) from each allelochemical in this study, judging from their structures, most of these compounds were likely to be detoxified via epoxidation or hydroxylation by the

TABLE 3. EFFECT OF ALLELOCHEMICALS ON OXIDATIVE METABOLISM OF ALLELOCHEMICALS IN FALL ARMYWORM LARVAE

Treatment (0.2% in diet)[a]	Allelochemical metabolized (% of control)[b]									
	Xanthotoxin	Flavone	Rotenone	Nicotine	Safrole	Indole 3-acetonitrile	*trans*-Anethole	Estragole	(+)-Pulegone	Myristicin
Control	100	100	100	100	100	100	100	100	100	100
Indole 3-carbinol	389**[c]	408*	156*	271*	223*	202*				
Indole 3-acetonitrile	296**	374*								
Menthol	203**	278*								
Peppermint oil	209*	264*					236*	273**	384*	301**
Flavone	292**									

[a]Newly molted sixth-instar larvae were fed diets containing the allelochemicals for two days prior to enzyme assays.
[b]Mean of two experiments, each assayed in duplicate.
[c]See Table 4 (footnotes) for the level of statistical difference from the control.

TABLE 4. OXIDATIVE METABOLISM OF TERPENOIDS BY FALL ARMYWORM MICROSOMES AS MEASURED BY NADPH OXIDATION

Enzyme substrate (50 μm)	Enzyme activity (nmol NADPH oxidized/min/mg protein)[a]			
		Indole 3-carbinol fed		
			% of control	
	Untreated	Control	PB (10^{-4} M)	CO[b]
Monoterpenes				
(+)-α-Pinene	3.18 ± 0.31	16.56 ± 0.93**[c]	11	0
(−)-α-Pinene	3.90 ± 0.21	21.66 ± 0.20***	0	9
(−)-β-Pinene	2.67 ± 0.21	15.94 ± 0.54***	11	6
(+)-Limonene	3.69 ± 0.41	27.86 ± 0.90***	7	7
(−)-Menthol	5.33 ± 0.41	24.08 ± 1.38**	10	8
l-Menthone	5.54 ± 0.62	32.18 ± 3.43**	12	7
β-Myrcene	4.72 ± 0.21	28.22 ± 0.54***	16	9
(+)-Pulegone	3.69 ± 0.41	22.11 ± 0.54***	6	12
d-Carvone	2.25 ± 0.22	13.90 ± 1.27**	10	10
(+)-Camphor	1.84 ± 0.20	12.04 ± 0.42**	13	12
(+)-Camphene	2.86 ± 0.83	17.86 ± 0.75**	5	2
(−)-Camphene	3.52 ± 0.26	11.28 ± 0.43**	10	8
Geraniol	2.05 ± 0.41	16.37 ± 0.37***	6	12
Sesquiterpenes				
Nerolidol	3.80 ± 0.11	35.95 ± 1.86***	10	10
Farnesol	3.70 ± 0.42	34.96 ± 2.51**	20	20
Santonin	0	0	—	—
Diterpenes				
Phytol	3.70 ± 0.42	29.97 ± 0.98**	18	10
Gibberelic acid	0	0	—	—
Triterpenes				
Stigmasterol	0	1.97 ± 0.19	0	0
Sitosterol	0	4.15 ± 0.54	0	0
Sequalene	0	1.12 ± 0.38	26	40
Cholesterol	0.62 ± 0.21	3.35 ± 0.37*	0	23
Ergosterol	0	3.51 ± 0.39	25	20
Tetraterpene				
β-Carotene	0	1.97 ± 0.19	20	7

[a] Newly molted sixth-instar larvae were fed an artificial diet containing 0.2% of the compound for two days prior to enzyme assays. Mean ± SE of two experiments, each assayed in duplicate.
[b] Bubbled for one minute prior to enzyme assays.
[c] Values significantly different from the untreated group based on Student's *t* test.
*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE 5. OXIDATIVE METABOLISM OF ALLELOCHEMICALS BY FALL ARMYWORM MICROSOMES AS MEASURED BY NADPH OXIDATION

	Enzyme activity (nmol NADPH oxidized/min/mg protein)[a]			
		Indole 3-carbinol fed		
			% of control	
Enzyme substrate	Untreated	Control	PB (10^{-4} M)	CO[b]
Alkaloids				
Atropine	0.39 ± 0.04	1.57 ± 0.17*[c]	20	13
Strychnine	0.73 ± 0.15	3.13 ± 0.35*	38	38
Caffeine	0.47 ± 0.12	1.22 ± 0.17**	40	20
Cytisine	0	0	—	—
Colchicine	0	0	—	—
Monocrotaline	0	1.47 ± 0.08	16	32
Morin	0	0	—	—
Indoles				
Indole 3-carbinol	0.46 ± 0.11	1.80 ± 0.20**	20	20
Indole 3-acetaldehyde	1.92 ± 0.18	4.20 ± 0.20**	19	27
Indole 3-lactic acid	0	0	—	—
Glucosinolates				
Sinigrin	0.30 ± 0.06	0.70 ± 0.10**	33	50
2-Phenylethyl isothiocyanate	0.55 ± 0.03	5.00 ± 0.70***	0	0
Flavonoid				
Myricetin	0	0	—	—
Coumarins				
Coumarin	0	0.86 ± 0.14	0	0
Umbelliferone	0	0	—	—
Scopoletin	0.99 ± 0.14	1.29 ± 0.15	0	30
Cardenolides				
Digitoxin	0	0.71 ± 0.15	0	0
Digitoxigenin	0	0	—	—
Digoxigenin	0	1.17 ± 0.06	28	32
Phenylpropenes				
Eugenol	1.02 ± 0.13	4.80 ± 0.80*	12	28
Isosafrole	2.06 ± 0.08	7.01 ± 0.65*	20	28
Ketohydrocarbon				
2-Tridecanone	5.81 ± 0.59	8.52 ± 0.09*	40	25

[a] Newly molted sixth-instar larvae were fed an artificial diet containing 0.2% of the compound for two days prior to enzyme assays. Mean ± SE of two experiments, each assayed in duplicate.
[b] Bubbled for one minute prior to enzyme assays.
[c] See Table 4 (footnotes) for the level of statistical difference from untreated group.

TABLE 6. OXIDATIVE METABOLISM OF ALLELOCHEMICALS BY VELVETBEAN CATERPILLAR MICROSOMES AS MEASURED BY NADPH OXIDATION

Enzyme substrate (50 μM)	Enzyme activity (nmol NADPH oxidized/min/mg protein)[a]
(+)-α-Pinene	1.82 ± 0.15
(−)-α-Pinene	2.42 ± 0.08
(−)-β-Pinene	1.73 ± 0.03
(+)-Limonene	3.44 ± 0.31
(−)-Menthol	3.13 ± 0.05
l-Menthone	4.06 ± 0.04
β-Myrcene	4.49 ± 0.02
(+)-Pulegone	2.58 ± 0.26
d-Carvone	2.46 ± 0.24
(+)-Camphor	1.55 ± 0.04
(+)-Camphene	1.98 ± 0.31
(−)-Camphene	1.95 ± 0.22
Geraniol	3.84 ± 0.60
Nerolidol	4.45 ± 0.09
Farnesol	3.91 ± 0.09
Phytol	0.69 ± 0.05
Cholesterol	1.33 ± 0.03
Eugenol	1.16 ± 0.11
Estragole	2.31 ± 0.08
trans-Anethole	2.17 ± 0.38
Indole 3-acetonitrile	1.19 ± 0.08
Indole 3-carbinol	0.53 ± 0.11
2-Tridecanone	4.68 ± 0.43

[a] Microsomes were prepared from 2- to 3-day-old sixth-instar larvae. Mean ± SE of two experiments, each assayed in duplicate.

MFO system. The findings provide strong evidence for the current belief that MFOs are important in the selection of host plants by herbivorous insects.

In support of these observations, it has been shown that the botanical insecticides, rotenone, nicotine and pyrethrins, are metabolized by cytochrome P-450 monooxygenase systems in insects. Rotenone is oxidatively metabolized to rotenolone I and II, 6′,7′-dihydro-6′,7′-dihydroxyrotenone, and 8′-hydroxyrotenone in house flies (Fukami et al., 1969). Pyrethrin I is hydroxylated at the *trans*-methyl group on the acid side chain in house flies (Casida et al., 1971). As to nicotine, it is hydroxylated by the MFO system at the C-2 position to produce 2-hydroxynicotine followed by alcohol dehydrogenation to yield continine in tobacco-feeding insects (Self et al., 1964; Matsumura, 1975; Hodgson and Dauterman, 1980).

The metabolism of the furanocoumarin, xanthotoxin, was studied in the black swallowtail butterfly by Ivie et al. (1983). Two metabolites, 7-hydroxy-

8-methoxy-2-oxo-2H-1-benzopyran-6-acetic acid and α,7-dihydro-8-methoxy-oxo-2H-1-benzopyran-6-acetic acid, which were believed to be formed through microsomal oxidation (Kolis et al., 1979), were found in larvae when they were fed xanthotoxin. The ability of the black swallowtail to detoxify this compound explains why this insect can feed on xanthotoxin-containing plants without being poisoned.

A variety of monoterpenes is oxidatively metabolized by insects. α-Pinene is hydroxylated to *trans*-verbenol (Hughes, 1975), myrcene to ipsdienol (Hughes, 1974), and camphene to 6-hydroxycamphene (Renwick et al., 1976) in bark beetles. That MFO is involved in the oxidation of monoterpenes was reported by White et al. (1979), who showed that α-pinene was oxidized to α-pinene epoxide by cytochrome P-450-dependent monooxygenases prepared from bark beetles.

Some plants, such as bracken ferns, were found to contain insect molting hormones (Kaplanis et al., 1967). The phytoecdysone, α-ecdysone, has been shown to be hydroxylated by microsomal oxidase at the C-20 position to become β-ecdysone in the African migratory locust (Feyerisen and Durst, 1978).

The juvenile hormone antagonist, precocene II, was shown to be primarily attacked by microsomal oxidases prepared from the fat body homogenates of cabbage looper larvae (Soderlund et al., 1980). The metabolites were 6-hydroxy, 7-hydroxy, 3,4-dihydro-3-hydroxy, and *cis*- and *trans*-3,4-dihydro-3,4-dihydroxy precocene II. Although precocene 3,4-epoxide was not detected in this study, its formation was believed to be essential as an intermediate for both diols. Because of the extreme reactivity of the epoxide, it was suggested that the epoxide is involved in the allatotoxication of precocene II.

As to the enzyme induction, the results of this study demonstrate that all the allelochemicals previously shown to be MFO inducers (e.g., α-pinene, β-pinene, menthol, menthone, myrcene, limonene, indole 3-carbinol, indole 3-acetonitrile, flavone, and xanthotoxin) (Brattsten et al., 1977, 1984; Yu et al., 1979; Yu, 1983, 1984) were substrates for the MFOs in fall armyworm larvae. Specifically, feeding indole 3-carbinol to fall armyworm larvae increased the MFO activity which oxidized this allelochemical as much as four-fold (Table 4). In this respect, enzyme induction caused by allelochemicals would help the insects detoxify plant toxins in their diets. As a result, they would potentially have a wider host-plant range if enzyme inducers happen to coexist with plant toxins in the same plants, or inducing plants and toxin-containing plants are all available in the same area.

Comparing the MFO activity toward various allelochemicals in the generalist (polyphagous) larvae and the semispecialist (oligophagous) larvae, it is clear that our results are generally in agreement with those of Krieger et al. (1971) showing the correlation between MFO activity and degree of feeding specialization. However, the fact that certain allelochemicals are better substrates for the MFO from velvetbean caterpillar larvae than from fall armyworm

larvae suggests that the MFO in the generalist may be quantitatively as well as qualitatively different from that of the semispecialist. In particular, the isozyme composition of cytochrome P-450 could be different in these two insects as in the case of insecticide-susceptible and resistant insects (Yu and Terriere, 1979). More data from various species of generalist and specialist insects are needed before a firm conclusion can be made.

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RADIAL DIFFUSION METHOD FOR DETERMINING TANNIN IN PLANT EXTRACTS

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Abstract—Tannin in plant extracts can be determined by reacting the tannin with a protein and quantitating the precipitated complex. In the new assay described here, a tannin-containing solution is placed in a well in a protein-containing agar slab. As the tannin diffuses into the gel and complexes with protein, a visible ring of precipitation develops. The area of the ring is proportional to the amount of tannin in the extract. The detection limit of the method is 0.025 mg tannic acid or condensed tannin and the precision is 6% (relative standard deviation). Tests with extracts of a variety of plants show that the new method gives results comparable to other precipitation methods and that the new method is superior for samples of unusual composition, such as aspen buds. The method has several advantages over other methods for determining tannin: The new method is very simple and requires neither complex reagents nor instruments. Components of the plant extract such as non-tannin phenolics or water-insoluble compounds do not interfere with the method. The assay is not subject to interference from the organic and aqueous solutions which are commonly used to extract tannin from plants.

Key Words—Tannin, proanthocyanidin, protein precipitation, digestibility-reducing substances, phenolic analysis.

INTRODUCTION

Tannins, like many other secondary compounds, are thought to defend plants from herbivores (Swain, 1979). To establish the role of tannin as a defensive compound, the tannin content of various plants must be correlated with patterns of herbivory. Sensitive, specific tannin assays which can easily be run on large numbers of samples are required for such studies of tannin as a defensive compound.

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The analytical methods currently available for determining tannin have several disadvantages. The functional group methods do not have satisfactory specificity. For example, the redox methods such as the Folin-Denis assay (Folin and Denis, 1915) are not specific for tannin, but detect any phenolic compound. On the other hand, the proanthocyanidin and vanillin assays (Bate-Smith, 1975; Price et al., 1978) are too selective. The hydrolyzable tannins, which are gallic acid derivatives (Haslam, 1979), do not react with acidic butanol or vanillin. Only the flavonoid-based condensed tannins (Haslam, 1979) can be detected with these reagents.

Precipitation assays also have disadvantages. Several methods for determining protein precipitated by the tannin have been described (Bate-Smith, 1973; Hagerman and Butler, 1980a; Martin and Martin, 1983). Although selective, these methods are inconvenient; they may include multiple steps for forming and isolating the precipitate, or they may require special materials such as radiolabeled compounds. Two simple precipitation methods have been described. In one method, a dye-labeled protein is used, and the amount of protein precipitated by the tannin is determined spectrophotometrically (Asquith and Butler, 1985). In another method, the tannin precipitated by excess protein is measured spectrophotometrically after reaction with ferric chloride (Hagerman and Butler, 1978). Although these methods are straightforward, sample preparation for these assays is complicated. Some solvents, such as acetone, interfere with the precipitation and must be removed from the extract before analysis. In addition, water-insoluble compounds frequently found in the tannin extract interfere with precipitation assays (Hagerman and Butler, 1978; Asquith and Butler, 1985).

A new protein precipitation assay that overcomes these problems is described here. In the assay, tannin diffuses through a protein-containing gel, and a visible disk-shaped precipitate develops as the tannin interacts with the protein. The method is simple, sensitive, and specific, and should be especially applicable to studies in which large numbers of samples are to be analyzed.

METHODS AND MATERIALS

Reagents. All reagents were analytical grade or the best grade available. Agarose (type I), bovine serum albumin (BSA) (fatty acid-free fraction V), and catechin were obtained from Sigma Chemical Co. (St. Louis, Missouri). Condensed tannin was prepared from *Sorghum vulgare* IS 4225 by the method of Hagerman and Butler (1980b). Hydrolyzable tannin was purified from commercial tannic acid as described by Hagerman and Klucher (1986). Buffer A consisted of 50 mM acetic acid and 60 μM ascorbic acid adjusted to pH 5.0.

Assay Method. A 1% (w/v) solution of agarose was prepared in buffer A

by heating the suspension of agarose to boiling while stirring. The solution was cooled to 45°C in a water bath, and the protein [0.1% (w/v) BSA] was added while the solution was gently stirred. The solution was dispensed in 9.5-ml aliquots into standard plastic Petri dishes (8.5 cm diameter) and allowed to cool. The agarose solidified on cooling; the plates were always cooled on a level surface to obtain slabs of uniform thickness. Once prepared, the plates were stored at 4°C to prevent bacterial growth.

Uniform wells were punched in the plates with a punch 4.0 mm in diameter (Biorad Co., Richmond, California). The wells were spaced 1.5 cm apart on the plates. The tannin-containing solutions were added to the wells with a Hamilton microsyringe; the capacity of 4.0-mm wells was slightly greater than 8 μl, so the samples were applied in 8-μl aliquots. For dilute samples, several successive 8-μl aliquots were added to a single well as the liquid was absorbed by the gel. Although any organic or aqueous solution could be applied to the wells, solutions containing at least 10% (v/v) water were easier to dispense than neat organic solvents.

After placing the samples in the wells, the Petri dishes were covered and sealed with Parafilm. The dishes were incubated at 30°C for 96–120 h. The diameters of the rings were then measured; for each ring, two diameters at right angles to one another were measured to minimize errors due to nonuniform ring development. Tannin concentration was calculated from the square of the average of the two diameters using an appropriate calibration curve.

Plant Extraction. Plant tissue was extracted for an hour at room temperature with 50% (v/v) aqueous methanol, using a solvent-to-tissue ratio of 0.5 ml solvent per 100 mg tissue. For diffusion assays, 100 mg tissue was an adequate sample size; if other assays were run, the sample size was increased to 300–500 mg. The extracts were applied directly to the diffusion gels without any sample cleanup. Two or three successive aliquots of 8 μl were sufficient to form rings for the plants tested.

If the extracts were to be analyzed with other precipitation tests, the samples were centrifuged (5000*g*, 15 min) and the extract removed from the pelleted tissue. For the protein precipitation assays (Hagerman and Butler, 1978, 1980a), 0.20 ml of the methanol extract was added to 7.0 mg iodine-125-labeled BSA in 2.00 ml acetate buffer. After 15 min, the mixtures were centrifuged. An aliquot of each supernatant was removed for radioactive counting, and the remainder of the supernatants discarded. The pellets were dissolved in triethanolamine reagent (Hagerman and Butler, 1978), ferric chloride was added, and the A_{510} was determined. The amount of protein precipitated was calculated from the radioactive counting data, using a calibration curve prepared with the labeled BSA. The amount of tannin precipitated was determined from a standard curve run with purified tannic acid (Hagerman and Klucher, 1986).

RESULTS

When a solution containing condensed or hydrolyzable tannin was placed in a protein-containing gel, the tannin diffused into the gel and formed a disk-shaped tannin–protein precipitate (Figure 1). No ring formed if the protein was omitted from the gel. Under the conditions described here, the ring was always clearly visible without any staining. The rings appeared similar for condensed tannin obtained from *Sorghum* seeds, for hydrolyzable tannin purified from commercial tannic acid, and for tannin-containing extracts from a variety of plants (Figures 1 and 2).

The rings expanded slowly, and reached their equilibrium sizes only after

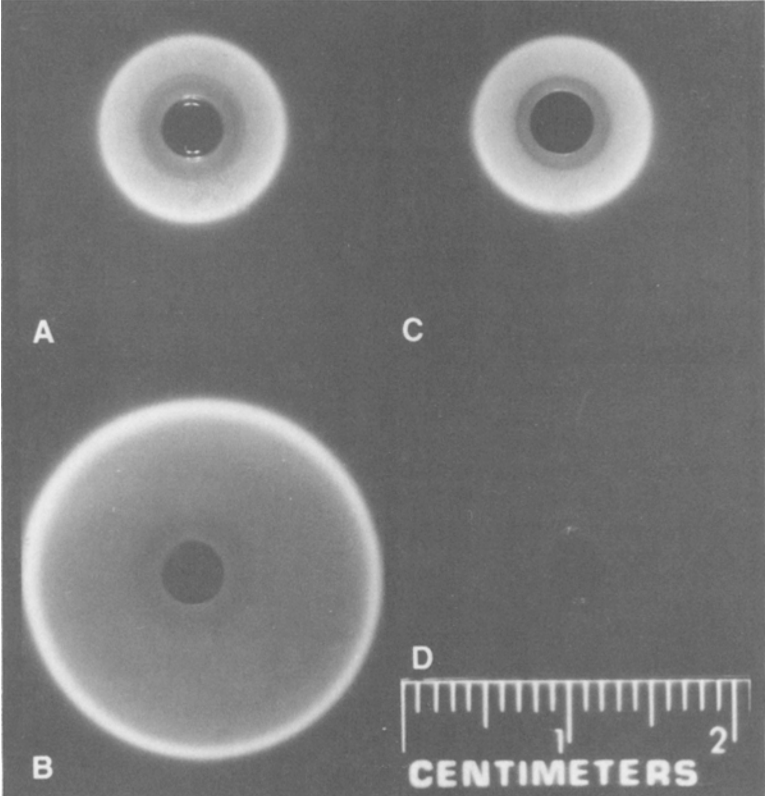

FIG. 1. Radial diffusion assay for tannin. An 8-μl aliquot of the sample dissolved in 70% acetone was placed in each well and photographed after the rings reached equilibrium. (A) 0.60 mg sorghum tannin; (B) 0.60 mg tannic acid; (C) 0.50 mg catechin plus 0.60 mg sorghum tannin; and (D) 0.50 mg catechin.

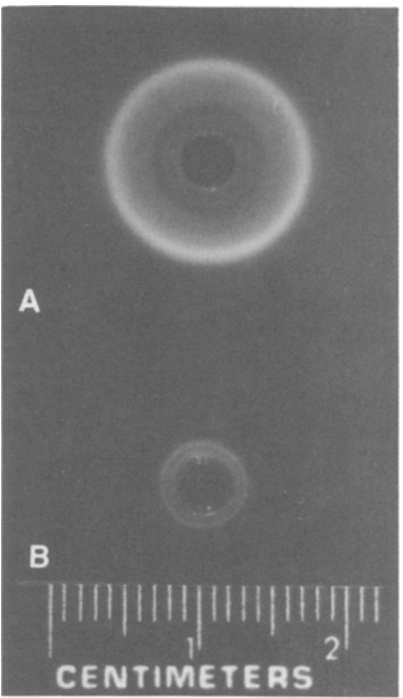

FIG. 2. Plant extracts in radial diffusion assay. Maple leaves (*Acer* sp.) or birch twigs (*Betula* sp.) were extracted with 50% methanol (300 mg tissue/1.5 ml solvent). Two 8-μl aliquots of the maple extract were placed in well A. Three 8-μl aliquots of the birch extract were placed in well B.

long incubation (Figure 3). The rings started to form immediately after the sample was placed in the well, but quantitative analysis was possible only after equilibrium was reached. The amount of time required for the rings to reach equilibrium increased with increasing tannin concentration (Figure 3). An incubation time of 96 hr after sample application was adequate for all samples tested here.

The area of the ring was linearly related to the amount of tannin placed in the well (Figure 4). To simplify calculations, the diameter squared was used instead of the area. The calibration lines had nonzero y intercepts because the wells into which the samples were dispensed had finite areas (0.16 cm^2). The detection limit of the assay was 0.025 mg tannin, and the precision was $\pm 6\%$ (relative standard deviation). The detection limit of the assay depended on the concentration of protein in the plates. The amount of protein used here was chosen to optimize the limit of detection.

A lower response was obtained with condensed tannin than with hydrolyz-

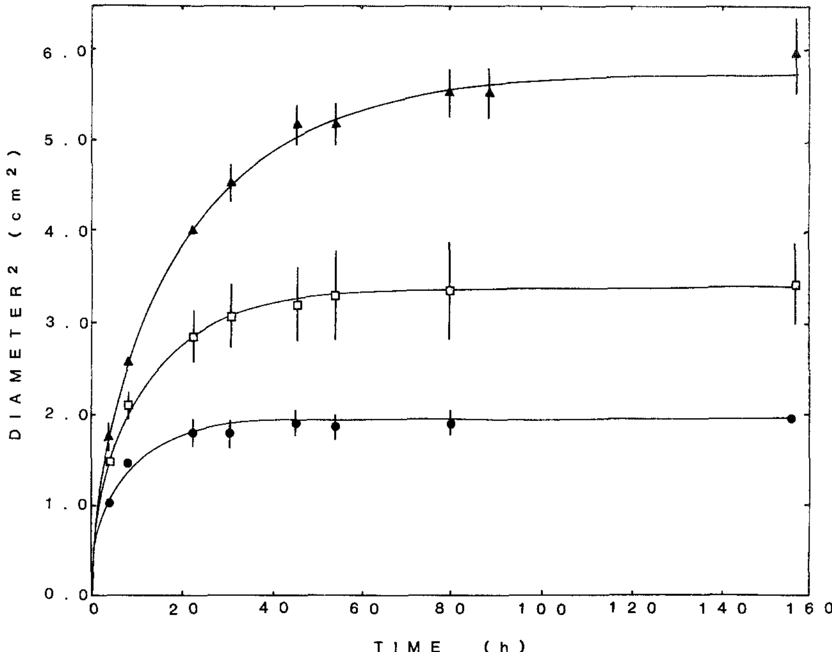

FIG. 3. Kinetics of ring formation. An 8-μl aliquot containing 1.0 mg tannic acid (▲), 0.50 mg tannic acid (□), or 0.25 mg tannic acid (●) was placed in a well and the diameters of the rings were measured at various times. Each point represents the mean ±SD for at least three replicates.

able tannin (Figure 4). Because the two types of tannin are not chemically identical, it is typical to obtain different sensitivities with the same test. For example, the response to condensed tannin in the protein precipitable phenolics assay is only half as large as the response to hydrolyzable tannin because the yield of colored complex differs for the two types of tannin (Hagerman and Butler, 1978). The amount of protein precipitated by the two types of tannin is different (Hagerman and Klucher, 1986). Therefore, it was not surprising that the two types of tannin responded differently in the diffusion assay.

The response of the two types of tannin to the assay was additive, even though the responses were not equivalent. The size of a ring formed by a mixture of tannins was equal to the sum of the sizes of rings formed by the individual samples (Figure 5). Analysis of an extract containing both types of tannin accurately reflected the total tannin content since the response was additive even when one component was present in excess. The method cannot be used to determine the relative amounts of the two types of tannin. Functional group assays are available for chemical characterization of mixtures of tannins.

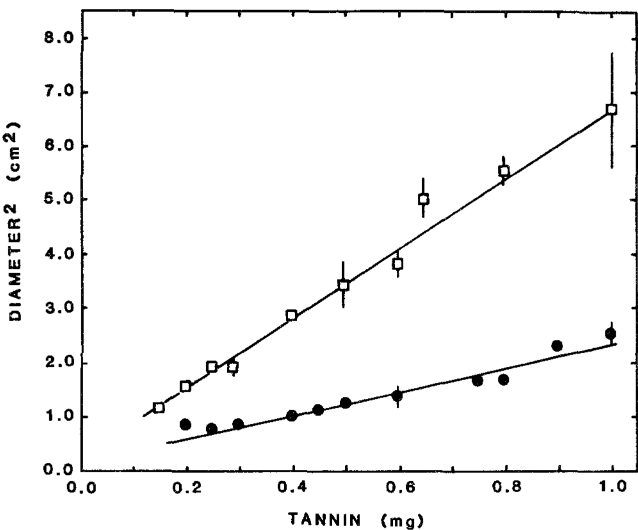

FIG. 4. Calibration curve for condensed and hydrolyzable tannin. An 8-μl aliquot of 70% acetone containing the purified tannin was placed in the well. The diameters of the rings were measured after equilibrium was reached, and the diameter squared was calculated. Each point represents the mean of at least three replicates $\pm$SD. The data was fit to a line using the method of least squares. The diameter^2 is in cm^2; the amount of tannin is in mg. For tannic acid (□): diameter^2 = 6.5 (amount of tannin) + 0.24 (r^2 = 0.94); for sorghum tannin (●): diameter^2 = 2.21 (amount of tannin) + 0.17 (r^2 = 0.92)

Nontannin phenolics such as flavonoids, benzoic acids, or hydroxycinnamic acids did not interfere with the assay. The addition of catechin or gallic acid to purified tannic acid or sorghum tannin had no effect on the ring size (Table 1). Similar data were obtained for hydroxycinnamic acids. If an aliquot of a saturated solution of catechin or gallic acid was applied to the well, a small ring formed rapidly. However, these rings disappeared within an hour and did not contribute to the final size of the ring. Plant extracts which do not contain tannin but do contain nontannin phenolics, such as extracts of leaves of the spider plant, did not form rings. Addition of such tannin-free leaf extracts to solutions of purified or condensed tannin did not affect the ring formation, indicating that nontannin components extracted from the leaf did not interfere with the tannin assay.

The solvent did not affect ring size. Common solvents used to prepare extracts include 50% methanol (methanol–water, 1 : 1 v/v), 70% acetone (acetone–water, 7 : 3 v/v), and 1% HCl in methanol (HCl–methanol, 1 : 99 v/v). None of these solvents affected the response to tannic acid obtained with the assay (Table 2). Similar data were obtained for condensed tannin in the same

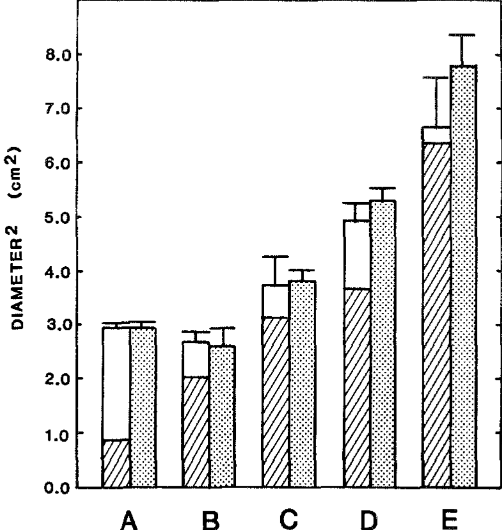

FIG. 5. Analysis of mixtures of condensed and hydrolyzable tannin. An 8-μl aliquot of 70% acetone containing either tannic acid (▨), sorghum tannin (□), or a mixture of the two (⬚) was placed in the well. The diameters of the rings were measured after equilibrium was reached. For each pair of bars, the bar to the left represents the sum of the diameters squared for the two individual solutions, and the bar on the right represents the diameter squared for the mixture. Each value is based on three replicate determinations, and error bars represent 1 SD. (A) 0.091 mg tannic acid, 0.91 mg sorghum tannin; (B) 0.33 mg tannic acid, 0.33 mg sorghum tannin; (C) 0.50 mg tannic acid, 0.25 mg sorghum tannin; (D) 0.50 mg tannic acid, 0.50 mg sorghum tannin; and (E) 0.91 mg tannic acid, 0.091 mg sorghum tannin.

solvents. The addition of antioxidants such as ascorbic acid did not interfere with the assay.

The results obtained with the radial diffusion assay were highly correlated (r^2 = 0.95) with the results obtained with precipitation assays (Figure 6). The radial diffusion assay, the protein precipitable phenolics assay, and the labeled BSA protein precipitation assay were used to analyze extracts of a variety of plants and two *Vaccinium*-containing diets (C. T. Robbins, personal communication). All the assays gave qualitatively similar results. One group of samples was classified as low tannin based on all of the assays (*Ceanothus*, *Sorghum*, 50% *Vaccinium*); another group was classified as intermediate tannin (oak, 75% *Vaccinium*, green tea, dogwood); and a third group was classified as high tannin (fireweed flowers). Quantitatively, the assays gave different values since, with each assay, a different chemical characteristic of tannin is measured. There was a twofold difference between the tannin content determined with the radial diffusion method and the content determined with the protein

TABLE 1. EFFECT OF NONTANNIN PHENOLICS ON SIZE OF RING IN RADIAL DIFFUSION ASSAY[a]

Tannin	Phenolic	Diameter^2 (cm^2)[b]
Tannic acid		
0.30 mg	none	1.98 + 0.04
0.30 mg	catechin	2.10 + 0.10
0.30 mg	gallic acid	2.10 + 0.15
Tannic acid		
0.60 mg	none	4.88 + 0.06
0.60 mg	catechin	4.66 + 0.22
0.60 mg	gallic acid	5.18 + 0.41
Sorghum tannin		
0.30 mg	none	0.83 + 0.03
0.30 mg	catechin	0.87 + 0.05
0.30 mg	gallic acid	0.83 + 0.03
Sorghum tannin		
0.06 mg	none	1.25 + 0.07
0.60 mg	catechin	1.27 + 0.10
0.60 mg	gallic acid	1.28 + 0.10

[a]The indicated amount of tannin or tannin plus 0.50 mg catechin or gallic acid was dissolved in 70% acetone. An 8-μl aliquot was placed in each well and the diameter was measured after equilibrium was reached.
[b]The values shown are the means of three replicates ± 1 SD.

precipitable phenolics method (Figure 6), although tannic acid was used as the standard for both assays. The radial diffusion assay depends only upon the ability of the tannin to interact with protein to form a visible precipitate. The protein precipitable phenolics assay depends both on interaction with protein to form a precipitate and on reaction with ferric chloride to form a colored complex. Be-

TABLE 2. EFFECT OF SOLVENT ON SIZE OF RING IN RADIAL DIFFUSION ASSAY

Solvent[a]	Diameter^2 (cm^2)[b]
50% acetone	3.49 + 0.21
50% methanol	3.74 + 0.23
Buffer A	3.13 + 0.51
1% HCl in methanol	3.49 + 0.21

[a]The indicated solvent, 8 μl, containing 0.50 mg purified tannic acid was placed in the well. The diameter of the ring was measured after the system was at equilibrium.
[b]Values are the mean of three replicates ± 1 SD.

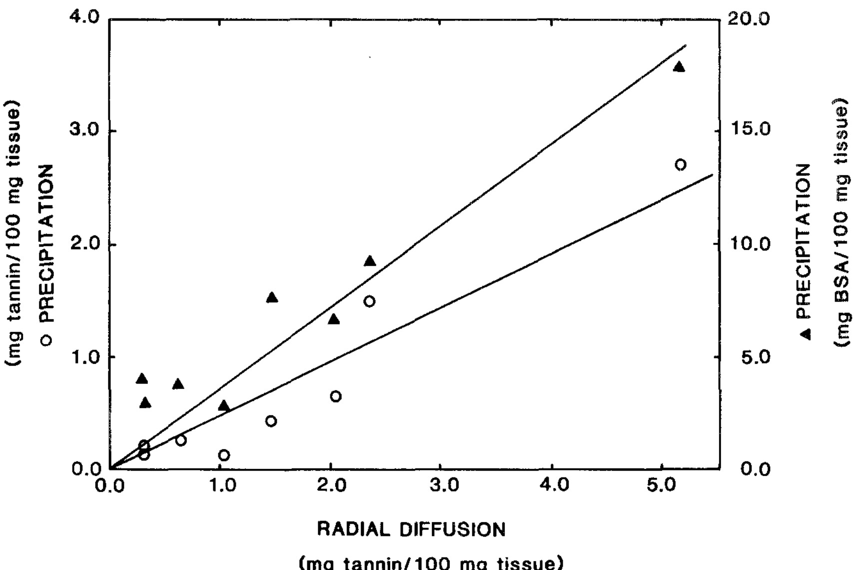

FIG. 6. Comparison of the radial diffusion assay to precipitation assays. A variety of plant tissues were extracted with 50% methanol (300 mg tissue/1.5 ml solvent). The extracts were analyzed by the radial diffusion assay, the protein precipitable phenolics assay, and the labeled protein precipitation assay as described in the text. The data was fit to a line using the method of least squares. For protein precipitable phenolics (○): $y = 0.49x$, $r^2 = 0.95$; for labeled protein precipitation (▲): $y = 4.56x$, $r^2 = 0.95$. The samples analyzed and their tannin content by the radial diffusion method are: *Ceanothus* leaves (0.308 mg tannin/100 mg tissue); *Sorghum vulgare* grain (0.313 mg tannin/100 mg tissue); 50% *Vaccinium* diet (0.642 mg tannin/100 mg tissue); oak leaves (*Quercus* sp.) (1.03 mg tannin/100 mg tissue); 75% *Vaccinium* diet (1.46 mg tannin/100 mg tissue); green tea leaves (2.03 mg tannin/100 mg tissue); red-osier dogwood leaves (*Cornus stolonifera*) (2.36 mg tannin/100 mg tissue); and fireweed (*Epilobium angustifolium*) (5.15 mg tannin/100 mg tissue).

cause the two assays are based on different chemistry, it was not surprising that the assays gave different responses with the plant extracts.

DISCUSSION

The radial diffusion assay is similar to radial immunodiffusion, in which antibodies are quantitated by forming a ring of precipitation in an antigen-containing agarose gel (Becker, 1969; Vaerman, 1981). Application of principles of immunoassays to tannin analysis was undertaken since tannin–protein interactions are similar in some respects to antigen–antibody interactions. In both reactions, a water-soluble precipitating reagent interacts specifically with pro-

teins to form multivalent, precipitable complexes (Hagerman and Butler, 1981). Radial diffusion assays are particularly suitable for tannin analysis. Tannin is water soluble and readily diffuses through the agarose gel. Tannin apparently does not interact to form precipitable complexes with the carbohydrate medium of the gel, since rings of precipitation are not observed if the protein is omitted from the gel.

BSA was selected as the protein to incorporate in the gel because it is homogeneous, soluble, and inexpensive. Tannin is a selective protein-precipitating agent, binding tightly to some proteins and more weakly to others (Hagerman and Butler, 1981; Hagerman and Klucher, 1986). Precipitation assays conducted with inhomogeneous proteins are ambiguous, since the tannin interacts selectively with the proteins in the mixture. Both condensed and hydrolyzable tannin have moderate affinities for BSA (Hagerman and Butler, 1981; Martin and Martin, 1983; Hagerman and Klucher, 1986).

The assay has several advantages in common with previously described precipitation assays (Hagerman and Butler, 1978, 1980a; Martin and Martin, 1983; Asquith and Butler, 1985). For example, the assay is not subject to interference by low-molecular-weight, nontannin phenolics. In addition, the method can be used to determine either condensed or hydrolyzable tannin or mixtures of the two. However, the assay is an improvement over other precipitation assays because it accommodates a wide variety of solvents including acetone; it can be used to assay extracts containing water-insoluble components; and it is especially appropriate for analyzing large numbers of samples.

Acetone does not affect the new assay, which simplifies analysis of extracts. Acetone is one of the most effective solvents for extracting tannin from plant tissue (Fletcher et al., 1977). Most protein precipitation assays for tannin are very sensitive to solvent. For example, as little as 4% acetone in a reaction mixture containing tannin and protein inhibits precipitation by 50%. Thus, acetone must be removed from extracts by evaporation under reduced pressure before attempting precipitation assays. Because acetone does not interfere with the new assay, plant extracts can be analyzed directly, without preliminary workup.

The new assay is more reliable than other assays for some unusual tissues, such as aspen buds or sorghum, which contain water-insoluble materials that interfere with most precipitation assays (Hagerman and Butler, 1978; Asquith and Butler, 1985; Hagerman, unpublished). The water-insoluble components, which are extracted into the aqueous organic solvents with the tannin, interfere with other assays by forming spurious precipitates in the aqueous buffers used in the assays. However, in the new assay, these water-insoluble materials do not enter the gel and thus do not form precipitation rings.

The assay can easily be used to determine tannin in a large number of samples, since extracts can be analyzed directly without any preliminary cleanup steps. Even centrifugation of the tissue extracts is unnecessary, although very

fine tissue samples may block the syringe used to dispense the extract into the wells. Allowing such samples to settle briefly or centrifuging at low speed clarifies the extract enough for radial diffusion analysis.

The assay could be modified to use proteins which are ecologically more significant than BSA. Although using BSA in the assay is analytically convenient, there may be advantages to using various plant or herbivore gut proteins for determining tannin (Martin and Martin, 1983). Soluble proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase could be incorporated into the agarose gel and used in a radial diffusion assay.

The assay described here provides a convenient, sensitive method for selectively determining tannin in crude plant extracts. The assay can be performed with as little as 100 mg tissue and can be conveniently used with large numbers of samples. Application of this method in studies of herbivory should enable ecologists to establish whether tannin defends plants from insects and other herbivores.

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ESTER AND KETONE COMPONENTS OF AGGREGATION PHEROMONE OF *Drosophila hydei* (DIPTERA: DROSOPHILIDAE)

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Abstract—Existence of an aggregation pheromone in *Drosophila hydei* was demonstrated by laboratory bioassay. The pheromone was produced by mature males, but both sexes responded. The nonpolar components consisted of three esters: the methyl, ethyl, and 1-methylethyl (isopropyl) esters of 2-methyl-(*E*)-2 butenoic (tiglic) acid, and two ketones: 2-tridecanone and 2-pentadecanone. The ketones and esters alone were only minimally active in the laboratory bioassay, but 2-tridecanone was highly synergistic with each of the esters, mixtures attracting 3–60 times more flies than the single components. 2-Pentadecanone was less active, but it did cause significant increases in activity when added to synthetic mixtures. The nonpolar portion of an extract of mature males and an equivalent mixture of the synthetic components were not significantly different in bioassay. Neither the esters nor the ketones were detected in sexually immature males or in females of any age. In extracts of mature males, ethyl tiglate was usually the most abundant ester component, with a mean of 8 $\pm$ 5 (SD) ng/male. The absolute and relative levels of the other esters were more variable. The mean level of methyl ketones in the extracts was 122 $\pm$ 106 (SD) ng/male, of which 85–93% was 2-tridecanone.

Key Words—*Drosophila hydei*, Diptera, Drosophilidae, aggregation, pheromone, 2-methyl-(*E*)-2-butenoic acid, ester, 2-tridecanone, 2-pentadecanone.

INTRODUCTION

Aggregation pheromone activity has been previously demonstrated by laboratory bioassay in two species of *Drosophila* (Diptera: Drosophilidae): *D. virilis*

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and *D. melanogaster*. In each species the pheromone was produced only by mature males, but was attractive to both sexes of any age. The chemical components of the pheromone systems were different. In *D. virilis* the active compounds were (*Z*)-10-heneicosene (Bartelt and Jackson, 1984) and a mixture of five esters: methyl, ethyl, and isopropyl tiglate [2-methyl-(*E*)-2-butenoate] and methyl and ethyl hexanoate (Bartelt et al., 1985a). The hydrocarbon and the ester components were synergistic, the combination showing 4–5 times more activity than either the hydrocarbon alone or the esters. In *D. melanogaster* a major pheromone component was (*Z*)-11-octadecenyl acetate (*cis*-vaccenyl acetate), but it had to be bioassayed in combination with food-related odors for its activity to be observed (Bartelt et al., 1985b).

Aggregation pheromone activity has now been demonstrated in *Drosophila hydei* Sturtevant as well. *D. hydei*, a cosmopolitan species, is a member of the *repleta* group (Wasserman, 1982). *D. hydei* was studied because there were no previous reports of aggregation pheromone activity in this species group, and we wish to sample a wide range of *Drosophila* species for pheromone investigations. We report here the isolation, identification, and bioassay characteristics of five pheromone components.

METHODS AND MATERIALS

Flies. The *D. hydei* culture was obtained from Mrs. M.E. Warren, Department of Genetics, University of Wales, Swansea, U.K. The flies were reared in 1-liter jars (ca. 500 per jar) on Formula 4-24 Instant Drosophila Medium (Carolina Biological Supply Co., Burlington, North Carolina) under existing laboratory temperature and light conditions.

Age of maturity. Based on previous results with *D. virilis* and *D. melanogaster*, only mature males of *D. hydei* were expected to produce pheromone. The age of sexual maturity was determined for *D. hydei* so that the extracts for initial bioassay experiments could be made from flies known to be mature. Also, only flies known to be immature would be used for bioassay tests because these would probably not contain the pheromones being evaluated by the experiments, and the potential for competition would be minimized.

To determine the age of maturity for males, groups of 5 virgin males (0–1 days old) were placed with five mature virgin females (10–16 days old) until the males reached specific ages ranging from 3 to 10 days old, after which the males were removed from the vials. The eventual appearance of larvae in the vials was evidence for the sexual maturity of the males. For females, groups of five virgin females (0–4 hr old) were placed with five mature males (10–16 days old) until the females reached specific ages ranging from 1 to 5 days old. The males were then removed from the vials and the vials observed for the appearance of larvae, indicative of the sexual maturity of the females. Each treatment was replicated four times.

Bioassay. The wind-tunnel olfactometer, paired-test bioassay method, and statistical analysis were described in detail previously (Bartelt and Jackson, 1984). Briefly, the olfactometer contained ca. 1000 flies. Pairs of treated vials were placed into the olfactometer. After each 3-min test, flies which had entered the vials were collected and later counted.

Extraction, Purification, and Identification of Pheromone Components. Flies to be extracted were separated according to sex at 0–5 days of age while immobilized by cold or Fly-Nap (Carolina Biological) and raised in 70-ml vials with ca. 75 flies per vial. At 10–16 days of age, the flies were extracted by soaking in hexane at room temperature for 24 hr. For use as a bioassay standard, 400 fly equivalents of a crude extract of mature males were added to a 9 × 1-cm ID open column of silicic acid (Bio-Sil A, Bio-Rad Laboratories, Richmond, California), and the column was eluted with two void volumes (15 ml) of 10% ether–hexane, to obtain all the less polar constituents in one fraction. Alternatively, for qualitative analysis of pheromone components, ca. 1000 fly equivalents of crude extracts were applied to the silicic acid columns, and the columns were eluted with 15 ml each of a series of solvents of increasing polarity: hexane and 2.5%, 5.0%, 7.5%, and 25% ether–hexane.

The silicic acid fractions which did possess aggregation activity in initial bioassays were further separated by HPLC using a size exclusion column (PLgel 10 μm, 50 Å, 30 cm × 7.7 mm ID, Polymer Laboratories, Shropshire, U.K.). The HPLC fractions which were active in the bioassay were examined by gas–liquid chromatography (GLC) on a Durabond DB-225 or DB-1 capillary column (both columns were 30 m × 0.25 mm ID, from J and W Scientific, Rancho Cordova, California). Details of chromatography were as reported previously (Bartelt et al., 1985a).

Electron impact mass spectra were obtained on a VG MM16 or VG 7070 mass spectrometer, using the DB-225 capillary GLC column for introduction of samples. The ionization energy was 70 eV.

Synthetic Tiglic Esters and Methyl Ketones. After tentative identification of pheromone components by mass spectrometry, synthetic compounds were obtained for comparison of mass spectra and chromatographic retentions and for bioassay tests. Synthetic methyl tiglate, ethyl tiglate, isopropyl tiglate, and propyl tiglate (>98% pure) were on hand from previous work (Bartelt et al., 1985a). Synthetic 2-tridecanone, 2-tetradecanone, and 2-pentadecanone were prepared by converting dodecanal, tridecanal, and tetradecanal to 2-tridecanol, 2-tetradecanol, and 2-pentadecanol, respectively, with methylmagnesium bromide, then oxidizing these alcohols with chromic acid to the corresponding methyl ketones. After silicic acid chromatography and preparative GLC, the methyl ketones were >98% pure. For bioassay, the ketones were diluted with hexane to 5 ng/μl and the esters, to 1 ng/μl. Alternatively, a mixture of ketones and esters was prepared to contain the same concentrations as one extract from mature males (details with Results).

Quantification of Tiglic Esters and Ketones. Six extracts of 1000–1500 mature (10- to 16-day-old) males were analyzed for tiglate content to determine the extent of variability among groups. In another experiment, the levels of both tiglic esters and methyl ketones were measured as a function of age of males. Here, flies were separated by sex within 24 hr of emergence and raised to various ages between 1 and 22 days (ca. 100 flies per sample, exact counts made after extraction). Finally, aliquots of the male-derived nonpolar bioassay standard (eluted from the silica column with 10% ether–hexane) were analyzed for the tiglates and methyl ketones so that an equivalent synthetic mixture could be prepared for bioassay against the fly-derived materials.

The tiglates were quantified for these extracts as described previously (Bartelt et al., 1985a) except that ethyl hexanoate was used as the internal standard. Ketones were quantified on the DB-1 capillary column with dodecanal as the internal standard. Two or three replicates were analyzed for each extract or age treatment.

RESULTS

Age at Maturity. Based on the appearance of progeny in the vials, males reached sexual maturity at 7–9 days of age and females at 2–3 days of age. Utilizing a different method for determining sexual maturity, Markow (1985) reported similar results. Thus aging the flies to 10–16 days ensured that they were mature when extracted, and bioassaying the flies at 0–5 days of age ensured that the males, at least, were immature.

Bioassay Characteristics. When the flies were first put into the olfactometer, they tended to form tight aggregations in the upper corners and flew little. After starvation for 14–16 hr, the flies became dispersed throughout the cage, with a large number on the olfactometer floor, and flight became more frequent. In response to an active preparation, the number of airborn flies increased markedly, and anemotactic behavior was observed. The flies flew slowly upwind, often exhibiting a zigzag motion, until alighting on the pheromone-treated vial. The flies then crawled about on the vial, eventually entering and drinking from the water drop at the bottom. Once inside a vial, flies usually remained for the duration of the test. The flies exhibited the same response behaviors toward both insect-derived and synthetic materials.

The absolute bioassay catches for a given treatment did vary from day to day, due primarily to differences in temperature and in the number and condition of the flies in the olfactometer. Nevertheless, as is apparent in the tables, the ratios of the means between any two treatments were reasonably consistent from day to day.

Isolation of Pheromone Components. As seen previously in the other species, the crude extracts of mature male *D. hydei* were clearly attractive in bioas-

say. For example, the mean catches for the mature male extract, mature virgin female extract, and controls were 20.2, 2.2, and 0.5, respectively ($N = 12$, 1 fly equivalent of extract/test). The male extract was significantly more attractive than the other treatments at the 0.001 level. Although the female extract was also significantly above the control level ($P < 0.05$), it was the more dramatic effect of the male extract which we investigated further. In this bioassay experiment (as well as all other bioassays reported here) both sexes responded in approximately equal numbers.

The crude extract of males contained both polar and nonpolar attractants. After passing a male extract through a silicic acid column with 10% ether–hexane, bioassay catches for this nonpolar fraction, the parent crude extract, and the controls were 17.3, 34.8, and 0.6, respectively ($N = 28$, 1 male equivalent per test, all means significantly different at the 0.01 level). The nonpolar fraction contained compounds with the polarities of hydrocarbons, esters, aldehydes, and ketones, and it accounted for ca. 50% of the activity of the crude extract. Since nonpolar pheromone components had already been identified in *D. virilis* and *D. melanogaster*, initial emphasis was placed on the less polar attractants, for comparative purposes. Work on polar attractants is still in progress.

After fractionation of the crude extract of males by polarity, only the 5.0% ether–hexane fraction and the 7.5% ether–hexane fraction showed activity by bioassay. Based on the elution of standards, the 5.0% and 7.5% ether–hexane fractions contained materials with the polarities of alkyl esters and ketones, respectively, and are referred to below as the "ester" and "ketone" fractions. While the response to either fraction alone was weak, the combination of the two fractions was reliably and dramatically active (Table 1). Unlike *D. virilis*, the hexane (hydrocarbon) fraction showed no hint of activity.

The synergistic activity of the ester and ketone fractions was used to advantage during their subsequent purification on the size-exclusion HPLC column. None of the HPLC fractions was active alone, but by combining each with the appropriate synergistic fraction, clear bioassay results were possible. Two regions of effluent from the ester fraction, 13.0–14.5 and 15.0–16.5 ml after injection, were highly synergistic with the ketone fraction, as the entire original ester fraction had been (Table 2). Similarly, one HPLC region of the ketone fraction, 12.0–13.5 ml after injection, was highly synergistic with the ester fraction in the bioassay (Table 2).

Ester Components. GLC peaks were observed in the active HPLC fractions which were absent from the inactive fractions. In fact, the active 13.5- to 14.0- and 15.5- to 16.0-ml fractions each contained only one detectable peak. Serendipitously, the mass spectra of these peaks corresponded to isopropyl tiglate and ethyl tiglate, respectively, which had been isolated previously from *D. virilis* (Bartelt et al., 1985b). Since *D. virilis* usually contained methyl tiglate and two hexanoic esters as well (methyl and ethyl), later extracts were also exam-

TABLE 1. SYNERGISTIC ACTIVITY OF ESTER AND KETONE FRACTIONS

Treatment[a]	Mean bioassay catch ($N = 12$)[b]
Ketone fraction	1.5 b
Ester fraction	2.3 b
Ketone + ester fractions	15.6 c
Control (solvent control)	0.3 a

[a]One fly equivalent per test.
[b]Means followed by the same letter not different at the 0.05 level.

ined for these esters. Methyl tiglate was detected in most extracts of mature male *D. hydei*, but the hexanoates were never found. The GLC retentions (DB-1 and DB-225) and mass spectra of the fly-derived esters and corresponding authentic tiglate standards were identical.

In six mature male extracts (Table 3), ethyl tiglate was always present and was usually the most abundant tiglate. The methyl and isopropyl esters varied greatly, both in absolute amount and relative to the other esters; in some cases one or the other of these was not detectable at all. Propyl tiglate was sometimes present in trace amounts. Replicated analyses indicated that the observed variability among groups of flies was a biological fact rather than an artifact of analytical technique. The biological significance of the variability in ester levels remains unknown, but tiglate production may be influenced by environmental factors such as food quality. For the purpose of evaluating the activity of synthetic compounds, 10 ng was believed a reasonable level for bioassay tests.

With respect to age, tiglates were absent from young males and first began to appear at 7–9 days old, as sexual maturity was attained. The mean level of ethyl tiglate measured in the sexually mature male flies (between 10 and 22 days old) was 8.6 ± 6.8 (SD), in the same range as the earlier results. Again, isopropyl and methyl tiglates were present at lower levels, and the ratios of esters were variable. Once the males reached maturity, neither the amounts nor ratios of esters appeared correlated with age. Tiglates were never detected in females of any age.

Ketone Components. The active HPLC fractions of the ketone material contained three GLC peaks which were absent from the inactive fractions. The mass spectra of these peaks indicated molecular weights of 198, 212, and 226. In all three spectra, the base peak was at m/e 58, and an intense peak at m/e 43 was also present. The data suggested a homologous series of saturated methyl ketones, with 13, 14, and 15 carbons. Comparison with published spectra (Heller and Milne, 1978) supported these structures. Synthetic 2-tridecanone, 2-tetradecanone, and 2-pentadecanone produced mass spectra and GLC retentions (DB-1 and DB-225) which were identical to the respective fly-derived compounds. (If branches had been present in the carbon chains, earlier GLC elution

TABLE 2. SYNERGISTIC BIOASSAY OF ESTER AND KETONE HPLC FRACTIONS FROM SIZE-EXCLUSION COLUMN

	Ester material[a]			Ketone material[a]	
	Mean bioassay catch (N = 4)			Mean bioassay catch (N = 4)	
Elution volume (ml)	HPLC fraction + ketone material	Control (ketone material)	Elution volume (ml)	HPLC fraction + ester material	Control (ester material)
8.0[b] -13.0	6.0	4.0	8.0-12.0	3.5	2.5
13.0-13.5	11.0	6.5	12.0-12.5	4.5	2.3
13.5-14.0	57.5	3.5	12.5-13.0	13.3	1.8
14.0-14.5	10.5	3.0	13.0-13.5	20.0	1.0
14.5-15.0	5.0	1.8	13.5-14.0	6.3	2.0
15.0-15.5	8.3	3.3	14.0-14.5	2.3	3.0
15.5-16.0	31.3	3.8	14.5-15.0	1.3	1.0
16.0-16.5	20.3	5.0	15.0-15.5	1.8	1.3
16.5-17.0	4.0	1.5	15.5-16.0	1.5	1.3
17.0-17.5	4.3	1.5			
17.5-18.0	7.3	2.0			

[a] HPLC fractions bioassayed in combination with the complementary silicic acid fraction (see text). One male equivalent per test for all preparations.
[b] Void volume of column was 8.0 ml.

TABLE 3. TIGLIC ESTER CONTENT (ng/male) FOR HEXANE EXTRACTS OF MATURE MALES (10–16 DAYS OLD)

Tiglic ester	Extract number 1	2	3	4	5	6	Mean
Methyl[a]	T[b]	9.0	1.3	T	1.0	1.4	2.1
Ethyl[a]	3.2	9.9	1.2	11.8	9.7	11.5	7.9
Isopropyl[a]	1.8	16.6	1.3	2.4	0.5	0.0	3.8
Propyl	T	T	0.0	T	0.0	0.0	T

[a] Fourteen analyses were conducted on the six extracts. One-way analysis of variance indicated significant differences among extracts for each ester ($P < 0.001$). In each extract, analytical error was correlated with the mean ester level; the overall coefficient of variation was 6%.
[b] T = trace (between 0.0 and 0.5 ng/male).

would have been expected, in addition to evidence of branching in the mass spectra).

As with the tiglic esters, these methyl ketones were never detected in females of any age, nor in newly emerged males. The ketones began to appear in males at 6–8 days of age and were always detected in mature males. Extracts of mature males contained 123 ± 106 (SD) ng/male (range: 36–308 ng/male). Although the total amounts of ketones varied considerably, the proportions were fairly constant. 2-Tridecanone was always most abundant, representing 85–93% of the total, while 2-tetradecanone and 2-pentadecanone accounted for 0–2% and 7–15% of the total, respectively.

For initial bioassays, each ketone was used at 50 ng/test. For 2-tridecanone, this was within the range found in mature males. Although the other ketones were less abundant in the flies, these were also tested at the 50-ng level so that relative activities of the ketones could be assessed.

Bioassay of Synthetic Compounds. The bioassay activity of the synthetic esters supported their identification as pheromone components. While the esters alone were only marginally active, all showed dramatic increases in activity when combined with the major ketone, 2-tridecanone (Table 4A). These results paralleled the synergistic effect of the original ester and ketone fractions. Since the experiments in Table 4A were done on different days (hence the means for different esters are not comparable), a further test was conducted to determine the relative activities of the esters on an equal weight basis (Table 4B). Ethyl tiglate and isopropyl tiglate were most active, and methyl tiglate, the least. Propyl tiglate was intermediate in activity, although all four esters clearly synergized the ketone. Since ethyl tiglate was present in all extracts and was usually the most abundant, it is probably the "key" ester under natural conditions. Conversely, propyl tiglate is probably of little importance in nature due to its

TABLE 4. BIOASSAY ACTIVITY OF SYNTHETIC TIGLIC ESTERS, ALONE AND WITH 2-TRIDECANONE

	Mean bioassay catch[b] (N = 12)			
Treatment[a]	Methyl tiglate	Ethyl tiglate	Isopropyl tiglate	Propyl tiglate
A. Experiments to measure activity of individual esters				
Ester	5.2 b	2.0 b	1.5 a	3.4 b
Ester + 2-tridecanone	13.7 c	21.7 c	11.3 b	12.2 c
2-tridecanone	0.3 a	0.6 a	0.7 a	0.2 a
Control (solvent blank)	0.7 a	0.1 a	0.5 a	0.1 a

Treatment[a]	Mean bioassay catch[b] (N = 16)
B. Relative activity of four tiglates in one experiment	
Methyl tiglate + 2-tridecanone	5.0 b
Ethyl tiglate + 2-tridecanone	15.0 c
Isopropyl tiglate + 2-tridecanone	16.1 c
Propyl tiglate + 2-tridecanone	8.2 bc
Control (2-tridecanone)	1.3 a

[a] Tiglates used at 10 ng/test, 2-tridecanone at 50 ng/test.
[b] Within each column, means followed by the same letter were not significantly different at the 0.05 level. Comparisons of means between columns are not meaningful because the experiments were done on different days, with different groups of flies.

very low level. The importance of methyl and isopropyl tiglates is less clear, but it is evident that even one ester is sufficient to demonstrate activity. The esters may be somewhat "interchangeable."

The previous results (Table 4) already demonstrated that 2-tridecanone had synergistic activity. A more comprehensive investigation with the methyl ketones is summarized in Table 5. None of the ketones was significantly above control levels alone (Table 5A), although in each case the mean catch for the ketone was numerically higher than the control. Only 2-tridecanone caused a clear (ca. eightfold) increase in the response to isopropyl tiglate. For 2-tetradecanone and 2-pentadecanone the increase was less than twofold, and only for the former was the increase significant. Thus the flies appeared far less sensitive to the 14- and 15-carbon ketones than to the 13-carbon homolog.

As with the tiglate experiments, the columns in Table 5A cannot be compared because the experiments were done on different days, but the relative activities of the ketones were determined directly (Table 5B). 2-Tridecanone

TABLE 5. BIOASSAY ACTIVITY OF SYNTHETIC KETONES, ALONE AND WITH ISOPROPYL TIGLATE

	Mean catch[b] (N = 12)		
Treatment[a]	2-Tridecanone	2-Tetradecanone	2-Pentadecanone
A. Experiments to measure activity of individual ketones			
Ketone	0.7 a	0.8 ab	2.3 ab
Ketone + isopropyl tiglate	11.3 b	3.4 c	6.4 c
Isopropyl tiglate	1.5 a	1.6 b	4.0 bc
Control (solvent blank)	0.5 a	0.4 a	1.3 a

Treatment[a]	Mean catch[b] (N = 16)
B. Relative activity of ketones in one experiment	
2-Tridecanone + isopropyl tiglate	8.5 c
2-Tetradecanone + isopropyl tiglate	2.3 ab
2-Pentadecanone + isopropyl tiglate	3.3 b
Isopropyl tiglate	1.7 ab
Control (solvent blank)	1.0 a

[a]Ketones used at 50 ng/test, isopropyl tiglate at 10 ng/test.
[b]Within each column, means followed by the same letter were not significantly different at the 0.05 level. Comparisons of means between columns are not appropriate because the experiments were done on different days, with different groups of flies.

was clearly the most active ketone, and neither larger ketone caused a significant increase in the activity of isopropyl tiglate. Due to the minor activity of the higher homologs at 50 ng/test and the small amounts of these compounds actually present in the flies, we anticipated that 2-tetradecanone and 2-pentadecanone would have, at most, subtle effects.

To further test for the effect of 2-pentadecanone (the second most abundant ketone), a mixture of synthetic esters and ketones was prepared in the concentrations found in one extract of mature males (see footnote of Table 6 for details). This was compared in bioassay to a second mixture, which did not contain 2-pentadecanone but was otherwise identical. The means for these mixtures and controls were 32.2, 24.5, and 1.7, respectively (N = 32, mixtures used at one "male equivalent" per test). The two mixtures differed at the 0.10 level, but not at the 0.05 level. We conclude that 2-pentadecanone can cause a subtle increase in response at natural levels and should be considered a pheromone component. Comparable tests for the even less-abundant 2-tetradecanone were not made.

Natural vs. Synthetic Components in Bioassay. In a bioassay experiment

TABLE 6. BIOASSAY COMPARISON OF MALE-DERIVED, NONPOLAR BIOASSAY STANDARD AND COMPARABLE SYNTHETIC MIXTURE[a]

Treatment	Mean bioassay catch[b] (N = 24)
Male-derived material	24.3 b
Synthetic mixture	21.6 b
Control (solvent blank)	2.0 a

[a] Male-derived material contained 1.4 ng of methyl tiglate, 11.5 ng of ethyl tiglate, 94.0 ng of 2-tridecanone, and 24.0 ng of 2-pentadecanone per fly equivalent. The concentrations of these components in the synthetic mixture were equivalent. No isopropyl tiglate was detected in this male-derived sample, and less than 1 ng/fly of 2-tetradecanone was present. Neither compound was added to the synthetic mixture. The materials were bioassayed at 1 male equivalent per test.
[b] Means followed by the same letter not significantly different at the 0.05 level (LSD).

(Table 6), the nonpolar portion of a mature male extract, containing all constituents with the polarities of hydrocarbons, esters, and ketones, was not significantly different ($P > 0.4$) from an equivalent synthetic mixture composed of a mixture of tiglic esters and methyl ketones (see footnote of Table 6). Both treatments were far above control levels in activity. Thus we had no evidence that additional, relatively nonpolar components remained to be discovered; the tiglic esters and ketones together accounted for the observed activity. When 2-pentadecanone was omitted from the synthetic mixture, the means for the natural nonpolar material, synthetic mixture, and controls were 44.6, 31.5, and 0.7, respectively ($N = 36$). Now the natural and synthetic materials differed at the 0.05 level, again indicating that 2-pentadecanone had a measurable effect at natural levels.

DISCUSSION

D. hydei, in possessing an aggregation pheromone produced only by sexually mature males but to which both sexes respond, follows the same pattern observed previously for *D. virilis* and *D. melanogaster*. Aggregation pheromones have now been studied in members of three distinct species groups, and we suspect they exist rather generally in the *Drosophila*.

As in *D. virilis*, the pheromone of *D. hydei* includes a number of components. One class of chemicals, the methyl ketones, has not been reported from *Drosophila* before, although these compounds have been detected in many insect species (for examples see Blum et al., 1983; Francke et al., 1983). The other class of compounds, the tiglic esters, has been detected previously in *D. virilis*. In both species, the tiglic esters exhibit pheromonal activity best when combined with another compound, 2-tridecanone in *D. hydei* but (*Z*)-10-heneicosene in *D. virilis*.

This investigation demonstrated the versatility and sensitivity of the bioassay method. Although no single compound caused clear behavioral responses, it was still possible to screen chromatographic fractions and isolate the active chemicals by bioassaying preparations in combination with appropriate synergistic fractions. Because the test duration was short (3 min), responses toward minute amounts of volatile compounds (e.g., tiglic esters) could be measured before the chemicals dissipated, and a large number of replications of an experiment could be run in a relatively short period of time.

Qualitatively, *D. hydei* responded more consistently and energetically in bioassay than either *D. virilis* or *D. melanogaster*. Instances of 200–300 flies responding to a pheromone source within 1 min did occur. Because these flies responded so strongly in the laboratory bioassay, *D. hydei* might be a good model species for studies on *Drosophila* pheromones in the field.

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IDENTIFICATION OF VOLATILE ALLELOCHEMICALS FROM *Amaranthus palmeri* S. WATS.[1]

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Abstract—Allelopathic volatiles associated with the weed Palmer amaranth (*Amaranthus palmeri* S. Wats.; AMAPA) were trapped on Tenax GC, thermally desorbed, and identified by gas chromatography-mass spectroscopy. Methyl ketones and alcohols (C_4–C_{11}) were the principal components of the volatiles mixture. Seedheads, stems, or roots were placed in a glass container and incubated at 31°C (10 hr)/21°C (14 hr) for three days prior to trapping the volatiles. Seedheads were rich in 2-heptanone which was consistently found, together with 2-heptanol, in all AMAPA tissues. Vapors of authentic 2-heptanone and (±)-2-heptanol at concentrations of 1 ppm or higher strongly inhibited the germination of onion and carrot and almost completely suppressed the germination of tomato and AMAPA seeds.

Key Words—*Amaranthus palmeri*, Palmer amaranth, allelopathy, seed germination, weed residue, volatiles, ketones, alcohols, 2-heptanone, 2-heptanol.

INTRODUCTION

Amaranthus palmeri S. Wats. (Palmer amaranth, AMAPA) is a hardy, fast-growing weed that commonly infests fields in the southwestern United States. Plants can grow to 9 ft or more and produce a large aerial biomass in the field.

Menges (1985) reported that, in the field, soil-incorporated residues of Palmer amaranth produced allelopathic inhibition in carrot and onion and were

[1] Names of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

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autotoxic. Laboratory seed germination studies of aqueous and organic solvent extracts of harvested AMAPA, as well as isolated and identified compounds from this weed (Bradow, 1985; Bradow and Connick, 1985; Fischer and Quijano, 1985), indicated the presence of both inhibitory and promotive allelochemicals. However, none of these exhibited a sufficiently strong or diverse negative activity to explain the field observations. Subsequently, Bradow and Connick (1985) demonstrated that volatile allelochemicals associated with Palmer amaranth residues were highly inhibitory of the germination of onion, carrot, tomato, and Palmer amaranth itself. It was clear from the magnitude of the inhibitory effects that these volatiles could be a major source of the allelopathic interactions associated with Palmer amaranth.

Our objectives in this study were to identify the major components of the volatiles mixture emitted by harvested Palmer amaranth and to test some of the authentic compounds to see if they inhibited germination of onion, carrot, tomato, and Palmer amaranth seeds.

METHODS AND MATERIALS

Plant Materials. AMAPA was grown at Weslaco, Texas, and harvested on August 16, 1984. Green (young) and mature plants were separated, divided further into seedheads, stems, and roots, and kept frozen until use. Thawed samples were cut into 1 to 2-cm pieces and air dried in the laboratory at 21 ± 2°C for one day, 15 days, or longer for some samples.

Sample Container. Samples were placed in a 1.2-liter glass bottle (Kontes No. K-323255) fitted with Teflon valves, an inlet tube that reached almost to the bottom, and a Teflon joint sleeve. Sample vapors contacted only glass and Teflon.

Tenax GC Trap Tubes. A 0.10-g quantity of Tenax GC (60–80 mesh) was added to glass tubes measuring 84 mm long × 9 mm OD × 1 mm wall thickness (TekLab, Baton Rouge, Louisiana) and held in place near the center with a glass wool plug on each side. Packed tubes were conditioned at 220°C for 24 hr while purging with helium at 15 ml/min. A mark was made near one end of each tube to designate the inlet end for sample volatiles. The tubes were reversed for subsequent thermal desorption so that volatiles would be backflushed from the Tenax GC into the gas chromatograph–mass spectrometer (GC-MS) system.

Volatiles Sampling Procedure. A sample of AMAPA weighing 80 g (if air dried for 1 day) or 20 g (if dried 15 days) was placed in the sample container. Ten milliliters of ultrapurified water (Water-1 apparatus, Barnstead) were added to the plant sample. The container was sealed, wrapped in aluminum foil to exclude light, and placed in an environmental chamber programmed for 10 hr at 31°C and 14 hr at 21°C. After three days, the container was removed and equilibrated at room temperature. A small amount of fungal growth was often

seen on the plant samples after incubation. A Tenax trap tube (sample tube) was placed on the exit port of the sample container with a short length of Tygon tubing. A second Tenax tube was affixed to the inlet port to serve as a final filter for the nitrogen purge gas which had already been passed through a molecular sieve–charcoal cartridge. The valves were opened and the purified nitrogen was swept through the sample container at 100 ± 4 ml/min for 20 min. Not all the volatiles present in the sample container were collected by this procedure, but a sufficient aliquot was trapped for qualitative analysis. Adsorption capacity of the sample tubes was not exceeded using this procedure with a typical plant sample. The Tenax sample tube was sealed with corks pressed through Teflon tape and stored in a freezer until GC-MS analysis. Blank runs gave very small background peaks.

GC-MS Analysis. Volatiles were separated and identified using a Finnigan MAT GC-MS-DS model 4000 instrument. The GC was interfaced with an external, closed inlet system (Scientific Instrument Service, River Ridge, Louisiana) as previously described by Legendre et al. (1979). The GC column was 1/8 in. × 10 ft nickel tubing packed with 60–80 mesh Tenax GC coated with 8% poly-*m*-phenoxylene (poly MPE). With the GC column at room temperature (30°C), the sample liner was secured in the external inlet apparatus and the six-port valve was switched to the inject position. Volatiles were stripped from the sample tube by heat (200°C) and a helium flow of 20 ml/min for 20 min. The valve was then switched to the run position, the mass spectrometer and data system were turned on, and the GC oven temperature was raised to 100°C at 25°/min. Once at 100°C, the column was programmed to 230°C at 5°/min and held at this temperature for 30 min. The mass spectrometer was repetitively scanned from 33 to 450 amu every 2 sec. Data acquistion and analysis were accomplished with a Finnigan-Incos Data System. Compounds were identified by a mass spectra library search in conjunction with a comparison of retention times and mass spectra obtained from authentic compounds (Aldrich Chemical Co.).

Seed Germination Bioassays. Germination of seeds of onion, *Allium cepa* L., cv. Texas Early Grano 502 (Baxter's, Weslaco, Texas); carrot, *Daucus carota* L., cv. Danvers Half-long (Burpee, Warminster, Pennsylvania); tomato, *Lycopersicon esculentum* Mill., cv. Homestead (Carolina Biological, Burlington, North Carolina) and Palmer amaranth grown in Weslaco, Texas, were used to compare the bioactivities of 2-heptanone and 2-heptanol with deionized water controls.

The procedure followed was a slight modification of that used by Bradow and Connick (1985) for the bioassay of volatiles from AMAPA residues. A 10-ml glass beaker was placed on 50 g of pure sand in a crystallizing dish (100 × 50 mm) and the sand was moistened with 10 ml of deionized water. A sample of 2-heptanone or (±)-2-heptanol (1.2, 2.4, 6.0, or 12.3 μl) was added to the beaker. The dish and contents were then placed in the bottom of a 2.5-liter desiccator (160 mm id). The nominal vapor concentrations were 0.5, 1.0, 2.4,

and 4.9 ppm by volume, respectively. These were not exact concentrations because of partitioning and adsorption effects that probably occur within the apparatus.

Seeds of a single species were placed on a porcelain desiccator plate that was covered with two sheets of Whatman No. 1 filter paper saturated with deionized water. Circles (22 mm diameter) had been cut in the center of the paper to facilitate the diffusion of vapors. The filter paper sheets were divided into eight equal segments containing the same number of seeds (20 of carrot, onion, or tomato; 25 of AMAPA), and each segment was treated as a replicate for purposes of statistical analysis (Bradow and Connick, 1985). Controls lacked only the beaker with the volatile sample. Desiccators were sealed and incubated the same as described above for the plant samples in the volatiles sampling procedure, and a germination count was taken after three days. Results of the germination assays of 2-heptanone and 2-heptanol activity (Figures 4 and 5) were compared separately for each seed species by two-way analyses of variance with 16 replicates (Sokal and Rohlf, 1981).

RESULTS AND DISCUSSION

Identification of Allelopathic Volatiles. Volatile organic compounds emitted from harvested AMAPA (green and mature) seedhead, stem, and root pieces were trapped and concentrated using Tenax GC adsorbent. The volatiles were thermally desorbed into a GC-MS instrument and the principal components were identified. Packed-column GC incompletely separated some of the compounds (Figures 1–3), but identifications could usually be made through use of the MS data system and authentic compounds. A list of identified compounds appears in Table 1. Methyl ketones and alcohols, particularly those with an odd number of carbon atoms, were the predominant components of the volatiles associated with AMAPA seedheads, stems, and roots. Although the sampling procedure and GC-MS method are effective for aldehydes (Legendre et al., 1979), the only ones detected were acetaldehyde and hexanal in trace amounts. Because the mechanism(s) and biochemical substrates responsible for the generation of these volatiles have not yet been determined, a satisfactory understanding of the significance of some of the following observations is not possible at this time.

Seedheads were particularly rich in 2-heptanone (Figure 1). Other methyl ketones, such as 2-butanone and 2-pentanone, and the alcohols 2-heptanol and 3-methyl-1-butanol were also found. The same qualitative results were obtained with green and mature seedheads and with samples that were dried one day and 15 days. However, the concentration of the volatiles was generally lower for the 15-day-dried samples. 2-Heptanol was present in low to moderate amounts and eluted on the leading edge of the 2-heptanone peak. Seedheads proved to be the plant tissue most able to release volatiles, particularly 2-heptanone, after

TABLE 1. VOLATILE COMPOUNDS ASSOCIATED WITH PALMER AMARANTH IDENTIFIED BY GC-MS

Compound	Source[a]	Comments
Ketones		
2-Butanone	SH, ST, R	
3-Hydroxy-2-butanone	ST, R	
3-Methyl-2-butanone	R	
2-Pentanone	SH, ST, R	
3-Pentanone	SH, ST, R	
2-Heptanone	SH, ST, R	Major component
2-Nonanone	R	
2-Undecanone	R	Trace
Alcohols		
Ethanol	SH, ST, R	
2-Propanol	SH, ST, R	Trace
2-Butanol	SH	Trace
3-Methyl-1-butanol	SH, ST, R	Major component
2-Heptanol	SH, ST, R	
Miscellaneous		
Acetaldehyde	SH, ST, R	Trace
Methyl acetate	SH, ST, R	
2-Ethylfuran	SH, ST, R	
2-Methylfuran	SH, ST, R	
Heptane	SH, ST, R	
Octane	ST, R	
Hexanal	SH, ST, R	Trace

[a] SH = seedheads, ST = stems, R = roots.

extended air drying. Under the test conditions, 2-heptanone was released from seedheads that had been dried for 99 days.

With stem samples, the strongest peaks were due to 3-methyl-1-butanol, 3-hydroxy-2-butanone (not detected in seedheads), and 2-heptanone (Figure 2). Green and mature stems dried for one day gave almost identical chromatograms. Drying the samples for 15 days led to a large reduction in the concentration of all the volatiles, especially 2-heptanone.

Roots, which constitute a very small portion of the AMAPA biomass, were rich in a variety of volatile compounds after drying for one day. Roots tissue emitted most of the volatiles found in seedheads and stems plus 3-methyl-2-butanone, 2-nonanone and, occasionally, 2-undecanone (Figure 3, Table 1). As was noted for stems, the quantity of volatiles trapped from roots that were dried 15 days was very low.

Volatiles emitted by seedheads (dried for one day) were collected after

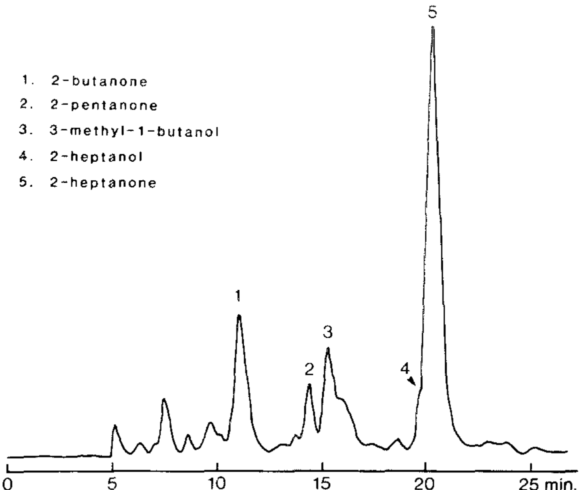

FIG. 1. Gas chromatogram of volatiles emitted by AMAPA seedheads (green, dried 1 day) after a 3-day incubation.

one, two, and three days in the environmental chamber. After just one day, the sample container headspace was already rich in volatiles, particularly 2-heptanol and 2-heptanone. The areas of all the peaks increased each succeeding day but, qualitatively, no new components appeared after the first day.

Water had a dramatic effect on increasing the emission of organic volatiles from AMAPA residues. When 0, 10, or 20 ml of water was added to a seedhead (20%)/stem (80%) mixture that had been air dried for seven days, the respective areas of the 2-heptanone peak were 0, 3.8×10^5, and 6.9×10^5 units, respectively.

Incubation of seedheads (air dried 42 and 99 days) for three days in a

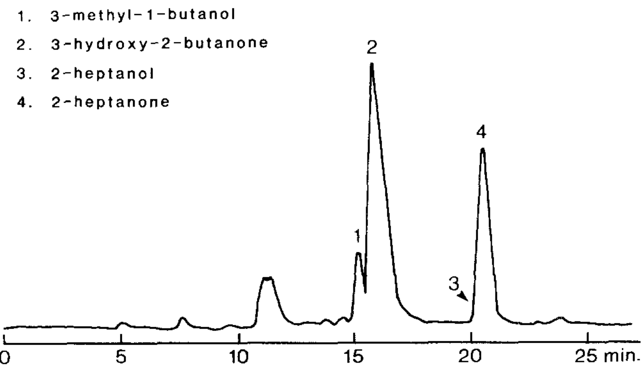

FIG. 2. Gas chromatogram of volatiles emitted by AMAPA stems (green, dried 1 day) after a 3-day incubation.

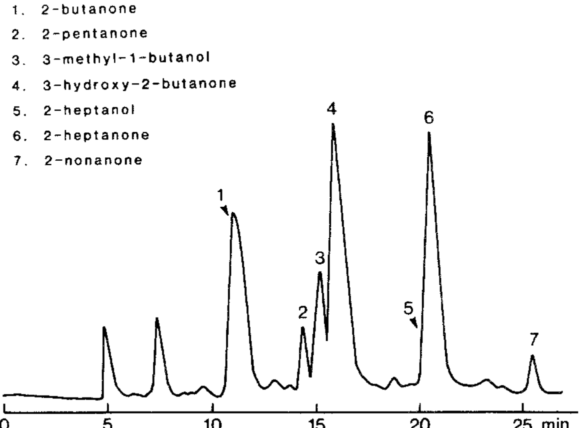

FIG. 3. Gas chromatogram of volatiles emitted by AMAPA roots (green, dried 1 day) after a 3-day incubation.

nitrogen atmosphere greatly increased the concentration of organic headspace volatiles compared with incubation in air. The 2-heptanone concentration (peak area) from the nitrogen atmosphere samples increased an average of 840% compared with the air atmosphere samples. The 2-pentanone and 3-pentanone concentrations increased more than 20 times. Although 2-pentanol increased about 990%, 2-heptanol increased only slightly. Small amounts of 2-hexanone, 2-octanone, and 2-nonanone were detected in the nitrogen headspace, but not in air.

The methyl ketones and alcohols that were identified in the volatiles mixture emitted by AMAPA residues are slightly water-soluble ($> C_4$), oily liquids with boiling points that are in the range 80–232°C. These compounds are widespread in nature and have been found in corn (Buttery and Ling, 1984; Buttery et al., 1978), wheat (Buttery et al., 1985), and in fruits such as strawberry (Schreier, 1980), loquat (Shaw and Wilson, 1982), guava (Idstein and Schreier, 1985), and passion fruit (Chen et al., 1982). Flath et al. (1984) identified numerous volatile compounds released by aqueous suspensions of fresh *Amaranthus retroflexus* plant tissue including 2-heptanol, 3-methyl-1-butanol, 2-butanone, and 3-pentanone. However, we believe that the present study is the first to report C_5–C_{11} 2-alkanones in association with a weed species.

Inhibition of Seed Germination by 2-Heptanone and 2-Heptanol. Reynolds (1977) reported that dilute aqueous solutions of 2-heptanol, 3-methyl-1-butanol, and C_4–C_9 2-alkanones inhibit lettuce fruit germination. French and Leather (1979) found that vapors of 2-heptanone, 2-octanone, and 2-nonanone inhibited the germination of several weeds including redroot pigweed (*Amaranthus retroflexus* L.). Interestingly, these compounds also stimulated germination of curly dock (*Rumex crispus* L.) and/or red sorrel (*Rumex acetosella* L.).

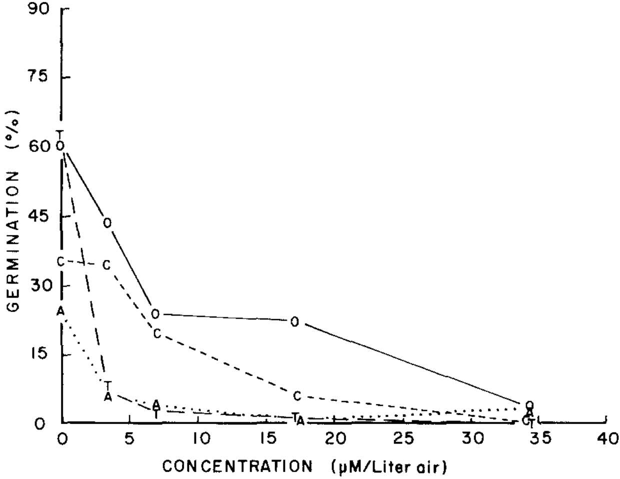

FIG. 4. Effects of 2-heptanone vapors (3-day exposure) on seed germination: (O) onion; (C) carrot; (T) tomato; (A) Palmer amaranth. Germination percentages are means of 16 replicates (standard errors of the means were ≤ 2.0%).

In the present study, 2-heptanone consistently was a principal component of the volatiles mixture and it was always accompanied by 2-heptanol. We decided to bioassay these two compounds as a representative 2-alkanone and a 2-alkanol associated with AMAPA residues.

2-Heptanone and (±)-2-heptanol were highly bioactive in the vapor state, and each was capable of severely inhibiting the germination of carrot, onion, tomato, and AMAPA seeds (Figures 4 and 5). There was a striking similarity in the results of bioassays conducted at several concentrations of the two compounds. Most affected were tomato seeds, which were greatly inhibited by each compound even at 3.4 μM/liter (0.5 ppm), the lowest concentration tested. Little or no germination occurred above 6.9 μM/liter (1 ppm). AMAPA seed in the control experiments germinated only 24.7% in three days. After incubation with 2-heptanone or 2-heptanol at concentrations above 6.9 μM/liter, the germination dropped to 6% or below.

Carrot and onion, the crops that showed the most severe allelopathic effects of AMAPA in the field, were also very sensitive to 2-heptanone and 2-heptanol vapors. Carrot seed germination was strongly inhibited at concentrations of 6.9 μM/liter and higher. The inhibitory effects on onion were evident as low as 3.4 μM/liter, especially with 2-heptanol which was a little more active than 2-heptanone. At 6.9 μM/liter, the germination of onion dropped from 61.1% (control) to 24.1% in 2-heptanone, and to 14.2% in 2-heptanol vapors. The more pronounced inhibition of onion seed by 2-heptanol persisted after the volatile source was removed and the seeds allowed to germinate for four additional

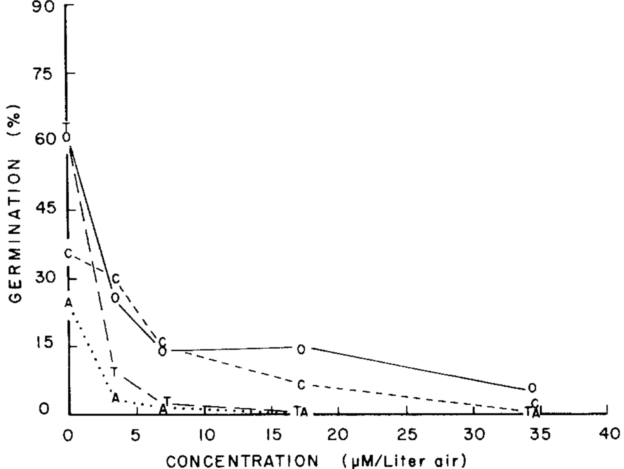

FIG. 5. Effects of 2-heptanol vapors (3-day exposure) on seed germination: (O) onion; (C) carrot; (T) tomato; (A) Palmer amaranth. Germination percentages are means of 16 replicates (standard errors of means were ≤ 2.1%).

days. At 34.4 μM/liter (5 ppm) of either compound, germination of carrot and onion was reduced to 5% or less. The two-way analyses of variance showed highly significant concentration effects in all four assay species exposed to 2-heptanone or 2-heptanol vapors. 2-Heptanol was significantly more inhibitory than 2-heptanone only in onion.

The effects of plant residue volatiles on agricultural crop seed germination have not been widely investigated but could be very important factors in effective weed and crop management. We believe that it is very significant and encouraging that the two compounds we happened to bioassay first were as inhibitory (at low concentrations) as were the volatiles from the most active plant samples (Bradow and Connick, 1985). A more thorough examination of all the volatiles identified in this study is underway. We hope this will lead to a better understanding of their potential as allelochemicals and their role in chemical ecology, as well as explain some fundamental aspects of the allelopathic activity of Palmer amaranth residues in the field.

Acknowledgments—We thank Mary P. Lovisa for her excellent technical assistance.

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FLAVONOID PIGMENTS IN CHALKHILL BLUE (*Lysandra coridon* Poda) AND OTHER LYCAENID BUTTERFLIES

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Abstract—Nine flavonoids, namely, kaempferol, kaempferol 7-rhamnoside, kaempferol 3-rhamnoside, kaempferol 3-glucoside, kaempferol 3-glucoside,7-rhamnoside, quercetin 3-glucoside, quercetin 3,7-diglucoside, isorhamnetin 3-glucoside, and isorhamnetin 3,7-diglucoside, have been identified in the body and wings of the chalkhill blue butterfly *Lysandra coridon* Poda. Flavonoids have also been found in 15 of a further 17 lycaenid species examined. Analysis of the two-dimensional paper chromatographic flavonoid patterns and aglycone results has shown that the flavonoid content of *L. coridon* and the other lycaenids is dependent on the flavonoid content of the larval diet. Differences in the flavonoid patterns of *L. coridon* and its leguminous larval food plants *Hippocrepis comosa, Anthyllis vulneraria*, and *Lotus corniculatus*, indicate that the ingested flavonoids are metabolized by *L. coridon* or its gut flora before sequestration. Despite the presence of flavones, glycoflavones, and isoflavones in the larval food plant species, only flavonols are sequestered by the lycaenid species examined. The relationship between lycaenid butterflies and their larval food plants, and the possible role(s) of flavonoids in lycaenids has been discussed. Interactions between ants, plants, flavonoids, and myrmecophilous lycaenids have also been considered.

Key Words—*Lysandra coridon*, Lepidoptera, Lycaenidae, blue butterflies, flavonoids, flavonols, sequestration, two-dimensional chromatography, wing pigmentation, chemical defense, myrmecophily, insect-plant interactions, chemical ecology.

INTRODUCTION

Although the occurrence of flavonoid pigments in plants has been well documented (Harborne and Mabry, 1982), little is known of their existence in animals. Flavonoids have been recognized in insects on a number of occasions

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(Thomson, 1926; Ford, 1941, 1944; Morris and Thomson, 1963, 1964; Fujimoto et al., 1959; Hayashiya *et al.*, 1959, Feltwell and Valadon, 1970; Wilson, 1985a,b, 1986a,b), but, in most cases identification of these pigments has either not been attempted or is subject to doubt.

Flavonoid pigments in the marbled white butterfly (*Melanargia galathea* L.) have recently been examined in some detail (Wilson, 1985a,b, 1986a). Eighteen flavonoids have been identified in *M. galathea*, and the same flavonoid pattern found in a number of other *Melanargia* species (Wilson, 1985a). In addition the dietary origin of insect flavonoids has been confirmed, and it has been shown that flavonoids are not merely sequestered from the diet but are also partly metabolized (Wilson, 1985b).

Flavonoids have been recognized in the scales on the underside of most British blue butterflies (Ford, 1945); however, only those in the common blue butterfly *Polyommatus icarus* Rott. have been examined further. From the wings of 800 *P. icarus* collected in France feeding on *Anthyllis* species, the free flavonol 3-*O*-methylkaempferol and the flavonol glycoside quercetin 3,4′-diglucoside have been tentatively identified (Feltwell and Valadon, 1970). An unidentified flavone glycoside and an unidentified isoflavone have also been reported.

In this paper, flavonoid pigments in the chalkhill blue butterfly (*Lysandra coridon* Poda) have been identified and the relationship between flavonoids in the butterfly and its leguminous larval food plants *Hippocrepis comosa* L., *Anthyllis vulneraria* L., and *Lotus corniculatus* L. examined. The flavonoids in a number of other lycaenids have also been examined, and the relationship between lycaenid butterflies and their larval food plants discussed.

METHODS AND MATERIALS

Lysandra coridon, *Lysandra bellargus* Rott., *Polyommatus icarus*, and *Cupido minimus* Fuessly were collected as wild individuals from downland at Aston Tirrold in Oxfordshire; *Syntarucus pirithous* L., *Lampides boeticus* L., and *Aricia cramera* Esch., from Grandola, Portugal; *Lycaeides idas* L., *Cyaniris semiargus* Rott., and *Plebicula thersites* Cantener from Ferrières-les-Verrières, Hérault, France; *Aricia agestis* Denis and Schiff. from the Mendips, England; and *Aricia artaxerxes* Fab., from Scotland.

Everes argiades Pallas, *Plebejus argus* L., *Glaucopsyche alexis* Poda, and *Maculinea arion* L., in the entomological collection at Reading Museum, were examined by fuming specimens with ammonia vapor; a reversible yellow coloration indicates the presence of flavonoids.

Leaf, stem, and flower material of *Hippocrepis comosa*, *Anthyllis vulneraria*, and *Lotus corniculatus* collected in May from downland at Aston Tirrold in Oxfordshire was rapidly air-dried and then stored in sealed paper bags until

required for analysis. Voucher specimens of plants have been deposited in the herbarium at Reading University.

The wings and body of each butterfly, including approximately 300 *L. coridon*, were separated, placed in small sample tubes, and the flavonoids extracted by soaking the crushed tissues in 1–2 ml of 70% ethanol at room temperature for 12 hr.

Extracts of the larval food plant species (*H. comosa*, *A. vulneraria*, and *L. corniculatus*) were made by immersing approximately 20 g of dried plant material in about 50 ml of boiling 70% ethanol, and boiling for 3 min. After cooling, the ethanolic plant material was soaked for 12 hr in a stoppered flask at room temperature.

Concentrated aliquots of the plant and insect extracts were applied to ¼ sheets of Whatman No. 1 chromatography paper, and run two dimensionally in BAW (*n*-butanol–acetic acid–water, 4 : 1 : 5, upper phase) and 15% aqueous HOAc (acetic acid) (Harborne, 1973). Dried chromatograms were examined in longwave UV light in the absence and presence of ammonia vapor and the position and color of each spot recorded.

The position of flavonoid spots on the two-dimensional chromatograms is a good indication of the nature of the flavonoids present (Harborne, 1973). Flavonoids occupying different positions on the chromatograms are different; however, flavonoids occupying the same positions are not necessarily the same, since some compounds have similar mobilities in BAW and 15% HOAc. The identification and cochromatography of flavonoids occupying similar positions on the two-dimensional chromatograms is required to confirm that they are the same compound.

Flavone aglycones, most flavone glycosides, and flavonol 3-glycosides appear as dark spots on the papers. After fuming with ammonia, most of these turn yellow or yellow green, but some flavonoids, including 6-hydroxy flavones and flavone 4′-glycosides remain dark. Flavonol aglycones and flavonol 7-glycosides are yellow and remain so when fumed with ammonia. Flavone 5-glycosides are light blue or white and turn fluorescent yellow or yellow green in the presence of ammonia.

A further aliquot of each plant and insect extract was hydrolyzed with 2 N HCl at 100°C for 30–40 min, the cooled hydrolysate extracted twice with ethyl acetate, the extracts combined, evaporated to dryness, and the residues dissolved in a few drops of 90% ethanol. Concentrated spots of each residue were applied to Whatman No. 1 chromatography paper and the aglycones identified by their R_f values in five solvents: BAW (4 : 1 : 5); PhOH (phenol–water, 4 : 1); CAW (chloroform–acetic acid–water, 30 : 15 : 2); FOR (acetic acid–conc. HCl–water, 30 : 3 : 1); and 15% HOAc; their colors in UV light; and by comparison with authentic samples (Harborne, 1967, 1973).

Hydrolysates of *P. icarus*, *L. coridon*, and *L. bellargus* were examined for isoflavones. A few drops of the ethyl acetate residues dissolved in 90%

ethanol were spotted onto fluorescent silica gel TLC plates along with authentic samples of the isoflavones genistein, formononetin, and daidzein, and the plates developed by ascent in 2% methanolic chloroform. Dried plates were examined in UV light; daidzein and formononetin appear as fluorescent blue spots, and genistein as a fluorescent blue-black spot. Spraying the plates with *p*-nitroaniline caused the isoflavones to appear as faint orange spots.

The body and wing extracts of about 300 *L. coridon* which produced similar chromatograms were combined to give one body and one wing extract. These two extracts were treated separately throughout due to the presence of substances in the body fraction that interfere with the chromatographic separation of flavonoids. Further extraction of the wing and body tissues was achieved by soaking them in approximately 500 ml of 70% ethanol at room temperature for 24 hr. After removal of this extract, the tissues were extracted a further five times with warm 70% ethanol, and the series of extracts combined. The flavonoids from 150 g of leaf material of *H. comosa* and *A. vulneraria* were extracted in a similar manner. Each plant and insect extract was then filtered, washed with petroleum ether (bp 40–60°C), and concentrated to a small volume in vacuo on a rotary evaporator.

Standard procedures were used for the separation, purification, and identification of flavonoids (Harborne, 1967, 1973; Markham, 1982). Preparative paper chromatography was used to separate and purify flavonoids. Known pigments were identified on the basis of R_f, UV spectral analysis, acid and enzymic hydrolyses to the aglycone and sugar(s), and by direct comparison with authentic samples where possible.

RESULTS

Nine flavonoids have been identified in the wings and body of *L. coridon*, with the concentration of pigments greatest in the former. The flavonoids in spots 10 and 11 on the two-dimensional chromatograms of this butterfly remain unidentified. The chromatographic and spectral properties, identities, and positions of these flavonoids on the chromatograms are given in Tables 1 and 2 and Figure 1. With the exception of quercetin 3-glucoside, which has been identified in the zebra swallowtail *Eurytides marcellus* (Cr.) (Wilson 1986b), the remaining eight flavonoids, namely, kaempferol, kaempferol 7-rhamnoside, kaempferol 3-rhamnoside, kaempferol 3-glucoside, kaempferol 3-glucoside,7-rhamnoside, isorhamnetin 3-glucoside, isorhamnetin 3,7-diglucoside, and quercetin 3,7-diglucoside have been reported from an insect for the first time. Where the flavonoids identified contain more than one sugar residue, it should be noted that although the number of residues is known, the nature of the glycosidic linkages have still to be determined. No flavones, glycoflavones, or isoflavones were detected in *L. coridon*.

TABLE 1. R_f AND SPECTRAL DATA FOR FLAVONOIDS OF *Lysandra coridon*[a]

	R_f (×100) in					UVΔλ(μm)					
Flav	BAW	15% HOAc	H_2O	PhOH	UVλ_{max} MeOH(nm)	+NaOH	+NaOAc	+H_3BO_3	+$AlCl_3$	Col	Flavonoid
1	84	02	00	60	268, 367	54	8	0	45	Y/Y	Kaempferol
2	81	18	02	80	266, 352	52	0	0	46	Y/Y	Kaempferol 7-rhamnoside
3	78	47	24	71	268, 350	50	7	0	24	D/Y	Kaempferol 3-rhamnoside
4	71	39	13	74	255, 268, 350	56	7	5	26	D/Y	Isorhamnetin 3-glucoside
5	68	43	14	76	267, 353	52	7	0	23	D/Y	Kaempferol 3-glucoside
6	55	38	07	53	258, 365	55	13	15	24	D/Y	Quercetin 3-glucoside
7	60	63	29	83	256, 270, 350	50	0	0	30	D/Y	Isorhamnetin 3,7-diglucoside
8	58	65	48	76	268, 350	46	0	0	26	D/Y	Kaempferol 3-glucoside, 7-rhamnoside
9	40	61	39	38	258, 360	54	0	20	41	D/Y	Quercetin 3,7-diglucoside

[a] Abbreviations: Flav, flavonoid; BAW, *n*-butanol–acetic acid–water, 4:1:5, upper phase; 15% HOAc, 15% aqueous acetic acid; H_2O, water; PhOH, phenol–water, 4:1; Col, color in UV light in the absence and presence of ammonia vapor; D, dark absorbing; Y, yellow.

TABLE 2. R_f DATA AND COLOR CHARACTERISTICS FOR FLAVONOID SPOTS ON TWO-DIMENSIONAL PAPER CHROMATOGRAMS OF *Hippocrepis comosa. Anthyllis vulneraria, Lotus corniculatus* AND LYCAENID BUTTERFLIES[a]

	R_f (×100) in		
Spot	BAW	15% HOAc	Col
Hippocrepis comosa			
1	54	63	D/Y
2	41	63	D/Y
3	32	66	D/Y
4	34	83	D/D
5	27	83	D/D
R	41	55	D/Y
Anthyllis vulneraria			
1	37	13	Y/Y
2	23	39	Y/Y
3	54	66	D/Y
4	45	67	D/Y
5	35	71	D/Y
6	27	71	D/Y
7	28	87	D/Y
R	39	57	D/Y
Lotus corniculatus			
1	73	17	Y/Y
2	72	48	D/Y
3	56	36	D/Y
4	44	72	D/Y
5	30	74	D/Y
R	38	53	D/Y
Lysandra coridon			
1	84	01	Y/Y
2	79	16	Y/Y
3	76	46	D/Y
4	71	42	D/Y
5	71	42	D/Y
6	58	37	D/Y
7	58	65	D/Y
8	54	63	D/Y
9	41	64	D/Y
10	55	09	Y/Y
11	35	06	Y/Y
R	46	58	D/Y

TABLE 2. (*Continued*)

Spot	R_f (×100) in BAW	15% HOAc	Col
Lysandra bellargus			
1	76	04	Y/Y
2	76	17	Y/Y
3	73	54	D/Y
4	59	46	D/Y
5	44	49	Y/Y
6	45	70	D/Y
7	39	70	D/Y
8	39	78	D/Y
9	54	14	Y/Y
10	32	05	Y/Y
R	39	60	D/Y
Polyommatus icarus			
1	77	03	Y/Y
2	78	42	D/Y
3	74	52	D/Y
4	60	44	D/Y
5	40	51	Y/Y
6	54	77	D/Y
7	43	74	D/Y
8	51	11	Y/Y
9	39	11	Y/Y
R	43	58	D/Y
Plebicula thersites			
1	73	51	D/Y
2	57	37	D/Y
3	44	55	D/Y
4	31	51	D/Y
5	36	64	D/Y
6	31	76	D/Y
R	41	55	D/Y
Plebicula amanda			
1	53	46	D/Y
R	44	59	D/Y
Aricia agestis			
1	55	07	Y/Y
2	64	26	D/Y

TABLE 2. (*Continued*)

Spot	R_f (×100) in BAW	15% HOAc	Col
3	75	47	D/Y
4	59	44	D/Y
5	45	47	Y/Y
6	47	62	Y/Y
7	52	71	Y/Y
8	37	56	D/Y
R	44	60	D/Y
Arica artaxerxes			
1	64	32	D/Y
2	75	50	D/Y
3	60	47	D/Y
4	53	61	D/Y
5	41	55	D/Y
6	44	68	D/Y
R	47	61	D/Y
Aricia cramera			
1	70	51	D/Y
2	52	44	D/Y
R	43	57	D/Y
Lampides boeticus			
1	66	06	Y/Y
2	54	41	D/Y
3	43	61	D/Y
4	31	06	Y/Y
R	45	59	D/Y
Syntarucus pirithous			
1	80	04	Y/Y
2	61	30	D/Y
3	50	34	D/Y
4	35	08	Y/Y
R	41	56	D/Y
Cupido minimus			
1	67	47	D/Y
2	61	70	D/Y
R	49	59	D/Y

TABLE 2. (*Continued*)

Spot	R_f (×100) in BAW	R_f (×100) in 15% HOAc	Col
Cyaniris semiargus			
1	56	41	D/Y
2	36	49	D/Y
R	38	59	D/Y
Lycaeides idas			
1	59	43	D/Y
R	39	58	D/Y

[a]Abbreviations: BAW, *n*-butanol-acetic acid–water, 4:1:5, upper phase; 15% HOAc, 15% aqueous acetic acid; Col, color in UV light in the absence and presence of ammonia vapor; D, dark absorbing; Y, yellow; R, rutin marker (quercetin 3-rutinoside).

The presence or absence of each flavonoid spot on the two-dimensional chromatograms of the wings and bodies of every *L. coridon* butterfly examined was recorded. Although some spots were more difficult to detect on the body chromatograms due to the lower concentration of flavonoids, the flavonoids in spots 1–11 were present in the wings and body of all butterflies. The flavonoid pattern of *L. coridon* at this site may therefore be considered constant.

Due to the difficulty and amount of time involved in the separation and purification of the complex mixtures of closely related flavonoid glycosides present in *H. comosa*, *A. vulneraria*, and *L. corniculatus*, the flavonoids in these plants were not identified. The flavonoid content of *L. coridon* was therefore compared with that of the plant species on the basis of their aglycone results and two-dimensional chromatographic flavonoid patterns.

The flavonols quercetin and kaempferol were identified in the hydrolyzates of all three plant species; isorhamnetin and apigenin were also identified in *A. vulneraria* and *L. corniculatus*, respectively (Table 3). The position and color of flavonoid spots on the two-dimensional chromatograms suggests that *H. comosa* contains a mixture of quercetin and kaempferol 3-, 3,7-, and 3,4′-glycosides; *A. vulneraria* various quercetin, kaempferol, and isorhamnetin 3-, 7-, and 3,7-glycosides; and *L. corniculatus* a mixture of apigenin 7-, and quercetin and kaempferol 3-, 7-, and 3,7-glycosides.

Quercetin and kaempferol were identified in *L. coridon* and the three plant species; however, it is clear from the two-dimensional chromatograms that flavonol glycosylation patterns differ in the plants and insect. Flavonol 3-, 3,4′-, and 3,7-glycosides were found in the plants whereas flavonol 3-, 7-, and 3,7-glycosides were found in the insect. In addition, the number of sugar residues

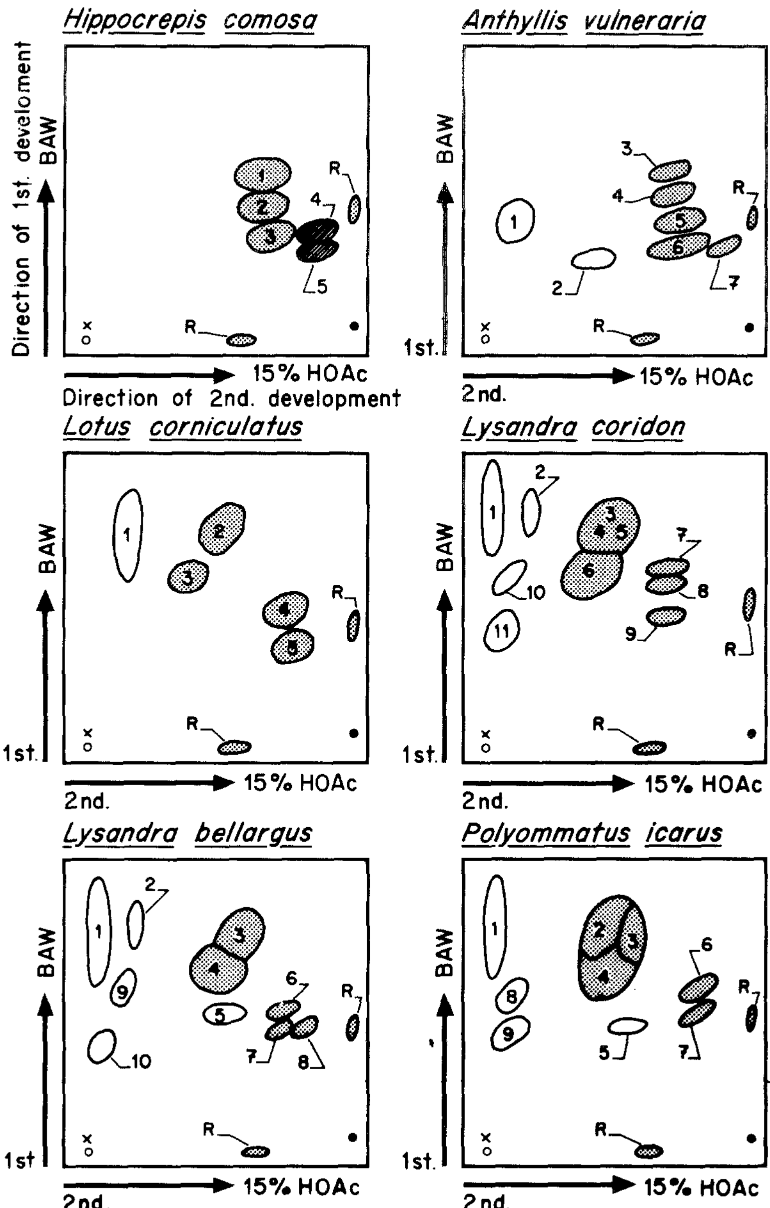

FIG. 1. Two-dimensional paper chromatograms of *H. comosa A. vulneraria*, *L. corniculatus* and the lycaenid butterflies. Chromatograms were developed first in BAW (4 : 1 : 5), then in 15% aqueous HOAc. Spot color characteristics in longwave ultraviolet light are represented as follows: yellow spots remaining so when fumed with ammonia, unstipled; dark absorbing spots changing to yellow in the presence of ammonia, stipled; dark absorbing spots remaining so when fumed with ammonia; hatched. x, represents the origin; •, the position at which the rutin marker was applied for development of the chromatogram in BAW; ○ the position at which rutin was applied for development of the chromatogram in 15% HOAc.

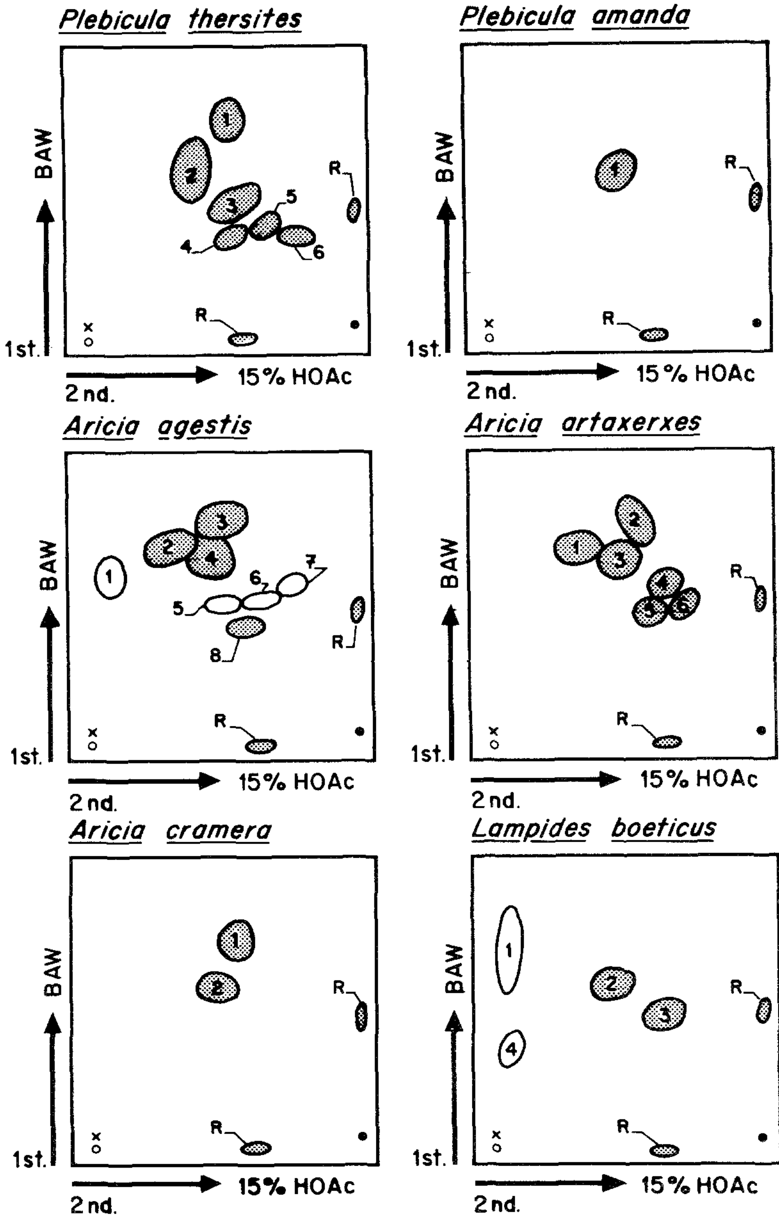

FIG. 1. Continued.

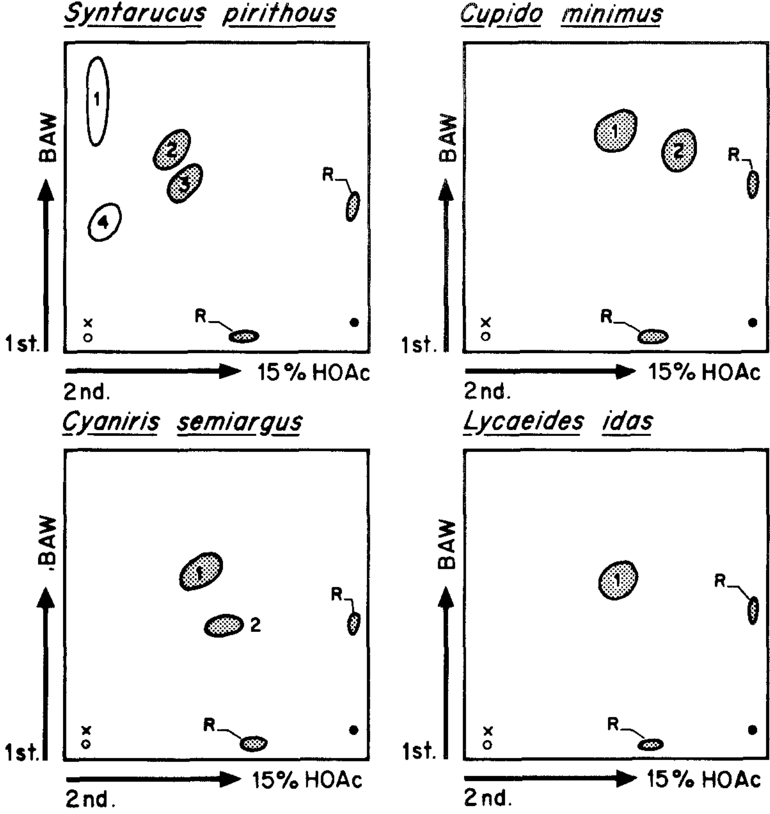

FIG. 1. Continued.

attached to flavonols in the plants is greater than that in *L. coridon*. This is shown by the greater mobility of many of the plant glycosides in 15% HOAc and their correspondingly lower mobility in BAW. Although some flavonoid spots occupy similar positions on the two-dimensional chromatograms of *L. coridon* and the plants, for example, spots 8 and 9 in the insect, spots 1 and 2 in *H. comosa*, and spots 3 and 4 in *A. vulneraria*, it is not possible to say that they contain the same compounds, as many flavonol glycosides have similar mobilities in BAW and 15% HOAc. The purification, identification, and co-chromatography of spot components is necessary to determine that they contain the same flavonoids.

The two-dimensional flavonoid pattern of *L. bellargus* is similar to that of *L. coridon* despite the absence of isorhamnetin from the former species (Figure 1). This difference in the butterflies' aglycone contents is reflected in the flavonoid contents of the larval diets; *H. comosa*, the larval food plant of *L. bellargus*, lacks isorhamnetin, whereas *A. vulneraria*, a recorded larval food plant

of *L. coridon*, contains isorhamnetin (Table 3). The position and color of flavonoid spots on the two-dimensional chromatograms indicates that the quercetin and kaempferol 3-, 7-, and 3,7-glycosides in *L. bellargus* contain fewer sugar residues than the quercetin and kaempferol 3-, 7-, and 3,4′-glycosides in *H. comosa*. No flavones, glycoflavones, or isoflavones were detected in *L. bellargus*.

The two-dimensional chromatogram of *P. icarus* is similar to that of the *Lysandra* species. From the aglycone results and the position and color of flavonoid spots on the two-dimensional chromatogram, it appears that *P. icarus* contains quercetin and kaempferol 3-, 7-, and 3,7-glycosides. No flavonoid spots were visible on the two-dimensional chromatograms that could represent quercetin 3,4′-diglucoside or 3-*O*-methylkaempferol, compounds previously tentatively identified in this butterfly (Feltwell and Valadon, 1970), nor was 3-*O*-methylkaempferol detected in the hydrolysates. Despite the range of flavonol aglycones reported in the larval food plants of this butterfly (Table 3) only quercetin and kaempferol were sequestered by *P. icarus*. Although flavones and isoflavones have previously been reported in *P. icarus*, none were detected in this study.

From the aglycone results and the position and color of flavonoid spots on the two-dimensional chromatograms, it appears that *Plebicula thersites* contains quercetin and kaempferol 3-, and 3,7-glycosides. *Onobrychis*, the larval food plant of *P. thersites*, is reported to contain quercetin and kaempferol (Jay et al., 1971).

Plebicula amanda has a much simpler flavonoid pattern than *P. thersites*. Only one flavonoid spot is present on the two-dimensional chromatogram which, from its chromatographic and color properties and the aglycone results, appears to contain a quercetin 3-glycoside. *Viccia cracca*, the larval food plant of this butterfly, is reported to contain quercetin, kaempferol, and apigenin (Jay et al., 1971).

The flavonoid pattern of *Aricia agestis* appears to consist of quercetin and kaempferol 3-, 7-, and 3,7-glycosides. Despite similarities in the flavonoid patterns of *A. agestis* and *L. coridon*, for example, spots 3, 1, and 4 on the two-dimensional chromatogram of the former species occupy similar positions to spots 3, 10, and 6 on that of the latter species; the larval food plants of *A. agestis* are not members of the Leguminosae but belong to the Geraniaceae and Cistaceae.

The flavonoid pattern of *A. artaxerxes* is similar to that of *A. agestis*. Flavonoid spots 1, 2, and 3 on the two-dimensional chromatogram of *A. artaxerxes* occupy similar positions to those of spots 2, 3, and 4 on the two-dimensional chromatogram of *A. agestis*. Although quercetin and kaempferol 3-, and 3,7-glycosides are found in both species, *A. agestis* also contains a flavonol 7-glycoside (represented by spot 1) (Table 2, Figure 1). The larval food plants of *A. artaxerxes* are reported to be the same as those for *A. agestis*.

TABLE 3. FLAVONOID AGLYCONES OF LYCAENID BUTTERFLIES AND LARVAL FOOD PLANT SPECIES[a]

Butterfly		Larval food plants[b]		
Species	Flavonoid aglycones	Species	Family	Flavonoid aglycones
Lysandra coridon	Q, K, I	*Hippocrepis comosa*	(Leg.)	Q, K
		Anthyllis vulneraria	(Leg.)	Q[c], K[c], I[c], R, Rc, F, Ge, Dk
		Lotus corniculatus	(Leg.)	Q, K, A
Lysandra bellargus	Q, K	*Hippocrepis comosa*	(Leg.)	Q, K
Polyommatus icarus	Q, K	*Lotus corniculatus*	(Leg.)	Q, K, A
		Medicago	(Leg.)	Q, K, T, G, C, Mg
		Ononis	(Leg.)	Q, K, D, Gn, Fo
		Trifolium	(Leg.)	M, Q, K, I, F, L
Plebicula thersites	Q, K	*Onobrychis*	(Leg.)	Q, K
Plebicula amanda	Q	*Vicia cracca*	(Leg.)	Q, K, A
Aricia agestis	Q, K	*Helianthemum*	(Cis.)	nd
		Erodium	(Ger.)	nd
Aricia artaxerxes	Q, K	*Helianthemum*	(Cis.)	nd
		Erodium	(Ger.)	nd
Aricia cramera	Q, K	Unknown	—	nd
Lampides boeticus	Q, K	*Colutea*	(Leg.)	Q, K
Syntarucus pirithous	Q, K	Legumes	(Leg.)	nd
Cupido minimus	Q, K	*Anthyllis vulneraria*	(Leg.)	Q[c], K[c], I[c], R, Rc, F, Ge, Dk
Cyaniris semiargus	Q, K	*Armeria maritima*	(Plu.)	nd
		Anthyllis vulneraria	(Leg.)	Q[c], K[c], I[c], R, Rc, F, Ge, Dk
		Trifolium	(Leg.)	M, Q, K, I, F, L
Lycaeides idas	Q	Legumes	(Leg.)	nd
Celastrina argiolus	None	*Ilex aquilifolium*	(Aqu.)	nd
		Hedera helix	(Ara.)	nd
Plebejus argus	+	Legumes	(Leg.)	nd
Everes argiades	+	Legumes	(Leg.)	nd
Glaucopsyche alexis	+	Legumes	(Leg.)	nd
Maculinea arion	–	*Thymus drucei*	(Lab.)	nd
		Myrmica larvae	–	nd

[a]Abbreviations: Q, quercetin; K, kaempferol; M, myricetin: R, rhamnetin; I, isorhamnetin; F, fisetin; G, gossypetin; Rc, rhamnocitrin; Mg, 7-*o*-methylgossypetin; Ge, geraldol; Dk, 5-deoxykaempferol; T, tricin; L, luteolin; A, apigenin; C, chrysoeriol; D, daidzein; Gn, genistein; Fo, formononetin; Leg., Leguminosae; Cis., Cistaceae; Ger., Geraniaceae; Plu., Plumbaginaceae; Aqu., Aquifoliaceae; Ara., Araliaceae; +, flavonoids present; –, flavonoids absent; nd, not determined.

[b]Larval food plant aglycone data compiled from Jay et al., 1971; Reynaud et al., 1982; Gonnet 1975; Gonnet and Jay, 1972, and confirmed where possible by the author.

[c]Only these aglycones were detected in *A. vulneraria* collected from the *L. coridon* site.

Aricia cramera has a much simpler flavonoid pattern than both of the former *Aricia* species; only two flavonoid spots which appear to contain quercetin and kaempferol 3-glycosides were present on the two-dimensional chromatograms. Although the larval food plants of *A. cramera* are unknown, one can speculate that they probably include flavonol-containing members of Leguminosae, Geraniaceae, or Cistaceae.

Four flavonoid spots were visible on the two-dimensional chromatogram of *Lampides boeticus*. These appear to contain free quercetin, quercetin or kaempferol 3-, and 7-glycosides and possibly a 3,7-glycoside. *Colutea*, a common larval food plant of this butterfly, also contains flavonols (Table 3).

The aglycone results and position and color of flavonoid spots on the two-dimensional chromatogram suggests that *Syntarucus pirithous* contains free kaempferol and quercetin and kaempferol 3-, and 7-glycosides. It is possible that spot 4 on the two-dimensional chromatograms of *S. pirithous* and *L. boeticus* contains the same flavonoid, probably a flavonol 7-glycoside. The larval food plants of *S. pirithous* are recorded as "small legumes" (Higgins and Riley, 1975) which are also flavonol containing.

Only two flavonoid spots were visible on the two-dimensional chromatogram of *Cupido minimus*. These appear to contain quercetin and kaempferol 3-glycosides. The absence of isorhamnetin from *C. minimus* but its presence in *A. vulneraria*, the larval food plant, may be due to its absence from the seed and seedpod tissues on which the larvae actually feed; the occurrence of isorhamnetin in *A. vulneraria* generally refers to its presence in the leaves.

The two flavonoid spots on the two-dimensional chromatogram of *Cyaniris semiargus* also appear to contain quercetin and kaempferol 3-glycosides. However, as indicated by their different positions on the two-dimensional chromatograms these glycosides are different to those in *C. minimus*.

The single flavonoid spot on the two-dimensional chromatogram of *Lycaeides idas* appears to contain a quercetin 3-glycoside. It is possible that this flavonoid is similar to, if not the same as, that in *P. amanda*. As in the case of *P. amanda*, the larval food plants of *L. idas* are flavonol-containing legumes.

Celastrina argiolus appears to lack flavonoids; none were detected on the two-dimensional chromatogram, nor were any aglycones produced by hydrolysis of the extract. Furthermore, no yellow coloration was detected when the wings were fumed with ammonia vapor. *C. argiolus* does not feed on legumes; alternate generations feed on *Ilex aquilifolium* (Aquifoliaceae) and *Hedera helix* (Araliaceae), respectively. As the date these specimens were collected is not known, it is not known to which generation the specimens belong and hence on which of these food plants they had fed.

Pale-colored scales on the wings of *Plebejus argus*, *Everes argiades*, and *Glaucopsyche alexis* changed reversibly to a yellow color when fumed with ammonia vapor, indicating that they contain flavonoids. The larval food plants of these species include legumes.

The large blue butterfly *Maculinea arion* did not respond to fuming with ammonia vapor; its color remained unchanged. Thus, it appears that this butterfly lacks flavonoids. In contrast to the other lycaenid species examined, *M. arion* does not feed on legumes, but feeds on thyme (*Thymus drucei*), a member of the Labiatae in the early stages, and is later tended by ants and reared on *Myrmica* larvae.

DISCUSSION

The results of this investigation have shown that the flavonoid content of *L. coridon* and the other lycaenids examined is dependent on the flavonoid content of their larval food plants. Flavonoid aglycones identified in the butterflies are always present in the larval food plants; however, flavonoid aglycones reported in the larval food plants do not always occur in the butterflies. Unfortunately, as the precise larval diets of the butterfly specimens examined are not known, it is not possible to say to what extent certain flavonoid aglycones in the recorded larval food plants are not sequestered by the butterflies. For example, where a number of larval food plant species have been recorded for a butterfly, the specimens examined in this study may have fed on only one or two of them containing derivatives of perhaps one or two flavonol aglycones. Consequently, by comparing the range of aglycones reported in the recorded larval food plants with those identified in the butterfly, it would appear that some aglycones are not sequestered by the insect, when in fact, their absence from the butterfly may be due to their absence from the diet. In spite of this, a clear relationship exists between the presence of flavonols and flavonol glycosides in lycaenid butterflies and their larval food plants.

During this investigation, the importance of examining both plants and animals from the same site when studying the relationships between insects and their host plants has been emphasized. Previously, the flavonol aglycones quercetin, kaempferol, isorhamnetin, rhamnetin (7-*O*-methylquercetin), rhamnocitrin (7-*O*-methylkaempferol), fisetin (5-deoxyquercetin), geraldol (fisetin 3′-methyl ether), and 5-deoxykaempferol have been identified in *A. vulneraria* (Gonnet and Jay, 1972). In addition, about 35 flavonol glycosides, the major constituents being quercetin 3-glucoside, quercetin 3-galactoside, quercetin 3-arabinoside, isorhamnetin 3-galactoside, and isorhamnetin 3-arabinoside have been reported in the leaves of *A. vulneraria* (Gonnet, 1975). In this study, the only flavonol aglycones detected in *A. vulneraria* were quercetin, kaempferol, and isorhamnetin, and no flavonoid spots were visible on the two-dimensional chromatograms that could represent the various flavonol 3-glycosides. As these flavonol 3-glycosides were reported as major constituents, it is unlikely that their presence was overlooked in this study, and rather, that flavonol di- and triglycosides are the major flavonoid constituents of *A. vulneraria* at this site.

That differences occur in the proportions of flavonoid mono- and diglycosides in plants from different populations has been demonstrated in *L. corniculatus* (Reynaud et al., 1982). In view of variation in the proportions of flavonoid mono-, di-, and triglycosides in the food plants of some lycaenids, it would be interesting to see if this is reflected in the flavonoid contents of butterflies feeding on plants from different populations.

Differences in glycosylation patterns of flavonols in *L. coridon* and its larval food plant species indicate that this butterfly or its gut flora is capable of metabolizing ingested flavonoids before sequestration. That this involves hydrolysis of at least some of the ingested glycosides to aglycones is suggested by the presence of free kaempferol in *L. coridon* but its absence from the food plants. The metabolism of ingested flavonoids prior to sequestration is not peculiar to *L. coridon*. This phenomenon has been recognized in the sequestration of flavonoids by *M. galathea* (Wilson, 1985a,b), and in the sequestration of other classes of plant secondary compounds, for example, the carotenoids (Harashima et al., 1972, 1976; Kayser, 1982); pyrrolizidine alkaloids (Rothschild and Aplin, 1972; Edgar and Culvenor, 1974; Edgar et al., 1974); and cardenolides (Roeske et al., 1976; Brower et al., 1982) by insects.

Although 3-*O*-methylkaempferol and quercetin 3,4′-diglucoside have been tentatively identified, and an unidentified flavone and isoflavone reported in *P. icarus* (Feltwell and Valadon, 1970), these compounds were not found in specimens examined in this study. However, this does not mean that these flavonoids are not sequestered by *P. icarus*, only that they were not sequestered by the specimens examined by the author. As the butterflies examined in the two studies were from different sites (and from different countries), it is possible that the larvae had fed on different plant species and hence sequestered different flavonoids.

A series of feeding experiments is desirable to determine whether or not lycaenid butterflies are capable of sequestering flavones, glycoflavones, and isoflavones. Although present in many of the larval food plants, these flavonoids were absent from all the lycaenids examined. Rearing batches of lycaenid larvae on diets containing these compounds and examining the flavonoid contents of larval feces and the resulting imagos will yield information on the fate of ingested flavonoids (Wilson, 1985b). The presence of flavones, glycoflavones, and/or isoflavones in imagos would indicate that lycaenids are capable of sequestering these compounds and therefore that their absence from the specimens examined is probably due to their absence from the larval diet; the presence of these flavonoids in the feces but not in the imago would indicate their excretion, while failure to detect them in the feces or in the imago would suggest their metabolism and excretion as nonflavonoid fragments.

The relationship between the *Aricia* species examined and their larval food plants is particularly interesting. These lycaenids do not feed on plants belonging to the Leguminosae, as do most of the other lycaenids examined, but on

species from the Geraniaceae and Cistaceae. Despite this difference in larval food plant families, flavonoid patterns in the *Aricia* species are similar to those in the legume-feeding lycaenids. That similarities exist between the larval food plant families is indicated by the Engler classification scheme in which the Geraniaceae in the Geraniales is placed next to the Leguminosae in the Order Rosales; the Cistaceae is placed slightly further away in the Parietales. The selection of plants from these families as larval food plants by lycaenid butterflies in itself suggests biochemical similarities, since the choice of larval food plants is influenced, at least in part, by their secondary chemistry (Erlich and Raven, 1964; Shapiro and Masuda, 1981). The presence of flavonols in these plants, and their subsequent sequestration by lycaenid butterflies, provides evidence for some similarity in their secondary chemistry.

The absence of flavonoids from *M. arion* is not surprising if one considers that it is only in the very early stages that larvae of this butterfly feed on plant material; for the major part, the larval diet consists of ant larvae. The absence of flavonoids from *C. argiolus* is more difficult to explain. It is possible that this butterfly's choice of larval food plants, species from the Araliaceae and Aquifoliaceae, is partly responsible. However, why this should be so, especially when both of these plant families contain flavonoids is not known.

It has been estimated that about a third of the species in the Lycaenidae associate with ants (Downey, 1962; Cottrell, 1983). As a result of these associations, the range of larval host-plant species is greater in myrmecophilous than in nonmyrmecophilous lycaenids (Pierce and Elgar, 1985). Because the flavonoid content of lycaenid butterflies is dependent on the flavonoid content of the larval diet, one can predict that there will be greater variation in the flavonoid content of myrmecophilous than nonmyrmecophilous phytophagous lycaenids. If lycaenid larvae sequester different flavonoids from different host plants, resulting in different flavonoid patterns in the imagos, then analysis of the flavonoid contents of butterflies belonging to a particular species could provide a means of estimating migrational interchange and gene flow between populations.

Hinton (1951) and others (Malicky, 1969; Eliot, 1973) have argued that ancestral lycaenids were myrmecophilous. Considering the protection from parasitoids and predators gained by lycaenid larvae in ant–butterfly associations (Pierce and Mead, 1981), dissociation of such a relationship would be disadvantageous to the butterfly, unless an alternative defense mechanism evolved. Although the role of flavonoids in butterfly defense has not been established, species containing flavonoids have been reported to be distasteful to a number of vertebrates (Lane, 1957; Pocock, 1911; Wilson 1986a). If the sequestration and storage of flavonoids by adult lycaenids represented a form of chemical defense similar to that encountered with cardenolides in *Danaus plexippus* L. (Brower, 1985), or alternatively, if they provided lycaenid larvae with increased protection against alien microbes rather than vertebrate predators (Wilson,

1986a), this may have reduced the dependence of lycaenids on ants, eventually resulting in the termination of some ant–butterfly associations. In the former case, the decreased mortality rate of adult lycaenids resulting in increased egg production may compensate for the increased mortality of larval and pupal stages concomitant with the loss of ant protection. Since the defense strategy involving ants is costly, in so much as it depends on lycaenid larvae secreting carbohydrate and amino acids (ant-appeasement substances), this may have restricted butterflies to plants rich in nitrogen and protein, for example, to legumes and other nitrogen-fixing or parasitic plants (Mattson, 1980; Pierce, 1985). Reducing the dependence of lycaenids on ants would permit some lycaenids to utilize plants less rich in nitrogen, for example, plants from the Geraniaceae and Cistaceae.

A further possibility in the relationship between ants and butterflies is that flavonoids are present in the secretions of some lycaenid larvae. Although ant attractants are carbohydrate and/or amino acid in nature, the considerable bacteriocidal and antimycetic properties exhibited by many flavonoids (Havsteen, 1983) may be of importance in maintaining infection-free ant colonies.

Flavonoid pigments are thought to be involved in wing coloration in the Lepidoptera (Ford, 1941, 1944; Wilson, 1986a,b). In particular, a relationship has been demonstrated between the flavonoid content and wing background color of *Melanargia galathea*; the greater the flavonoid content, the yellower the wings (Wilson, 1986a). Even in those butterflies with the whitest wing background color, flavonoids represent on average 0.83% by weight of the wing materials and contributed "body" as well as some color to the wings. Hence, it is possible that flavonoids are responsible for the white, cream, and yellow coloring on the underside of the wings of many lycaenids; the blue coloring on the upperside is structural, whereas the brown is melanic. Interestingly *C. argiolus*, which lacks flavonoids, also lacks any cream or white patterning; this butterfly has blue structural color on both the upper and underside.

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INTERMALE SEXUAL EFFECT ELICITED BY VOLATILE URINARY ETHER EXTRACT IN *Microcebus murinus* (PROSIMIAN, PRIMATES)

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Abstract—The effect of volatiles of diethyl ether-extracted dominant male mouse lemur urine on conspecific testosterone plasma concentrations was determined in an apparatus permitting olfactory isolation of the experimental subjects. Two experimental groups were exposed to air odorized with either ether or aqueous extracts of dominant male urine, while a third group received nonodorant air. Testosterone concentrations were measured before, during, and after the period of stimulation. The group submitted to the lipid fraction of dominant urine ($N = 15$) showed a significant decrease in testosterone concentrations. In contrast, no difference was observed between the group submitted to the aqueous fraction ($N = 10$) and the controls ($N = 11$). This physiological effect, which occurs in response to conditions of persistent stress, appears to be due to lipophilic components present in the urine of dominant males. The results are discussed in terms of the social structure of this primitive primate species.

Key Words—Primates, *Microcebus murinus*, olfactory signal, urine, organic fraction, dominance, sexual inhibition, testosterone.

INTRODUCTION

Chemical signals mediated through olfactory or vomeronasal endocrine pathways appear to be fundamental to many aspects of mammalian reproductive and social relationships (reviewed in Brown, 1979; Breipohl *et al.*, 1982; Johnston, 1983).

In fact, reproduction and social status are generally linked in mammals since dominant individuals seem to reproduce more often than subordinates (Huck and Banks, 1982; Fedigan, 1983). This correlation is not restricted to a

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behavioral issue (e.g., priority access to females) but influences the physiological state of all animals within a hierarchical structure (Henry and Stephens, 1977; Keverne *et al.*, 1982; Dantzer, 1984). More precisely, it has been demonstrated in several species, including primates, that in both sexes reproductive functions are inhibited or reduced in subordinate individuals (Christian, 1964; Andrews, 1979; Coe and Levine, 1983; Abbott, 1984; Perret, 1985a).

In some mammals, chemical signals used for social communication, provided by the secretion of specialized odoriferous skin glands, are dispersed passively or actively by "scent marking" behaviors (Mykytowycz and Goodrich, 1974; Schilling, 1979; Epple, 1980; Walro and Svendsen, 1982). Other mammals use secreta of nonspecialized glands and excreta such as urine (reviewed in Adams, 1980) or feces (MacDonald, 1979; Schilling, 1980a). Urine has been shown to be an important mammalian chemosignal system, particularly in species which lack specialized scent-marking glands (Candland *et al.*, 1980; Schilling, 1980b). The voided urine is a complex chemical mixture (Beruter *et al.*, 1973; Preti *et al.*, 1977; Zechman *et al.*, 1984), many components of which are volatiles (reviewed in Albone, 1984). Therefore, it is not surprising that urine is involved in a large number of social functions through either physiological (reviewed in Marchlewska-Koj, 1984) or behavioral responses (Beauchamp, 1973; Nyby *et al.*, 1977; Dunbar and Carmichael, 1981).

As already suggested, these responses can be elicited by the same urinary signal in different reproductive or social contexts. For instance, male dominant urine deters other males but attracts females in mice (Jones and Nowell, 1974) and bank voles (Hoffmeyer, 1982). Moreover, the male mouse androgen-dependent urinary primary pheromone that accelerates sexual development in juvenile females (Bronson and Whitten, 1968) is significantly more active in dominants than in subordinates (Lombardi and Vandenbergh, 1977).

In primates, several studies have suggested a correlation between social rank and either scent deposition (Epple, 1970; Castell and Heinrich, 1971) or endocrine status (Keverne *et al.*, 1982; Coe and Levine, 1983). In the lesser mouse lemur, similar correlations were demonstrated. Indeed, when several males of this prosimian species are kept together, a social hierarchy develops and dominant males in groups mark more frequently than other males and perform almost all mating and sexually associated behaviors (Lebec, 1984). Furthermore, plasma testosterone levels are significantly reduced in all nondominant males (Perret, 1985a). The sensory mechanism of this physiological response has been provided only recently (Schilling *et al.*, 1984). Indeed, a decrease in plasma testosterone concentrations may be induced when males kept in isolation are exposed to the dominant urine odor. This effect, observed even in males which had had no previous contact with the dominant urine donor, was described as resulting from a pheromone-like mechanism (Schilling *et al.*, 1984).

Since chemical investigations concerning urinary material involved in social status or intraspecific agonistic behavior refer to active substances found in the organic (i.e., lipid) fraction of urine (Lee, 1976; Lee *et al.*, 1980; Hoffmeyer, 1982), the present study was conducted in order to determine if the volatile responsible for intermale sexual effect observed in our primate species is present in the lipid or in the aqueous fraction of the dominant urine. As in our previous study, the physiological effect of the odorant factor was measured by the variations of plasma testosterone concentrations of experimental subjects.

METHODS AND MATERIALS

Animals. We used 36 male lesser mouse lemurs (*Microcebus murinus*, Cheirogaleidae, Strepsirrhini), 2–5 years old. The animals were born in the laboratory, from stock originating from the dry forest of south west Madagascar. They were caged singly in experimental conditions described elsewhere (Perret, 1985a) and had no previous contact with the dominant urine donor. The lesser mouse lemur displays a clear photoperiod-dependent cycle of sexual activity (Petter-Rousseaux and Picon, 1981). Sexual activity may be artifically induced by a long photoperiod rhythm and maintained when three- or six-month experimental rhythms are imposed (Petter-Rousseaux, 1975). Our first study showed that the hormonal response to the odorant dominant factor seems to vary according to the seasonal period of the sexual activity cycle (Schilling *et al.*, 1984). Consequently, an artificial photoperiodic schedule consisting of three months of short photoperiod (8 hr light–16 hr dark) followed by five months of long photoperiod (14 hr light–10 hr dark) was used. All the subjects of the present study were tested 60 days after the beginning of the 5-month long-daylight period of reversed lighting cycle.

Urinary Material and Experimental Procedures. The odorant stimulus was obtained from dominant male urine. Dominance status was determined in three heterosexual groups (3 or 4 females with 3 or 4 males) of *M. murinus* according to a method detailed elsewhere (Perret, 1985a). Urine of the dominant males was collected by isolating the animal in special cages placed over a freezing system, thus avoiding bacterial fermentation. Urine was pooled and stored at −25 °C. Separation of whole urine in lipid and aqueous fractions was performed in a Soxhlet apparatus allowing continuous extraction (12–16 hr) of urine to twice its volume of redistilled pure diethylether (peroxide free). The organic and aqueous portions were then separated in 0.5-ml daily samples stored at −25 °C in sealed vials until testing.

Animals are isolated in individual chambers which are composed of a top-closed Perspex cylinder (24 cm in diameter and 42 cm in high) fixed on a wood support. The animals have access to a feeding compartment in a removable

sliding drawer under the chamber. This airtight unit is ventilated by an independent air circuit assuring olfactory isolation. The present experimental device includes six of these units which are separated from each other by wooden partitions ensuring visual isolation. The ventilation is provided by a compressor for intake of external air (pressure regulated to 0.7 bars), through one-way valves and flowmeters (regulated to 50 ml/min) located at the entry of each experimental chamber. An additional exhaust fan expels air outside the experimental room. A digital programmer controls solenoid valves, which direct, alternatively, the air flow through polyethylene or glass tubings to either a nonodorized or an odorized air circuit for each unit. The control animal is only submitted to nonodorized air. In the odorized circuit, air is initially passed through a vial containing the dry residue of 0.5 ml ether extract or aqueous fraction of the dominant urine.

Six animals were tested in each experiment according the following procedure:

1. *Prestimulation Phase (15 Days).* Males, maintained under conditions of visual and olfactory isolation, were submitted to nonodorized air.

2. *Stimulation Phase (30 Days).* Except for controls, males were submitted daily to air odorized by introducing 0.5 ml of the lipid or aqueous fraction of dominant urine in the stimulus vial. The stimulation program was 30 sec of odorized air followed by 4 min of fresh nonodorized air.

3. *Poststimulation Phase (15 Days).* Conditions similar to those of the prestimulation phase. Three series (ether extract group, $N = 15$) of different subjects were submitted to the lipid fraction of the urine of three different dominant male lesser mouse lemurs and two series (aqueous group, $N = 10$) of other subjects to the aqueous fraction of the urine of two of the same dominant males. Since the results for the experimental control group ($N = 5$) showed no statistical difference with six sampled reference animals left in their home cage during the experiment, we summarized the results of the measures as a single control group ($N = 11$).

Blood Collection and Testosterone Radioimmunoassay. Blood samplings began 15 days before the 5-month long-photoperiod cycle and lasted 15 days after. Because the lesser mouse is a very small animal (body weight about 80 g), blood collection ($N = 14$ for each animal) was not made at regular intervals to avoid changes in the packed cell volumes of the animals studied. Thus blood collections (100 μl) were made every two weeks before the stimulation phase, every week during stimulation and post stimulation phases, and then every three weeks (Figure 1).

Animals were sampled during their daily sleeping period without prior anesthesia, at a fixed time (8 hr after onset of the light period), according to studies on circadian testosterone variations (Perret, 1985b). Blood was collected from the saphenous vein within 5 min of removing the animal from its nest box. After centrifugation, plasma was stored at $-20°C$ until assayed. Testosterone

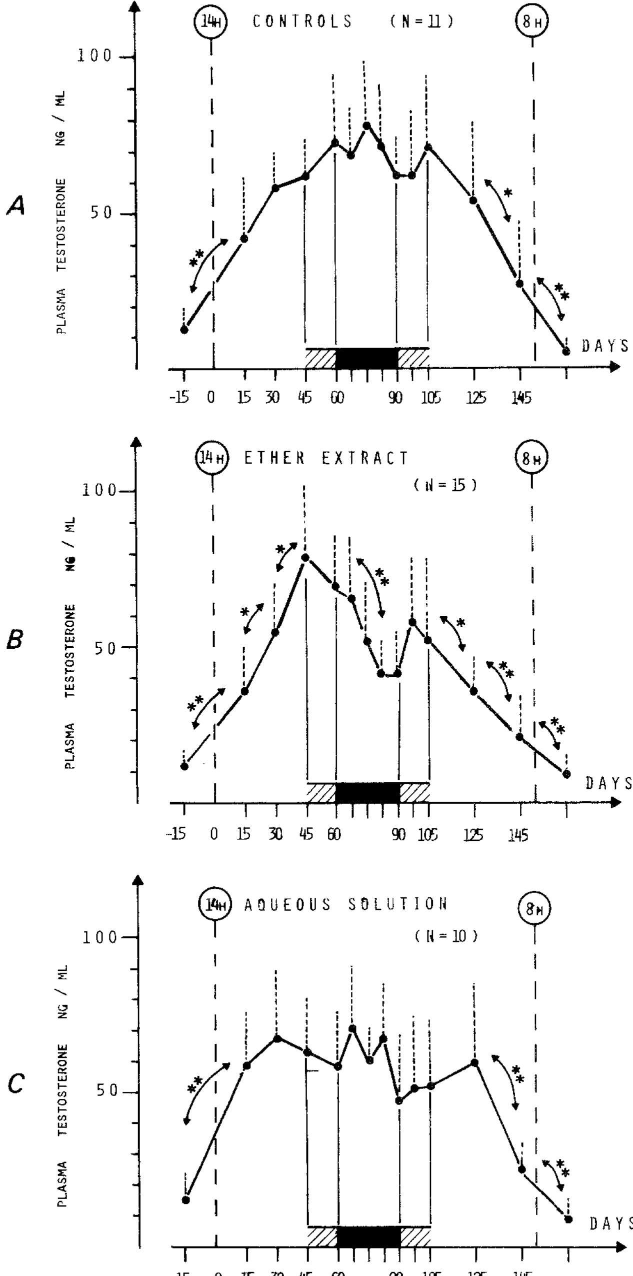

FIG. 1. Mean plasma testosterone concentrations (±SD) throughout the whole experiment for each experimental group. Significant differences between testosterone values within a group are indicated: $*P < 0.05$ and $**P < 0.01$ (F test). The successive experimental phases are indicated: hatched areas: pre- and poststimulation phases; black area: stimulation phase.

concentrations were measured in 20 μl plasma using the radioimmunoassay procedure described in Schilling *et al.* (1984). The antiserum diluted 1/200,000 was raised from 15-carboxymethyl-testosterone coupled to bovine serum albumin (purchased from Pasteur Institute). Radioimmunoassay was performed on extracted aliquots corresponding to 1.6 and 8 μl plasma. The percentages of cross-reactivity were: testosterone, 100%; dihydrotestosterone, 36%; dihydroandrosterone, 3%; Δ^4-androstenedione, 1.5%; other steroids tested, $\leq 1\%$. The intra- and interassay coefficients of variation were less than 14%. The sensitivity of the radioimmunoassay was 10 pg and the minimum detectable level in plasma was 0.6 ng/ml.

[3H]Testosterone was purchased from Amersham Int Plc (Bucks, U.K.) and nonradioactive steroids from Sigma (St. Louis, Missouri).

Statistical Analysis. All data are mean $\pm$ SD, and statistical differences in plasma testosterone concentrations were tested using variance analysis (F test). To compare groups during pre-, post-, and stimulation phases, we used two weeks-mean of testosterone concentrations.

RESULTS

Control Group. Under the experimental photoperiod schedule, testosterone concentration increased rapidly in control animals during the first month of long-daylight stimulation, followed by a spontaneous decrease four months later (Figure 1A). The mean value of testosterone concentration of this control group remained constantly elevated (mean 67 $\pm$ 13.8 ng/ml, N = 11) during the period between 30 and 120 days after the beginning of light stimulation. The apparent variations observed during the different phases of the experiment (Figure 1A) were not significantly different.

Ether Extract Group. As in control subjects, this experimental group responded rapidly to the change in photoperiod conditions by an increase of testosterone concentration which reached a maximum (75 ng/ml) after 1.5 months of long-daylight stimulation, during the beginning of the prestimulation phase of the experiment (Figure 1B). There was no statistical difference with the control group (Figure 2).

During the stimulation phase, animals submitted to dominant male urinary ether extracts demonstrated a significant decrease ($P < 0.01$) in plasma testosterone levels. This decrease was statistically significant within the first two weeks of stimulation and then stabilized after another two weeks (Figure 1B). The increase of values which appeared during the poststimulation phase, probably due to large individual variations of hormonal concentrations, is not statistically significant. This increase in testosterone concentration is followed by a rapid and marked decrease occurring three weeks earlier than the spontaneous decrease observed in control animals. Indeed, at the 125th day of long photo-

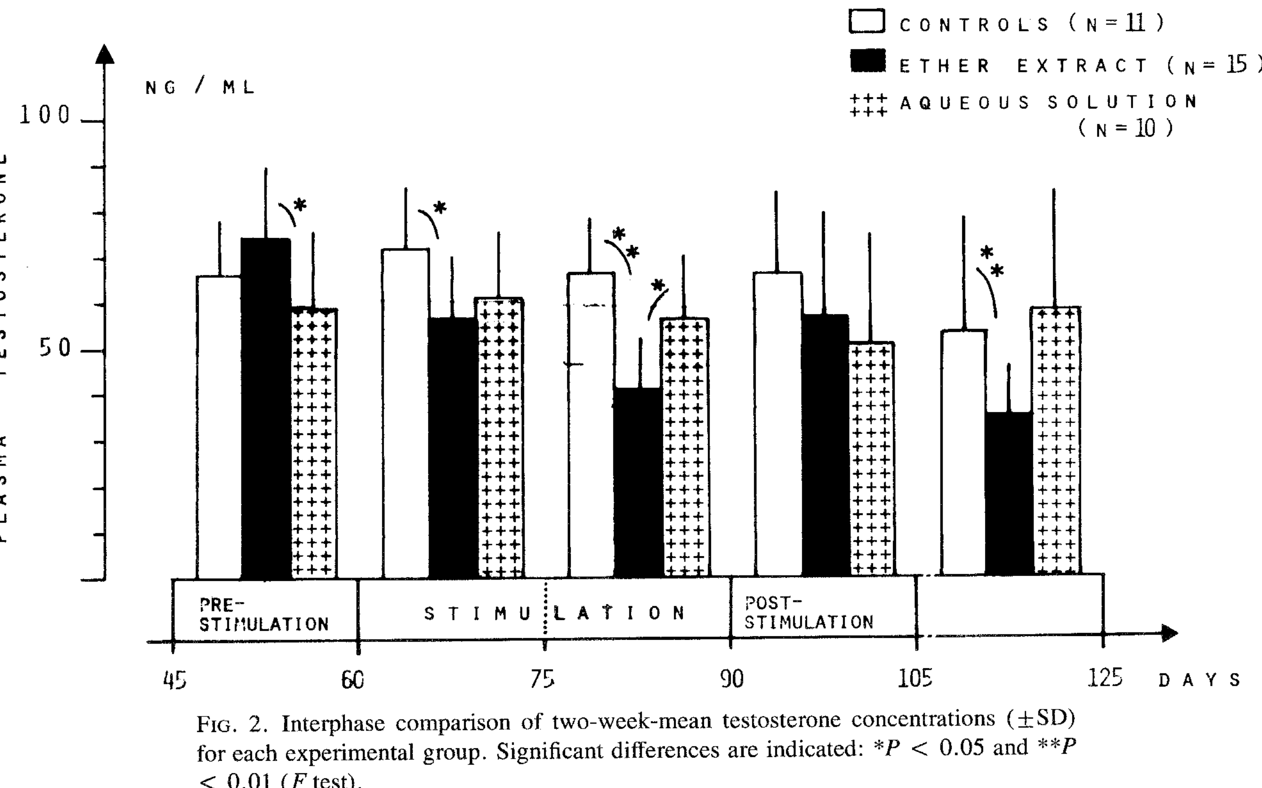

FIG. 2. Interphase comparison of two-week-mean testosterone concentrations (±SD) for each experimental group. Significant differences are indicated: $*P < 0.05$ and $**P < 0.01$ (F test).

period, control animals still showed higher significant testosterone concentrations (i.e., >50 ng/ml) than animals in the ether extract group (i.e., <40 ng/ml).

The interphase comparison of mean plasma testosterone level for each experimental group during 15-day periods (Figure 2) more clearly demonstrates the sexual inhibiting effect of the ether extracts since males exhibited, after 30 days, a significant decrease of the hormone compared with either the control or the aqueous phase group. Twenty days after the poststimulation phase, more than one month after stimulation was stopped, the hormonal level of the ether extract group remained significantly depressed, as if sexual rest had been induced earlier.

Aqueous Fraction Group. In contrast to the effect of ether extracts, the odor of the aqueous fractions of dominant male urine produced no significant changes in plasma testosterone level of conspecifics, although a decrease apparently occurs at the end of the stimulation phase (Figure 1C). In fact, the intergroup (Figure 1) and the interphase (Figure 2) comparisons of mean testosterone concentrations show that the aqueous fraction group reacted similarly to the control group, although at a slightly lower level.

DISCUSSION

The present study supports our previous findings that urine of dominant male *Microcebus murinus* contains a volatile chemosignal which influences testosterone plasma level in male conspecifics (Schilling *et al.*, 1984). This active volatile factor seems to be related to the status of the male, since subordinate male urines fail to induce significant variations of plasma testosterone concentrations in experimental males (Schilling and Perret, unpublished data). The mean value of testosterone decrease is similar in this experiment to previous measures observed in either subordinate males kept in groups (Perret, 1985a) or isolated subjects submitted to the whole dominant urine volatiles (Schilling *et al.*, 1984).

Since the active fraction of this chemosignal is extracted by an organic solvent and is not soluble in the aqueous fraction, it is suggested that the volatiles responsible for the physiological response of the lesser mouse lemur are lipophilic in nature. This result agrees with some other investigations concerning volatile chemicals in mammalian chemoreception. For instance, dichloromethane extracts shorten the estrous cycle of females, whereas the aqueous portions of the male mouse urine have no effect (Monder and Lee, 1978). Nevertheless, studies of urinary mouse pheromones have shown that the question of the volatility of the active chemical is rather complex. On the one hand, even if free urinary odorant active substances are lipophilic in nature, others possessing latent chemosignal properties are conjugated in the aqueous portion

of urine (Ingersoll *et al.*, 1982). On the other hand, volatile active compounds could possibly be bound to large nonvolatile carrier molecules from which they would be progressively released (Vandenbergh *et al.*, 1976; Novotny *et al.*, 1980). This could explain why mouse lemur urine marks, even when dried, retain almost indefinitely a strong characteristic odor.

This chemical complexity is further complicated by the existence of multiple neurosensory pathways involved in the reception of chemosignals. Firstly, depending on the physicochemical state of the material, the chemosignal may stimulate several sensory systems (gustatory, olfactory, vomeronasal, trigeminal, and perhaps terminal). Secondly, a chemical compound, even under the same phase, may, in addition to the main olfactory pathway, trigger different sensory organs. For instance, some of the neurons of the gustatory nucleus of the solitary tract have been shown to respond to various sets of olfactants (VanBuskirk and Erickson, 1977). In particular, the mechanism of stimulation of the vomeronasal system is also not completely resolved. Although several studies have shown that only a liquid-containing compound of high molecular weight (i.e., nonvolatile) activates the system (Johns *et al.*, 1978; Wysocki *et al.*, 1980; Singer *et al.*, 1984), others have shown that the vomeronasal cerebral center (accessory olfactory bulb) is able to respond to an airborne odorant stimulation (Meredith and O'Connell, 1979; O'Connell and Meredith, 1984). Moreover, physiological or behavioral responses to chemical cues may require a process of neuroendocrine integration which simultaneously involves several sensory chanels (Murphy, 1980; Beauchamp *et al.*, 1982; Meredith, 1983; Clancy *et al.*, 1984).

The lipophilic properties of the active urinary extract in male mouse lemurs may suggest a steroid-like nature of the chemosignal. Indeed several steroid components are odoriferous and have been implicated in chemosignals (Gower, 1976; Albone, 1984). Steroid excreted in the voided urine may originate either from the male or female reproductive tract or from the pituitary corticoadrenal system. Many of the sex hormone steroids, being conjugated before kidney filtration, are odorless when excreted. However, some of them, such as 3β-androstenol in pig urine, could be secondarily released by microorganism action and act as a pheromone (Booth and Baldwin, 1980). However, sexual steroids do not appear to be responsible for the effect described in male mouse lemurs since castration of a dominant active male does not reduce its capacity to decrease testosterone levels in conspecifics (Schilling and Perret, in preparation).

It is likely that social chemosignals may act at different physiological levels through different neuroendocrine pathways. Signals eliciting active responses (attraction between conspecifics) are often controlled by genital glands (Michael *et al.*, 1976; Epple, 1979; Dunbar *et al.*, 1980; Yarh, 1981; Macrides *et al.*, 1984). In contrast, some inhibitory signals could be modulated by adrenocortical function (Drickamer *et al.*, 1978; Marchlewska-Koj *et al.*, 1983; Novotny *et al.*, 1986). Indeed, urinary catabolites from corticoadrenals, especially 17-

hydroxycorticosteroids, are important since they reflect how an individual is affected, such as stress and arousal, and copes with psychosocial situations, such as sexual competition and hierarchy (reviewed in Dantzer, 1984), in which olfactory signals and olfactory pathways appeared to play an increasing role (Hoffmeyer, 1982; Feldman and Conforti, 1982; White *et al.*, 1984). Previous experiments have shown that in *Microcebus murinus*, plasma cortisol is increased in animals submitted to long-term social pressure (Perret and Predine, 1984) and that this increase is sustained by exposure to the urine odor of a dominant male (Schilling *et al.*, 1984).

Lastly, considering the importance of chemical signals in nocturnal solitary prosimian primates (Charles-Dominique, 1977a,b; Schilling, 1980a,b), it is assumed that some chemical components dispersed by urinary marking behaviors by dominant male mouse lemurs may possibly serve a double function to enhance the reproductive potential of the species (Perret, 1985a). Although male mouse lemurs with depressed testicular function are not necessarily sexually inhibited (Lebec, 1984), subordinate individuals are actually eliminated from the competition for females, as has been demonstrated for other mammals with similar social structure (Charles-Dominique, 1978). This effect could be achieved, firstly, through a physiological (depressed sexual function) and behavioral (deterent) effect on competitive males and, secondly, through a stimulating effect on the reproductive function of females which in turn would preferentially mate with dominant individuals.

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QUANTITATIVE COMPARISON OF BEHAVIORAL AND NEUROPHYSIOLOGICAL RESPONSES OF INSECTS TO ODORANTS: Inferences about Central Nervous System Processes

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Abstract—A consistent pattern of relationships emerges from comparisons of insect electroantennograms, peripheral olfactory receptor neuron responses, and behavioral responses to quantified concentrations of odorants. One consistency is that all of the different response measurements can be described by stimulus–response curves of the same form. Another is that the responses have characteristic groupings when they are plotted against odorant concentration. The pattern of relationships is exemplified in the responses of *Trichoplusia ni* (Hübner), *Heliothis zea* (Boddie), and *Plodia interpunctella* (Hübner) to several pheromone components and analogs. To quantify the relevant stimulus parameters for the response comparisons, the emission rates of the stimulus delivery system were calibrated for several 12 to 17-carbon pheromone components. The stimulus–response relationships determined for *T. ni*, *H. zea*, and *P. interpunctella* are combined with relationships reported for other insects in the literature, and applications are discussed for the interpretation of pheromone trapping and laboratory bioassays.

Key Words—Pheromone, olfaction, electroantennogram, neurophysiology, *Trichoplusia ni*, *Heliothis zea*, *Plodia interpunctella*, Lepidoptera, Noctuidae, Pyralidae.

INTRODUCTION

The ability of insects to detect odors and to distinguish differences in stimulus levels derives from peripheral and central nervous system (CNS) mechanisms

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that may operate similarly in many animals. Consider, for example, that the rate of action potentials elicited in a peripheral olfactory receptor neuron frequently is found to be graded proportionally to the odorant concentration, irrespective of the insect. This proportionality provides a neurophysiological basis whereby the elicited behavioral responses also can be graded with odorant concentration. (We do not imply that all behavior is graded in this way, because CNS integration and other processes may significantly modify the response.) The concept of the graded behavioral response has been a useful interpretive tool in vertebrate physiology, leading to the development of animal and human psychophysics (Stevens, 1975). Our objective is to quantify and compare several behavioral and neurophysiological stimulus–response relationships and then to demonstrate how the concept of graded response can be applied for interpreting laboratory and field bioassays.

A second focus of this report is to address the problem of pheromone quantification by measuring pheromone emission rates and concentrations.

We analyze the dose–emission rate relationship of a glass-tube odorant dispenser and apply the calibration in quantitative comparisons of neurophysiology and behavior for *Trichoplusia ni* (Hübner), *Heliothis zea* (Boddie), and *Plodia interpunctella* (Hübner).

METHODS AND MATERIALS

Insects and Chemicals

Male *T. ni*, *P. interpunctella*, and *H. zea* pupae were obtained from colonies maintained at this laboratory and held at 70–80% relative humidity, 24–27°C, on a 14:10 light–dark cycle. Adults were 48–72 hr old when tested.

Sex pheromone components of *T. ni*, (Z)-7-dodecen-1-ol acetate (Z-7:12Ac); *P. interpunctella*, (*Z,E*)-9,12-tetradecadien-1-ol acetate (*Z,E*-9,12:14Ac); *H. zea*, (Z)-11-hexadecenal (Z-11:16Al); and *Heliothis virescens* (F.), (Z)-9-tetradecenal (Z-9:14Al) and tetradecanal (14Al) were provided by Drs. J.H. Tumlinson and K.W. Vick of this laboratory. They were analyzed by gas–liquid chromatography (GLC) either on a 3% OV-1® 1.8-m packed column or 36-m capillary column and found to be >99% pure. The *Z*7:12Ac also was analyzed on a CPS-2 50-m liquid crystal capillary column by R.R. Heath of this laboratory. To calibrate dispenser release rates at low doses, samples of two of the pheromone components, *Z*-7:12Ac and *Z,E*-9,12:14Ac, were tritiated. The tritiated pheromone components were additionally purified by thin-layer chromatography and then by silicic-acid–column chromatography just before use because their lability increased after tritiation. Their specific activities were 0.804 and 0.951 Ci/mmol, respectively, as determined by GLC and liquid scintillation counting (LSC) using external standards.

Dispensers

Two types of pheromone delivery system were used, a glass-tube and a rubber-septa dispenser. The glass-tube dispensers were assemblies of ground-glass joints described in Mayer (1973) and Mayer et al. (1984). The desired quantity of a pheromone component diluted in 0.5 ml of hexane was pipetted into the assembly. The dispenser was rotated under aeration at 200 cm^3/min for 30 sec to coat the inside uniformly and to evaporate the hexane.

The rubber-septa system was one similar to that developed by Heath and Tumlinson (1986). Prior to loading, the 5-mm-ID × 9-mm-long rubber septa (A.H. Thomas Co.) were Soxhlet extracted in methylene chloride for 24 hr and air dried. The *Z*-7 : 12Ac was applied to the well of a septum in 50-μl aliquots of hexane, and the septum was aired in a hood for two days before use. For testing, the septa were placed in any one of three different delivery assemblies. One was a five-part assembly consisting of a brass Swagelok® 1.587-cm to 0.635-cm reducer, a 1.587-cm to 0.952-cm reducing union, and a 0.952-cm to 0.635-cm reducing port connector, with a 0.635-cm knurled nut at the inlet and a 0.635-cm Teflon® ferrule at the outlet. Another assembly was identical except that the metal was stainless steel. The third delivery system was the glass-tube assembly described above (without an initial pheromone coating).

Prior to pheromone collection or to use in a bioassay, the dispensers were purged for 30 sec with 200 cm^3/min of filtered dry air. The pheromone was delivered by passing filtered dry air through the glass-tube assembly at either 50 or 200 cm^3/min.

Collection and Analysis of Emissions

Three different methods were used in determining the glass-tube dispenser emission rates. Radiolabel analysis of pheromone components collected onto cotton filters was used for emissions at the 50 cm^3/min flow rate. GLC analysis of pheromone components collected in capillaries was used for emissions at 200 cm^3/min with doses between 10 and 1000 μg (0.044–4.4 μmol). The third method used a set of electroantennogram (EAG) bioassays combined with data from the radiolabeled calibration and from a calibration of rubber-septa emission rates by R.R. Heath of this laboratory.

Radiolabeled Pheromone Components. Emission rates of *Z*-7 : 12Ac and *Z*,*E*-9,12 : 14Ac at 50 cm^3/min were determined for doses between 0.08 and 10 μg (3.2×10^{-4}–4×10^{-2} μmol) by LSC analysis of radiolabeled pheromone components collected onto cotton filters. The dispensers were attached with Swagelok connectors to air samplers consisting of 5-mm-ID × 10-cm-long brass tubes stuffed with 35-mg cotton filters at each end. The filtering efficiency of the air samplers ranged from 0.9 to 0.97 as determined by comparing the radioactivity collected on the front and back filters. After the tritiated com-

pounds were collected, the filters were combusted in a Packard model 306® sample oxidizer. The oxidizer distilled the resultant tritiated water vapor into 10 ml of Packard Monophase-40® scintillation fluid cocktail. The efficiency of the oxidizer was monitored by external standards that indicated a negligible loss of radioactivity in this stage of the analysis. (Some of the emissions were collected independently by the capillary trap method described below, in which case the collected pheromone was rinsed with hexane directly into the scintillation cocktail.) The radioactivity of the cocktails was counted for 1 hr in a Packard model 2450® liquid scintillation counter calibrated by external standards.

GLC Analysis of Emissions of Unlabeled Pheromone Components at 200 cm^3/min. For pheromone component doses of 10–1000 μg (0.044–4.4 μmol), the collection device used was that described by Brownlee and Silverstein (1968). The emissions were collected in glass or stainless-steel capillaries connected to the dispenser by glass or Swagelok adaptors. The capillary was inserted into a metal tube heated at the dispenser end and chilled with acetone and Dry Ice at the outlet end. After a 3-min collection interval, the capillary was rinsed with 300 μl of hexane or diethyl ether into a calibrated centrifuge tube. In one series of tests the capillary effluent was passed through 3 ml of hexane to determine the collection efficiency of the capillary (about 80%). In another series of tests emissions from 100-μg doses of *Z*-7 : 12Ac were collected for 15-sec increments from 15- to 180-sec periods to determine a regression of quantity released on time.

The centrifuge tube contents were reduced for quantitative analysis by evaporation under dry nitrogen to remove excess hexane. Evaporative loss was calibrated in several cases by external standards using related compounds of similar volatilities but different GLC retention times. Aliquots from the centrifuge tubes were analyzed on a Varian model 210 GLC system with a flame ionization detector. The 1.8-m × 2-mm-ID glass column was packed with 3% OV-1 on 100–120 mesh Gas-Chrom-Q®, and the flow of nitrogen carrier was 30 cm^3/min. All analyses were isothermal, with the injector and detector at 200°C and the oven at 130–150°C. A Spectra-Physics model 4100 computing integrator was used for recording and quantitating the amounts recovered.

EAG Analysis of Z-7 : 12Ac Emission at 200 cm^3/min. The glass-tube dispenser emission rates at the 200 cm^3/min flow rate for doses of Z-7 : 12Ac between 0.1 and 10 μg (4.4×10^{-4}–4.4×10^{-2} μmol) were estimated by performing a set of three separate EAG bioassays on *T. ni* males. Two of the bioassays were done to determine a regression of EAG on concentration. In the third bioassay, EAGs performed at 200 cm^3/min were correlated with the concentrations predicted from the regression. The expected emission rate from a given dose then was determined by multiplying the predicted concentration by the total air flow in the plume passing over the antenna.

The first bioassay was done primarily at the 50 cm^3/min flow rate using the glass-tube dispenser. Here, the *Z*-7 : 12Ac concentrations delivered to the antenna were calculated from the radiolabeled pheromone calibration. The GLC measurements described above, calibrating *Z*-7 : 12Ac emission rates from the glass-tube dispenser at doses of 10, 31.6 and 100 μg, were used with EAGs of these three doses at the 200 cm^3/min flow rate.

The second EAG bioassay was done using a rubber-septa formulation, calibrated by R.R. Heath of this laboratory. The emission rates were estimated by interpolating between two curves of emission rate against dose, one at a 250 cm^3/min rate of flow through the brass assembly, and one at 100 cm^3/min.

The equation chosen to regress the EAG response magnitude against concentration was a power function (see, e.g., Mayer and Mankin, 1985) of the form:

$$\log(R) = A + B\log(C) \qquad (1)$$

where R is the EAG response in $-$mV, A and B are regression constants, and C is the pheromone concentration in μmol/cm^3. This equation was applied to the uncalibrated, low-dose EAG responses at 200 cm^3/min to estimate what concentrations had been present at the antenna when the responses were elicited. Finally, the release rates were calculated by multiplying the predicted concentration at each dose by the total flow rate.

Bioassays

Neurophysiological. Electroantennogram responses of *T. ni* and *H. zea* to *Z*-7 : 12Ac and *Z*-11 : 16Al, respectively, were measured by procedures similar to those described in Mayer et al. (1984). The concentrations delivered to the antennae for the *T. ni* EAGs were estimated from the 50 cm^3/min emission rate calibration and the rubber-septa calibration described above. The concentrations delivered in the *H. zea* bioassay were determined from the 200 cm^3/min emission rate calibration for doses of *Z*-11 : 16Al above 3 μg. The concentrations for lower doses were estimated by extrapolating from the curve for higher doses and using the same slope as for the *Z*-7 : 12Ac emission rates, as described in the Results section.

Single-sensillum recordings from *T. ni*, and *H. zea* were obtained as in Mayer (1968) using electrodes made from tungsten wire, sharpened electrolytically to less than a 1-μm tip diameter. Adult males were secured with wax, and the recording electrode was placed at the base of the sensillum. Signals were amplified and filtered (bandpass 1–10 kHz) with a Grass P15 AC preamplifier. The signals were monitored with a Tektronix 5113 dual-beam storage oscilloscope. Analysis and storage of data were performed on a Digital Equipment Corporation MINC Declab-23® microcomputer by a procedure similar to that described in O'Connell et al. (1973).

Flight Tunnel. The flight-tunnel bioassays for *T. ni* and *P. interpunctella* are reanalyses of data from two earlier reports by Mayer (1973) and Mankin et al. (1980). In those reports the concentration of pheromone in the tunnel had been estimated by dividing the dispenser output by the total tunnel airflow. However, this estimate of concentration is correct only if complete mixing of pheromone with air occurs. Results from subsequent flight and EAG bioassays had suggested that complete mixing was not occurring, even though $TiCl_4$ smoke released from the dispenser into the tunnel appeared to be well-mixed when the smoke reached the downwind end.

To avoid relying on visual estimates of the completeness of mixing in the tunnel, we determined the downwind pheromone concentration at the insect release point by an EAG bioassay. Electroantennograms were recorded from 10 *T. ni* males in the tunnel with the stimulus airflow through the dispensers set to 50 cm^3/min. Each male was tested with Z-7:12Ac at doses of 3.16, 10, and 31.6 μg (0.014–0.14 μmol). The upwind concentration at the dispenser release point was calculated by dividing the emission rate by the dispenser flow rate. The downwind concentration to which each male had been exposed was calculated from the regression of EAG on concentration described in the previous section. To estimate the mean dilution factor, the upwind concentration was divided by the downwind concentration. The mean dilution factor determined from the three different doses in the EAG bioassay then was applied to all dispenser doses to determine the concentrations in the tunnel.

RESULTS

Although the dispensers were calibrated primarily to relate responses from the behavioral bioassays to responses from the neurophysiological bioassays, the calibrations are interesting in themselves and will be described first. The calibrations then are compared with those of other types of dispensers, and the most significant features of the release pattern are generalized to other release systems.

Two of the findings in this section warrant particular emphasis. First, the emission rate of pheromone from the glass-tube dispenser (and probably from many other types of dispenser as well) is not linearly proportional to dose. The greatest deviations from linearity occurred at the upper and lower ends of the dosage range. Such deviations lead to overestimation of stimulus levels at both high and low doses of pheromone. This can lead to errors of interpretation when a dose–response bioassay is not referenced to the concentration in the airstream. Second, the plume of pheromone in a wind tunnel remains relatively undiluted under most conditions. Measurements by EAG indicate that the mean dilution factor may be no larger than 10–20, even when a visual indicator, e.g., a smoke plume, appears well dispersed. The instantaneous dilution factor varied consid-

erably about the mean in a pattern intermediate to the EAG pattern described for the point source and the continuous cloud source by Baker et al. (1985).

Glass-Tube Dispenser Emission Rates. The relationship between dose and emission rate for the glass-tube dispenser was similar for all of the pheromone components tested at both 50 and 200 cm^3/min flow rates and appeared to follow a general pattern predicted by current theories of molecule-surface dynamics (see Tully and Cardillo 1984). At doses from $1 \times 10^{-3.5}$–1×10^{-1} μmol, the emission rate increased with increasing dose. Above this range the emission rates reached a constant level, independent of dose. Thus we found it necessary to divide the calibration curves for each chemical into multiple segments. The regression equations of best fit for doses of less than 100 μg are listed in Table 1, and the data are shown in Figures 1 and 2.

The inset in Figure 2 shows the *Z*-7 : 12Ac emission rates calculated for the EAG calibration at 200 cm^3/min for doses below 10 μg. The segment of the *Z*-7 : 12Ac line labeled GC is redrawn from the GLC calibration on the main graph, and the segment labeled EAG is the part calibrated by EAG. In the EAG segment, the emission rates are calculated from the concentrations predicted at different EAG levels by the *T. ni* EAG–concentration regression line, TNE, in Figure 3. The top part of the dotted curve in the inset of Figure 2, labeled *Z*-11 : 16Al, also is redrawn from the main graph. The extension of this curve, the segment with larger dots, is an assumption that the curve for *Z*-11 : 16Al is parallel to the curve for *Z*-7 : 12Ac. The *Z*-11 : 16Al emission rate curve was used to estimate the concentrations for the regression of *H. zea* EAG on concentration, HZE, in Figure 3.

The amounts of *Z*-7 : 12Ac release over 15-sec periods from 15 to 180 sec yielded a statistically linear regression over the 3-min period (Table 2). (Note: theoretically, the release rate decreases exponentially, but most of the decrease occurs during the initial 30-sec purge.)

Comparison of Glass Tube with Other Dispensers. Because pheromones are not usually dispensed from glass, it is worthwhile to compare the glass tubes with other substrates and to generalize some of the findings, if possible. The (initial) rate of emission of pheromone from the glass tube is higher than from most slow-release formulations. For example, the half-life of *Z*-7 : 12Ac on rubber septa is 34.8–35.9 days (Butler and McDonough, 1979). Based on this half-life, the initial emission rate from a septum dosed with 1 mg is 6.1×10^{-5} μmol/min. The same initial emission rate of *Z*-7 : 12Ac from the glass tube is achieved by a dose of 3.98×10^{-3} μmol (890 ng), according to the inset in Figure 2. This also can be compared with the measurement by Baker et al. (1981) of a 1.62×10^{-5} μmol/min emission rate from a septum dosed with 1 mg of *Z*-8 : 12Ac.

Comparisons of *Z,E*-9,12 : 14Ac released from different substrates reveal a pattern similar to that shown by *Z*-7 : 12Ac. A polyethylene cap loaded with 33.7 μmol (8.5 mg) of *Z,E*-9,12 : 14Ac emits at the rate of 3.10×10^{-5} μmol/

TABLE 1. REGRESSION EQUATIONS FOR RELEASE RATES OF SEX PHEROMONES AND RELATED CHEMICALS AT DOSES BELOW 100 μg^a

Chemical	Flow rate (cm^3/min)	Regression constant	Estimate	Standard error of estimate	F	R^2
Z-1,12:17	200	A	−0.72	0.35	43.24[b]	0.73
		B	1.51	0.23		
Z-11:16Al	200	A	−2.01	0.21	65.11[b]	0.80
		B	1.38	0.17		
14Al	200	A	−1.29	0.34	19.51[b]	0.55
		B	1.63	0.37		
Z-9:14Al	200	A	−1.35	0.18	55.33[b]	0.58
		B	1.34	0.18		
Z-9:14For	200	A	−1.95	0.17	94.30[b]	0.78
		B	1.18	0.12		
Z,E-9,12:14Ac	50	A	−3.94	0.68	17.82[c]	0.78
		B	1.09	0.26		
Z-7:12Ac	50	A	−1.30	0.42	80.46[b]	0.82
		B	1.80	0.20		
Z-7:12Ac (by GLC)	200	A	−1.12	0.04	1057.03[b]	0.87
		B	1.37	0.04		
Z-7:12Ac (by EAG)[d]	200	A	0.30	0.44	186.72[b]	0.80
		B	2.05	0.15		

[a]The regression equations have the form: log(emission rate) = $A + B$ log(dose), where A and B are regression constants in the table, the units of emission rate are μmol/min, and the units of dose are μmol.
[b]Statistically significant at the 0.001 probability level.
[c]Statistically significant at the 0.01 probability level.
[d]See inset in Figure 2.

min (Mankin et al., 1980), which compares with a glass tube loaded with 0.18 μmol (45 μg) (Figure 1). A Fiberglas®-coated screen dosed with 0.397 μmol (0.1 mg) of *Z,E*-9,12:14Ac emits 1.33×10^{-6} μmol/min (Mankin et al., 1983), which is slightly less than the emission rate from the glass-tube dispenser.

More important for general comparison, the theory of molecule–surface dynamics (e.g., Tully and Cardillo, 1984) predicts that the emission of pheromone from any dispenser, except an ideal inert matrix, follows a pattern similar to that found for the glass tubes. At some point near the upper end of the dosage range of behavioral relevance, the low vapor pressure of pheromone limits the rate of pheromone release. At low doses the pheromone remains adsorbed to low-energy sites on the substrate. The actual doses where such effects occur

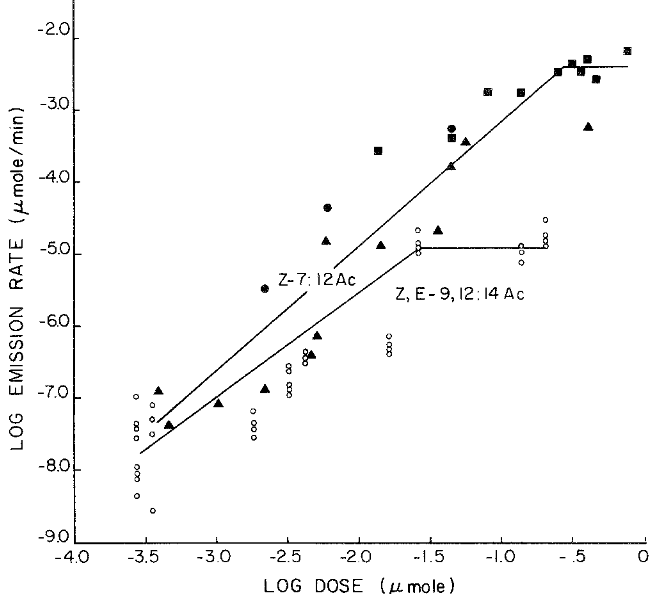

FIG. 1. Release rates of Z-7 : 12Ac and Z,E-9,12 : 14Ac from glass dispensers at a flow rate of 50 cm^3/min: Triangles, means of four to seven replications with ^{3}H-labeled Z-7 : 12Ac collected by the air sampler; closed circles, mean of four replications with ^{3}H-labeled Z-7 : 12Ac collected by cold trap; squares, means of 8–19 replications with unlabeled Z-7 : 12Ac collected by cold trap; open circles, single measurements with ^{3}H-labeled Z,E-9,12 : 14Ac collected by the air sampler.

depend on the substrate, the molecular weight, and the functional groups of the pheromone molecule.

Neurophysiological Bioassays. In the single-neuron study, the responses of pheromone-sensitive receptor neurons in *T. ni* occurred at concentrations only above $1 \times 10^{-4.6}$ μmol/cm^3, which is about the same as the EAG threshold. Similarly, pheromone-sensitive neurons in the long, sexually dimorphic sensilla spaced in rows on the proximal 40–50 subsegments of the male *H. zea* antennae responded to doses of Z-11 : 16Al above about $1 \times 10^{-3.4}$ μmol (0.1 μg). The emission rate of Z-11 : 16Al was not calibrated at this level; however, by extrapolation from the inset of Figure 2, this dose appears to correspond to an emission rate of about 1×10^{-8} μmol/min, and a concentration at the antenna of about $1 \times 10^{-11.1}$ μmol/cm^3 (Figure 3). The *H. zea* and *T. ni* receptor neurons, which are sensitive to their major pheromone components thus appear to have similar sensitivities, and the similarity of the two EAG response curves is in agreement with this finding (Figures 3–5).

The EAG responses of *T. ni* and *H. zea* to Z-7 : 12Ac and Z-11 : 16Al,

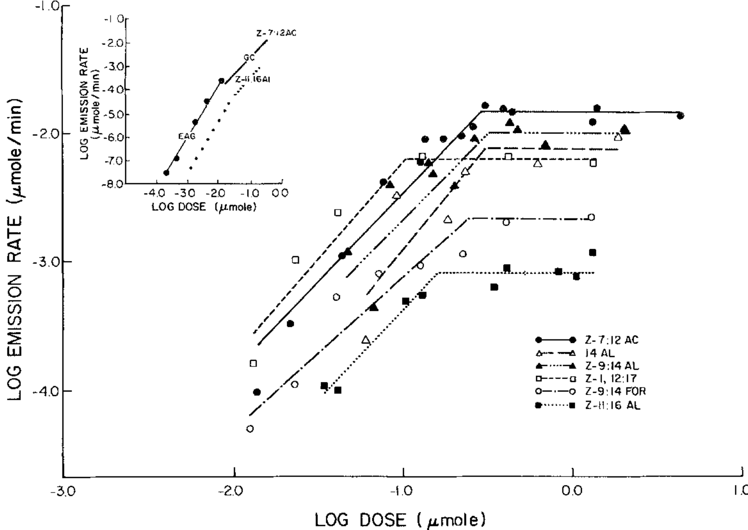

FIG. 2. Release rates of pheromone components from glass dispensers at a flow rate of 200 cm^3/min. All collections were by cold trap and analyses were by GLC. Closed circles, Z-7:12Ac; open triangle, Z-9:14Al; closed triangle, 14Al; open circle, Z-9:14For; closed square, Z-11:16Al; open square, Z-1,12:heptadecadiene. Inset shows EAG calibration of release rates of Z-7:12Ac for doses below 10 μg and the extrapolation of the emission rate of Z-11:16Al for doses below 10 μg.

respectively, are shown in Figure 3. No significant differences in the *T. ni* EAG response were noted in tests with the three different rubber-septa assemblies (brass, stainless steel, or glass). We note also that the *T. ni* EAG levels are somewhat lower than those given in Mayer et al. (1984) because improvements in the mixing chamber of the stimulus delivery system lowered the peak concentrations delivered to the antenna. The regression of *T. ni* EAG response on concentration was used to calculate the emission rate curve in the inset of Figure 2 that estimates emission rates of low doses of Z-7:12Ac at the 200 cm^3/min flow rate. The *T. ni* regression also was used to determine the dilution factor in Table 3 for the wind-tunnel bioassays in Figure 4. The regression equations are listed in Tables 4 and 5.

Wind Tunnel Bioassays. The dilution ratios measured by the wind-tunnel EAGs show that, contrary to earlier assumptions, there was not enough turbulence to cause complete mixing of pheromone in the bioassays of anemotaxis in *T. ni* (Mayer, 1973) and *P. interpunctella* (Mankin et al., 1980). The mean dilution ratio was 23.7 (Table 3). If the Z-7:12Ac plume in the EAG tests had mixed completely with the tunnel air, the mean dilution ratio would have been 51,200, three orders of magnitude higher than the EAG-derived ratio. Conse-

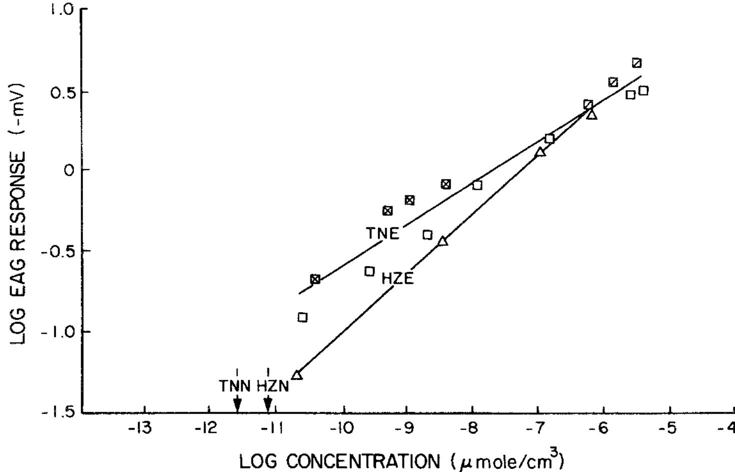

FIG. 3. Concentration–response relationships for *T. ni* and *H. zea* neurophysiological bioassays. TNE: regression of *T. ni* EAG response on Z7–12 : Ac concentration; open squares are means of 10 replications with glass-tube dispenser at 50 cm^3/min flow rate, squares with slash are means of 10 replications with glass-tube dispenser at 200 cm^3/min flow rate, squares with cross are means of 30 replications with rubber septa at 200 cm^3/min flow rate. HZE: regression of *H. zea* EAG response on Z-11 : 16Al concentration; triangles are means of 13 replications with glass-tube dispenser at 200 cm^3/min flow rate. TNN: lowest concentration eliciting detectable response by *T. ni* pheromone receptor neurons sensitive to Z-7 : 12Ac. HZN: lowest concentration eliciting detectable response by *H. zea* pheromone receptor neurons sensitive to Z-11 : 16Al. Regression equations are listed in Table 4.

TABLE 2. REGRESSION EQUATIONS FOR RELEASE RATES FROM DISPENSERS[a]

Flow rate (cm^3/min)	Regression coefficient ($\times 10^{-5}$)	Standard error of estimate ($\times 10^{-5}$)	F	R^2
50	1.10	.08	211.54[b]	0.99
100	3.86	.30	168.29[b]	0.99
200	9.67	.89	119.18[b]	0.98

[a] Dosed with 0.44 μmol (100 μg) Z-7:12Ac at flow rates of 50, 100, and 200 cm^3/min. The regression equations have the form: emission = $K \times$ time, where K is the regression coefficient, emission is given in μmol and time is in seconds.
[b] Statistically significant at the 0.01 probability level.

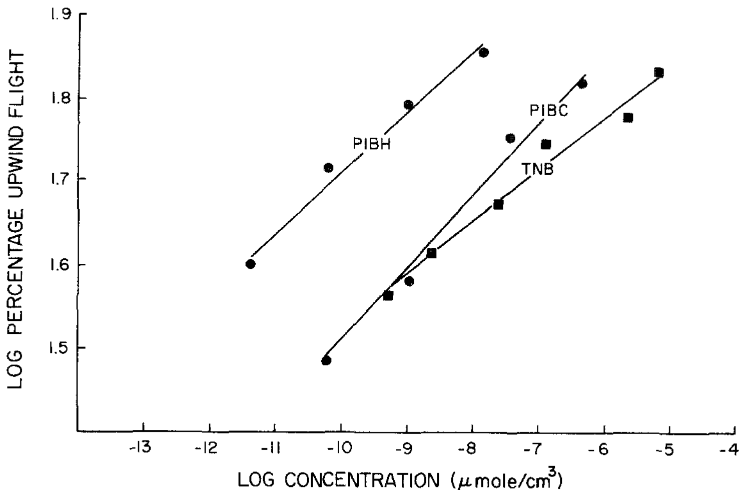

FIG. 4. Concentration–response relationships for wind-tunnel bioassays. The regression equations are given in Table 4. TNB line with squares, anemotactic response to *Z*-7 : 12Ac by *T. ni*; PIBC line with circles, anemotactic response to *Z,E*-9,12 : 14Ac by *P. interpunctella* at 23°C.; PIBH line with circles, anemotactic response to *Z,E*-9,12 : 14Ac by *P. interpunctella* at 34°C.

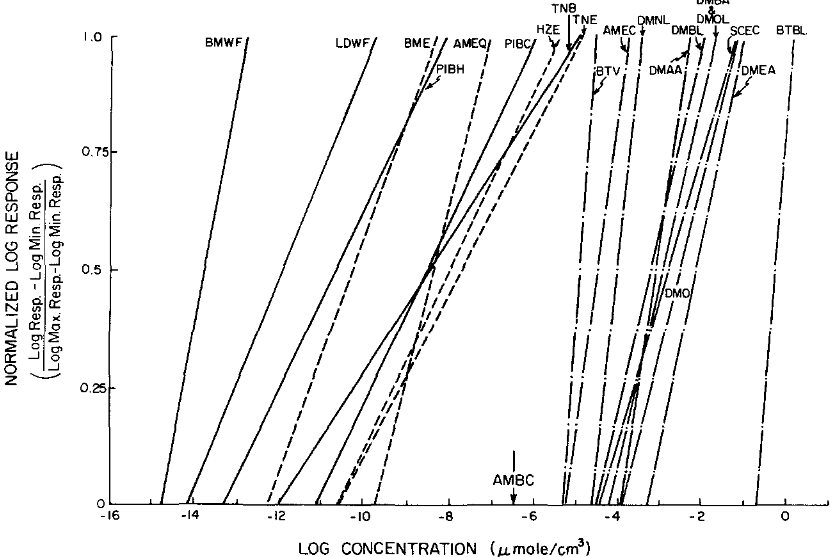

FIG. 5. Comparison of normalized behavioral and neurophysiological responses of several insect species to various odorants. Solid lines indicate behavioral response. Dashed lines indicate EAGs in response to a sex pheromone component. Dot-dashed lines indicate EAGs in response to a non-pheromonal odorant. AMBC is the behavioral threshold of female *A. mellifera* to caproic acid. See Table 5 for key to abbreviations.

TABLE 3. EAG BIOASSAY OF Z-7:12AC DILUTION RATIO IN WIND TUNNEL[a]

Dispenser dose (μg)	log EAG response ($-$mV)	Log concentration (μmol/cm^3) At dispenser outlet	At end of tunnel	Dilution ratio
3.16	-0.056	-6.70	-8.0	20.0
10.0	0.223	-5.95	-7.0	11.2
31.6	0.422	-4.50	-6.1	39.8

[a]The concentration at the end of the tunnel was estimated from Figure 3. The concentration at the dispenser outlet was estimated from the release rates in Figure 1, with the release rate divided by 50 cm^3/min to calculate concentration.

quently, we have revised upward the estimates of pheromone concentration in the two bioassays and recalculated the stimulus–response regression equations.

The pheromone concentration in the wind tunnel was recalculated from the new emission rate curves listed in Table 1 and the mean wind-tunnel dilution factor in Table 3. The regression constants of Eq. 1, based on the revised concentrations, are listed in Table 4, and the new concentration–response regressions are shown in Figure 4. The new estimates indicate that 50% upwind anemotaxis was elicited in *T. ni* at concentrations above about 1×10^{-7} μmol/cm^3 of Z-7:12Ac. Extrapolating the curve to the 25% level, at log percentage upwind flight = 1.4, we find that the threshold occurs near 1×10^{-12} μmol/cm^3. The range of anemotactic response by *P. interpunctella* at 23°C was similar to that for *T. ni.* At 34°C (PIBH), the magnitude of response of *P. interpunctella* was higher than at 23°C (PIBC), but the slopes of the two curves were statistically indistinguishable.

DISCUSSION

The remainder of this report applies a quantitative perspective to problems of behavioral and neurophysiological response. We begin with some comparisons of pheromone emission rates from glass-tube dispensers and natural emitters, and apply the dispenser calibrations to interpretations of behavioral responses in a wind tunnel. Then we discuss how sensation magnitude theory combines the results of this and a number of other studies in the literature into a common framework.

One additional point must be addressed briefly, i.e., the choice of odorant concentration as the common parameter for interrelating the behavioral and neurophysiological responses. It has been noted elsewhere (Elkinton and Cardé,

TABLE 4. REGRESSION EQUATIONS FOR BEHAVIORAL AND EAG RESPONSES OF INSECTS TO BEHAVIORALLY ACTIVE CHEMICALS[a]

Insect	Bioassay key	Regression constant	Estimate	Standard error of estimate	F	R^2
A. mell. male	AMEC	A	2.38	0.001	9999.9	0.99
		B	0.38	0.0003		
A. mell. male	AMEQ	A	4.46	0.002	9999.9	0.99
		B	0.47	0.0002		
B. terr. female	BTBL	A	−0.30	0.01	13265.3	0.99
		B	1.66	0.01		
B. terr. female	BTV	A	2.63	0.21	219.5	0.99
		B	0.64	0.04		
B. mori male	BMWF	A	6.64	0.67	53.9	0.95
		B	0.36	0.05		
B. mori male	BME	A	1.91	0.17	126.8	0.98
		B	0.19	0.02		
D. mela. female	DMAA	A	1.35	0.11	210.5	0.99
		B	0.52	0.04		
D. mela. female	DMBA	A	1.01	0.04	747.7	0.99
		B	0.40	0.01		
D. mela. female	DMBL	A	1.29	0.09	256.1	0.99
		B	0.46	0.03		
D. mela. female	DMEA	A	0.86	0.04	710.9	0.99
		B	0.55	0.02		
D. mela. female	DMNL	A	2.59	0.23	132.7	0.99
		B	0.63	0.05		
D. mela. female	DMO	A	0.20	0.06	289.3	0.98
		B	0.36	0.02		
D. mela. female	DMOL	A	1.58	0.09	144.6	0.95
		B	0.36	0.03		
H. zea male	HZE	A	2.90	0.21	238.4	0.83
		B	0.39	0.03		
L. dispar male	LDWF	A	4.31	0.50	29.2	0.88
		B	0.18	0.03		
P. inter. male	PIBC	A	2.37	0.05	191.3	0.99
		B	0.09	0.006		
P. inter. male	PIBH	A	2.51	0.07	120.1	0.98
		B	0.08	0.007		
Sc. scol. female	SCEC	A	2.61	0.0005	9999.9	1.00
		B	0.54	0.0002		

TABLE 4. Continued

Insect	Bioassay key	Regression constant	Estimate	Standard error of estimate	F	R^2
T. ni male	TNB	A	2.16	0.05	95.1	0.96
		B	0.06	0.006		
T. ni male	TNE	A	2.01	0.06	1443.7	0.86
		B	0.26	0.007		

[a]The regression equations have the form: log(response) = $A + B$ log(concentration), where A and B are regression constants in the table. The concentration is specified in μmol/cm^3. Details of the response measures and references are specified in the bioassay keys in Table 5.

1984; Mankin and Mayer, 1983a) that an odor receptor neuron reacts to the rate of adsorption of odorant onto its receptor sites rather than to the odorant concentration. The rate of adsorption, however, is directly proportional to the concentration because the final approach of odorant molecules to the antenna is controlled by molecular diffusion over a thin boundary layer. The rate of adsorption (μmol/sec) is equal to the overall flux [μmol/(cm^2 sec)] times the collecting surface area for the receptor site (cm^2), which is the sensillum surface. The flux in turn is equal to the concentration (μmol/cm^3) times a parameter called the deposition velocity, which for pheromonal molecular diffusion is about 1 cm/sec (Mankin and Mayer, 1984). Because the receptor surface area and the deposition velocity vary negligibly relative to the concentration, it is convenient to use the more familiar parameter of concentration as the unit of stimulation.

Comparisons between Pheromone Emission Rates of Glass-Tube Dispensers and Natural Emitters. The evaporation of pheromone from a relatively inert surface like glass or from a pheromone gland both occur by similar physical processes; consequently, any differences in the range of release rates between glass dispensers and natural emitters is due primarily to differences in the exposed surface area. There are examples in the literature where the emission rate of pheromone from a female insect is comparable to the maximal emission from a glass-tube dispenser. The emission rate of Z-7 : 12Ac from *T. ni* virgin females has been measured at 3.1×10^{-5} μmol/min by Sower et al. (1971), 5.3×10^{-5}–9.7×10^{-5} μmol/min by Bjöstad et al. (1980), and 1.06×10^{-5} μmol/min by Baker et al. (1981). These rates are about an order of magnitude less than the maximum rates for the glass dispensers in Figures 1 and 2.

Another example where the maximal emission rate from a glass tube and from a female are likely to be similar is the measurement of the emission rate of (+)-*cis*-7,8-epoxy-2-methyloctadecane from *Lymantria dispar* L. females. Charlton and Cardé (1982) reported that the maximal emission rate from a virgin female was 3.8×10^{-3} μmol/min and the mean rate was 1.2×10^{-3} μmol/min. In contrast the pheromone emission rate from *P. interpunctella* females,

TABLE 5. LIST OF KEYS TO CURVES IN FIGURE 4 AND REGRESSION EQUATIONS IN TABLE 4.

Key	Reference	Chemical	Response Measure
AMBC	Schwarz (1955)	Caproic acid	50% Behavioral response
AMEC	Kaissling (1971)	Caproic acid	EAG (−mV)
AMEQ	Kaissling (1971)	*E*-2, oxo-9:10OOH	EAG (−mV)
BME	Kaissling (1971)	*E*,*Z*-10,12:16OH	EAG (−mV) from Figure 42
BMWF	Kaissling (1971)	*E*,*Z*-10,12:16OH	% Wing flutter
BTBL	Fonta and Masson (1984)	Butanol	EAG (−mV)
BTV	Fonta and Masson (1984)	Vanillin	EAG (−mV)
DMAA	Venard and Pichon (1984)	Amyl acetate	EAG (−mV)
DMBA	Venard and Pichon (1984)	Butyl acetate	EAG (−mV)
DMBL	Venard and Pichon (1984)	Butanol	EAG (−mV)
DMEA	Venard and Pichon (1984)	Ethyl acetate	EAG (−mV)
DMNL	Borst (1984)	3-Nonanol	EAG (−mV)
DMO	Venard and Pichon (1981)	Octane	EAG (% of butanol standard)
DMOL	Borst (1984)	3-Octanol	EAG (−mV)
HZE	This report	*Z*-11:16Al	EAG (−mV)
LDWF	Elkinton et al. (1984)	(+)-Disparlure	% Wing flutter
PIBC	Mankin et al. (1980)	*Z*,*E*-9,12:14Ac	% Upwind flight at 23°C
PIBH	Mankin et al. (1980)	*Z*,*E*-9,12:14Ac	% Upwind flight at 34°C
SCEC	Wadhams (1982)	α-Cubebene	EAG [% of (+)-*threo*-4-methyl-3-heptanol standard]
TNB	Mayer (1973)	*Z*-7:12Ac	% Upwind flight at 22°C
TNE	Mayer et al. (1984) and this report	*Z*-7:12Ac	EAG (−mV)

1.95×10^{-7} μmol/min (Sower and Fish, 1975), falls near the lower limit of the dispenser release rate calibration in Figure 1 at $10^{-6.7}$ μmol/min.

General Comparisons of Pheromone Concentrations in Plumes Emitted by Dispensers and in Plumes from Natural Emitters. It is somewhat misleading to compare the dispensers with the females solely on the basis of emission rate. Typically, the emitting surface of the dispenser is greater than the area of the pheromone gland, so the dispenser can have a higher overall emission rate than the gland. But the pheromone concentrations in the emitted plumes of the dispenser and the female cannot exceed a limit imposed by the saturated vapor concentration. This concentration limit is often at the upper levels of emission rates of either pheromone glands or pheromone dispensers.

Consider, for example, the case of a hemispherical pheromone gland with a radius between $r = 0.03$ and $r = 0.3$ cm. The maximum physically possible release rate, *ER*, can be estimated from the equation (Hirooka and Suwanai, 1976):

$$ER = 2\pi r D C_v \tag{2}$$

where D is the diffusion coefficient (about 0.04 cm^2/sec), and C_v is the saturated vapor concentration. Values for C_v can be estimated from the literature. Hirooka and Suwanai (1976) list saturated concentrations of 7.0×10^{-5} μmol/cm^3 for 12Ac and 2.8×10^{-5} μmol/cm^3 for 14Ac. Olsson et al. (1983) found the saturated vapor pressure for Z-7 : 12Ac to be 0.562 *Pa*. This can be converted to saturated vapor concentration by applying the Ideal Gas Law, $PV = nRT$: for 1 $Pa = 0.987 \times 10^{-5}$ atm, 1 atm $= 1.01 \times 10^5$ newton/m^2, and $R = 8.31$ newton m/°K/mol, the saturated vapor concentration at $T = 290$°K (= 20°C) is 2.30×10^{-4} μmol/cm^3 ($1 \times 10^{-3.64}$ μmol/cm^3). For 12Ac with $r = 0.3$ cm, this release rate is about $1 \times 10^{-3.0}$ to $1 \times 10^{-3.5}$ μmol/min, and for 14Ac with $r = 0.03$ cm, it is about $1 \times 10^{-4.9}$ μmol/min. The maximum possible release rate for *T. ni* females is near the measured release rate. Based on the above, we conclude that *T. ni*, but not *P. interpunctella*, females release sex pheromone at nearly the highest possible rate.

As with the female *T. ni*, the concentration of Z-7 : 12Ac in the plume from the glass dispensers approaches but does not completely reach saturation. When the release rate is $1 \times 10^{-2.0}$ μmol/min (about the upper limit in Figure 2), the concentration of Z-7 : 12Ac in the dispenser plume is 5×10^{-5} μmol/cm^3 at a dispenser flow rate of 200 cm^3/min, an order of magnitude less than the saturated vapor concentration of about 2.3×10^{-4} μmol/cm^3. The release rates of pheromone from *T. ni* and *L. dispar* females, and from the dispenser, are limited physically as the concentrations approach the saturation vapor concentration.

We expect that similar physical limitations occur for any dispenser operating at physiological temperatures. Thus, when the moth emits pheromone at its highest possible rate, the concentration in its emitted plume will be about

the same as in the plume from the most efficient artificial dispenser. The dispenser can release more pheromone into a larger volume, e.g., by increasing either the surface area or the air flow through the dispenser, but the concentration can be no higher than saturation. It is thus no surprise that calling insects often compete well with field traps.

Comparison of Anemotactic and EAG Response in a Wind Tunnel. The utility of combining dispenser calibration data with stimulus–response data for EAG and behavioral bioassays into a common interpretive framework is illustrated by a reexamination of the anemotactic flight bioassays of *T. ni* and *P. interpunctella* in Mayer (1973) and Mankin et al. (1980). In the original reports, data were presented for pheromone doses up to about 1 μmol (100 μg). This dosage is above the transition point where the emission rate from the dispenser becomes independent of dose (Figure 2). In these assays, the response to increasing doses had seemed anomalous because, in contrast with lower doses, the percentage of upwind flight to the pheromone source failed to increase with dose. On the assumption that the emission rate was proportional to dose, it was hypothesized that a qualitative change in the behavioral response had occurred, i.e., that the moths were induced to switch to a more intensive search pattern in the presence of high pheromone concentrations. Similar hypotheses have been proposed to explain reductions in pheromone trap captures at high pheromone concentrations (e.g., Helland et al., 1985).

Although the original hypothesis indeed may still be valid, the calibration results in this report suggest an alternate explanation for the stimulus–response curves in the *T. ni* and *P. interpunctella* bioassays. Both the behavioral and EAG responses level off when the dose is increased above a critical level. The apparent anomaly disappears when either measure of response is referenced to the actual pheromone concentrations.

General Comparisons of Behavioral and Neurophysiological Responses. To proceed into a more general discussion of interrelationships between insect behavioral and neurophysiological responses requires an assumption that usually has been applied only to vertebrates. The assumption is that a graded olfactory input leads to the production of a graded "sensation" in the CNS that mediates a graded output or behavioral response.

The idea of sensation has considerable importance for the interpretation of insect behavioral and neurophysiological responses because it can be used to generate a number of testable hypotheses. One hypothesis in particular, proposed by Stevens (1975), has been validated many times in vertebrate physiology. Stevens proposed that the magnitude of sensation, and consequently of behavioral response, is a power function of the stimulus intensity. One form of the power function is Eq. 1, which also can be expressed as (Mankin and Mayer, 1983b):

$$R = R_0(C - T)^b \tag{3}$$

where R is the chosen measure of response intensity, R_0, T, and b are regression constants, and C is the odorant concentration. The regression constant, T is analogous to a response threshold, and b is analogous to the conversion factor of an electrical transducer. The parameters T and b are of particular interest in comparisons across bioassays because they are independent of the method of measuring the response, and they may provide a measure of the transduction and central processes that result in behavior.

The power function relationship appears to be valid for all of the insect neurophysiological and behavioral stimulus–response relationships that we have measured or found in the literature, as can be seen in Figure 5. Because a number of different responses were examined, each with a different scale, we transformed the different responses in Figure 5 into a common vertical scale. The transformation procedure makes use of the fact that the concentration, C, and the exponent, b, in the power function (Eq. 3) are independent of the response measure. This permits the different responses to be compared on an equal basis by using a normalizing function (cf. Mankin et al., 1980):

$$\text{normalized log resp.} = \frac{\text{log resp.} - \text{min. log resp.}}{\text{max. log resp.} - \text{min. log resp.}} \tag{4}$$

It should be noted that stimulation must be given over the full range from zero to maximal response to obtain the denominator in Eq. 4. Otherwise the range will be truncated. The regression constants of the stimulus–response relationships in Figure 5 are shown in Table 4.

There are two patterns that appear upon inspection of Figure 5. One is that neurophysiological responses occur at concentrations no more than about two orders of magnitude above the behavioral responses. The second is that responses to the most behaviorally significant pheromone components and responses to other odors, such as host-plant compounds, fall into two distinct groups. Behavioral, EAG, and single-unit responses to sex pheromone components are in the range 1×10^{-9}–1×10^{-15} μmol/cm^3. Responses of EAGs to other chemicals are four or more orders of magnitude less sensitive. An estimate of the threshold can be obtained from the points where the regressions cross the axis. This is the point of 25% response (log = 1.4).

Interpretation of Relationships among Behavioral and Neurophysiological Responses. The two patterns of consistent relationships between behavioral and neurophysiological responses in Figures 3 and 4 may reflect some underlying similarities in the way these different insect olfactory systems operate. A simple model of the olfactory process can be constructed to explain these relationships from concepts of sensation and signal detection theory. The details of such a model are presented in Mankin and Mayer (1983a), Mayer et al. (1984), and Mayer and Mankin (1985).

In brief, the model comprises: (1) a number of detectors that individually

respond at a level that is a power function of the stimulus intensity; (2) a central processor that linearly sums the individual responses to give a sensation; and (3) a behavioral output that is proportional to the intensity of sensation. The summing at a hypothetical central processor explains how a behavioral response can be measured at stimulus levels where a corresponding response cannot be measured at a single receptor neuron, i.e., why the behavioral response can be detected at concentrations up to two orders of magnitude below the single-unit response. This idea is supported by experimental evidence that the pheromone receptor neurons converge into a macroglomerulus from which interneurons emerge that respond to peripheral stimulation at levels below what can be detected in single-unit recordings (Boeckh and Boeckh, 1979).

The EAG fits into the model as a correlative of the summed input to the central processor. The EAG is presumed to be a bulk measure of the summed generator potentials that drive the action potential responses of the receptor neurons; consequently, the EAG is predicted to be a power function of the stimulus intensity (Mayer et al., 1984).

If there were no uncertainties in the detection of the odorant or in the behavioral and neurophysiological measures of the response, the different behavioral and neurophysiological response curves for each insect in Figure 5 would merge; i.e., they would simply be different but equivalent measures of the hypothetical curve for sensation intensity. However, there are many internal uncertainties in the insect's detection of the stimulus, as well as external measurement uncertainties in behavioral and electrophysiological bioassays. According to signal detection theory, increases in the level of uncertainty will shift the response curve to higher stimulus levels (Green and Swets, 1974). Consequently, we can consider each of the response curves in Figure 5 to represent a curve for sensation intensity that has been shifted to the right by an amount that depends on the external and internal uncertainties. The curve for a single olfactory neuron will be shifted, for example, partly because summation occurs at the central processor. The EAG curve will be shifted because of electrical losses in the antenna that decrease the sensitivity of external measurements.

We can apply the model to two sets of data in Figures 3–5 where EAG and behavioral responses to pheromone were measured. For convenience, consider the horizontal intercept of each response curve to be a type of threshold (although this is not strictly equivalent to T in Eq. 3). According to the model, the EAG thresholds for both *T. ni* and *B. mori* are higher than the behavioral thresholds for pheromone because of differences in the (combined) external and internal uncertainties.

Next, suppose that these relationships hold for other insects. If the activation or the flight response of *H. zea* to *Z*-11:16Al correlates with the neurophysiological response in the same way that the behavioral and neurophysiological responses to Z-7:12Ac correlate in *T. ni*, then the behavioral threshold for *H. zea* should be about 1×10^{-12} μmol/cm^3, the apparent threshold for *T.*

ni. The correlation seems plausible because the measurement procedures and uncertainties are similar. The EAG curve for the queen substance, a sex pheromone component of *A. mellifera* (Kaissling, 1971), is similar to the curve for *T. ni*. Thus, it can be expected that the behavioral response occurs at about 1×10^{-12} μmol/cm^3 also.

Finally, we can apply the idea of sensation to interpretation of the EAG responses to non-pheromonal compounds. In Figure 5, EAGs for non-pheromonal compounds do not begin to occur until much higher concentrations than for pheromones, 1×10^{-6}–1×10^{-4} μmol/cm^3. By analogy with the responses for pheromones, behavioral thresholds for these chemicals perhaps occur at concentrations no lower than two orders of magnitude below the EAG threshold, about 1×10^{-6} μmol/cm^3. The measured behavioral threshold of *A. mellifera* to caproic acid (Schwarz, 1955), AMBC in Figure 5, is in general agreement with such a hypothesis.

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MODIFICATION OF OLFACTORY-RELATED BEHAVIOR IN JUVENILE ATLANTIC SALMON BY CHANGES IN pH

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Abstract—The hypothesis that low pH modifies the response of salmonids to certain olfactory stimuli was tested. An interactive video–computer system was used to monitor the behavior of juvenile Atlantic salmon (*Salmo salar*). At a pH of 7.6, animals were attracted to glycine and avoided L-alanine. These effects were dose-dependent, with a threshold of 10^{-7} M. The response of the fish to both amino acids changed when the pH of the test chamber was gradually lowered from 7.6 to 5.1; they became attracted to L-alanine and indifferent to glycine. These effects were reversible with a return to pH 7.6. Our findings suggest that acid rain may contribute to reductions in salmonid populations in acidified rivers by impairing the recognition of olfactory cues by salmon during their spawning migration.

Key Words—Acid rain, salmon, *Salmo salar*, pH, olfaction, computer, video, migration, glycine, L-alanine.

INTRODUCTION

Acidification of rivers and streams throughout Europe and North America has been implicated in the decline of anadromous fish populations (Wright et al., 1975; Leivestad et al., 1976; Schofield, 1980). Population decreases have even been observed in rivers where the pH is above levels considered to be lethal[1] (Haines, 1981; Muniz, 1981; Watt, 1981; Watt et al., 1983). Many factors associated with moderately acidified waters may play a role in reducing or, in some cases, eliminating the recruitment of new animals into the population

[1] The lethal pH level for adult Atlantic salmon is 4.5, parr, 4.0, eggs, 3.5 (Daye and Garside, 1977).

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(Neville, 1979a,b; Daye and Garside, 1980; McDonald, 1983; McDonald et al., 1983). We have been testing the hypothesis that changes in pH alter salmonids' sense of smell and thus may modify their normal migratory behavior.

It is widely accepted that the freshwater spawning migration of salmonids, like many species of anadromous fishes, is controlled to a great extent by olfactory cues in the environment (Hasler, 1957, 1960a,b; Groves et al., 1968). If the olfactory sense of these animals is altered due to a moderate reduction in the pH of the water (pH 4.5–6.5), migrating fish may not be able to respond appropriately to necessary olfactory cues (Sutterlin, 1974; Fritz, 1980). Although there are some field data available which suggest that adult salmon halt upstream spawning migration and may return downstream when presented with copper and zinc pollution (Saunders and Sprague, 1967), little data are presently available concerning the effect of acidic precipitation on homing behavior or salmonid olfaction (Hara, 1976b; Sutterlin, 1974). We have examined the behavioral response of juvenile Atlantic salmon to amino acids and taurocholic acid, and the modification of their responses by changes in the ambient pH. Our results indicate that pH does have a significant impact on the behavioral response of salmonids to certain olfactory stimuli, and thus it may be a significant factor contributing to the reductions in salmonid populations reported in many moderately acidified rivers.

METHODS AND MATERIALS

Parr-stage Atlantic salmon (*Salmo salar*), approximately 15–20 cm long and 10.0–15.0 g in weight were used in all experiments. Fish were obtained from the Milford State Hatchery in Milford, New Hampshire. They were held in closed-recirculating holding systems at 10°C. Water quality was checked weekly, and the water source was the same as that used in the behavioral experiments.

A two-channel preference–avoidance chamber (Figure 1) that included a common area (Greer and Kasokoski, 1978) was used to monitor the behavioral responses of the juveniles to specific olfactory stimuli. At the beginning of each experiment, four fish were placed in the common area for a 15-min acclimation period. The experiment was initiated by removing the barrier and allowing the animals to explore the chamber (Figure 1). The pH was monitored throughout the experiments with an Orion pH meter equipped with an automatic temperature compensation probe. The response to specific olfactory stimuli was determined by recording movements on videotape and then converting these movements to digital form using an interactive video–computer system designed in our laboratory (Royce-Malmgren and Watson, 1986). To create a digitized track of the fish, the videotaped experiment was viewed on a high-resolution color monitor with computer generated targeting cross hairs overlayed on the image of the chamber by an Ambico special effects generator (ASEG, Figure 1). The

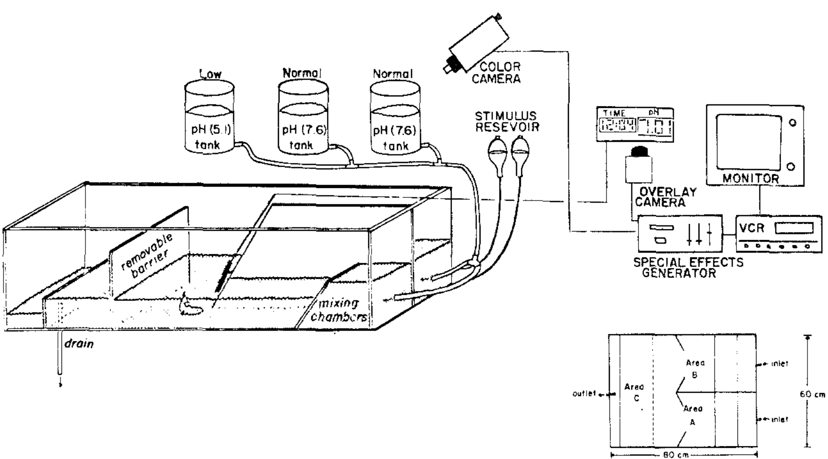

FIG. 1. Test chamber and video–computer data collection system. Fish were introduced into area C of the test chamber, behind the removable barrier. Dechlorinated, aerated tap water (pH 7.6) from overhead storage tanks flowed continuously through the chamber, at a rate of 3 liters/min. Following an acclimation period, the removable screen was lifted to start the experiment. During the control period, no olfactory stimulus was added. During the test period, stimulus was added to area A or B from the stimulus reservoirs. The concentration of stimulus declines as the water from area A and area B mix in area C. This creates a concentration gradient around the tank from the stimulus input to the opposite side. This gradient has been confirmed using dyes with fish present. After 30 min, pH was reduced over a 15-min period, using water from overhead storage tanks whose pH was lowered with a solution of 50% H_2SO_4 and 50% HNO_3 (0.5 M) to simulate the approximate composition of acid rain. Following the acidification, the water in the overhead holding tanks was thoroughly aerated to drive off excess CO_2 and equilibrate O_2 levels between tanks. The pH was maintained for 30 min and then returned to the original level over another 15-min period and held at that level for the remaining 30 min. The pH and temperature of the water were monitored throughout the experiment and stored on videotape by means of an overlay camera. Other physical parameters were determined at the end of the experiment and recorded on the videotape. A Hitachi/Everex model 8A color video camera mounted above the chamber was used to record the experiment, and this video signal along with that of the overlay camera were combined by a special-effects generator and transmitted to a Panasonic NV-8399 video casette recorder (VCR) for permanent storage. Each fish carried a color coded tag for identification.

targeting cross hairs, controlled by the pen of a digitizer tablet, were used to track individual fish in the experiment.

The computer calculated x–y coordinates, based on information from the digitizer tablet and individual test chamber calibration factors. These coordinates were recorded on data disks at time intervals specified by the technician. The pH information for each experiment was entered into a separate data disk

file. Stimulus concentration, channel with stimulus, and other pertinent information[2] about the experiment were added to the end of the tape to ensure that the technician analyzing the experiment was unaware of the presence, location, quality, and quantity of the stimulus. The digitized tracks of individual animals were plotted and the animal's distance from the source of stimuli, time per area of chamber (both measures of attraction or avoidance) and swimming velocity were measured, at user-determined intervals.

Amino acids have been used successfully to study the olfactory system of various fish (Idler et al., 1956, 1961; Sutterlin and Sutterlin, 1971; Hara, 1973, 1976a) and crustacean chemoreception (Mackie and Shelton, 1972; Derby and Atema, 1978). Initially, we tested the following seven amino acids to determine if they would elicit a consistent response from the Atlantic salmon in our test chamber: L-alanine, glycine, L-leucine, L-serine, L-cysteine, L-glutamine, and L-methionine. All amino acid solutions were prepared fresh daily with double-distilled, deionized water in acid-washed glassware. These compounds were chosen for two reasons: (1) when applied to the nares of Atlantic salmon (*Salmo salar*), they produce consistently large responses recorded from the olfactory bulb (Sutterlin and Sutterlin, 1971); and (2) certain of these amino acids are known to elicit behavioral responses in fish (Hoese and Hoese, 1967; Sutterlin and Sutterlin, 1971; Caprio, 1977; Goh et al., 1979; Goh and Tamura, 1980; Atema et al., 1980) and a variety of other animals (McLeese, 1973; Fuzessery and Childress, 1975; Allison and Dorsett, 1977). Taurocholic acid was also screened due to its implication as a naturally occurring olfactory attractant for Arctic char (*Salvelinus alpinus*) and other salmonids (Doving et al., 1980; Stabell, 1984).

Nasal occlusion experiments were conducted to determine the sensory modality involved in the observed behaviors. After running a screening experiment under normal conditions with a stimulus concentration of 10^{-4} M, or 10^{-6} M, the fish were removed and two of the four fish had their nares occluded with Vaseline. The other two fish were sham controls and had distilled water injected into their nares. All four fish were allowed to recover for 1 hr and retested under circumstances identical to the initial testing. This protocol was repeated with five groups of four fish.

The data were analyzed using Student's *t* tests and chi-square tests. Chi-square tests for homogenicity of replicates were performed and accepted for replicates of all tests with the four compounds that elicited reproducible behavioral responses; thus replicates were pooled. Two way analyses of variance (ANOVAs) were performed on the velocity data in order to determine homogenicity of activity levels both between experimental protocols and between fish within a single experiment.

[2]Physical properties of the water. Temperature 10°C ± 1°, Ca^{2+} concentration 6.8 ppm ± 2.3; alkalinity 12.3 ppm ± 3.2.

RESULTS

Eight compounds were screened to determine if they had an effect on the behavior of salmonids. Fish were attracted to glycine ($>10^{-6}$ M). L-alanine, L-glutamine ($>10^{-6}$ M), and taurocholic acid ($>10^{-6}$ M), all evoked significant ($P \leq 0.001$) avoidance responses when compared to controls. Taurocholic acid elicited the strongest avoidance response followed by L-glutamine and then L-alanine. L-Leucine, L-methionine, and L-cysteine elicited no detectable response (neither attraction or avoidance), and L-serine elicited inconsistent behavior.

We conducted most of our experiments with one compound that produced attraction at a normal pH (pH 7.6, glycine, Figure 2A) and one that fish avoided at a normal pH (pH 7.6, L-alanine, Figure 2B). When glycine was added to one of the channels in the test chamber, fish were attracted to the area of the test chamber containing the highest concentration of this amino acid (Figure 2A). The threshold for this effect was 10^{-7} M ($P \leq 0.001$, Figure 2A, bottom). As the concentration of glycine was increased, the preference of the fish for the side of the chamber containing glycine also increased, up to a maximal preference response at a concentration of 10^{-4} M glycine ($P \leq 0.001$, Figure 2A, bottom). In contrast, L-alanine evoked an avoidance response. The threshold for this effect and the concentration that gave maximal avoidance were very similar to the behavioral threshold and maximal preference response obtained with glycine (Figure 2B).

After having their nares occluded with vaseline, fish which had previously been attracted to glycine spent equal amounts of time in all three areas of the test chamber. Therefore, the amino acids required access to the olfactory receptors in the nares in order to elicit behavioral responses (Figure 3).

The behavioral response of the salmon to L-alanine and glycine changed markedly when the pH of the test chamber was gradually lowered from 7.6 to 5.1 (Figure 4). The initial attraction of fish to glycine changed to a neutral response (Figure 4A). Furthermore, fish that avoided L-alanine at a normal pH were attracted to L-alanine at an acidic pH of 5.1 (Figure 4B). The effect of pH was reversible in all experiments.

Behavior was not altered in response to changes in pH alone. The behavioral parameters of fish in near-neutral water (pH 7.6) was comparable to that in acidic water (pH 5.1, Table 1). When acidic water (pH 5.1) was perfused into one side of the test chamber and near-neutral water (pH 7.6) was perfused into the other side, fish displayed area preference behavior comparable to that observed under control conditions, when identical water was entering both channels (Table 2). In both cases, there was no significant difference in the behavioral parameters of the fish in near-neutral (pH 7.6) and acidic (pH 5.1) water.

The fish did not appear to prefer one area of the chamber over another

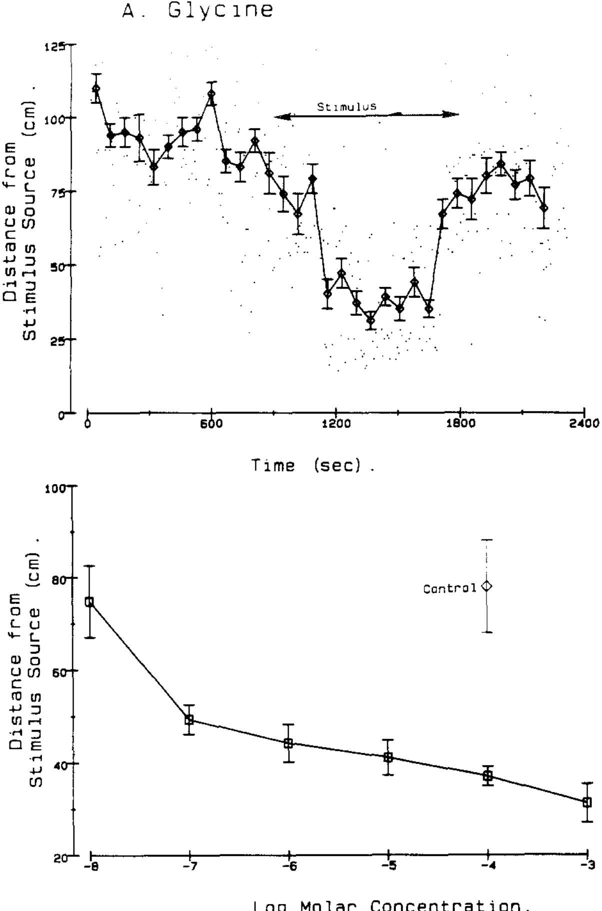

FIG. 2. Response of fish to specific amino acids. (A) Top: Response of eight fish to glycine (10^{-5} M, stimulus bar). Distance from the stimulus is used as an indication of preference for, or avoidance of, a particular olfactory stimulant. Diamonds ($\pm$SEM) are means of eight fish, averaged over 200 sec. Dots are mean values of eight fish, averaged every 9 sec. Prior to stimulant introduction, fish did not show a preference for either side of the chamber, but when glycine was added to one side, the fish tended to move into that area and remain there for longer periods of time. Bottom: The attraction to glycine was dose-dependent, with a threshold of between 10^{-8} M and 10^{-7} M (N = 6–10 fish/concentration). (B) Top: Response of eight fish ($\pm$SEM) to L-alanine (10^{-5} M). The fish showed no preference for either side of the chamber until L-alanine was added. Fish then moved away from the source of the stimulus. Bottom: The avoidance response was dose-dependent (N = 6–10 fish/concentration).

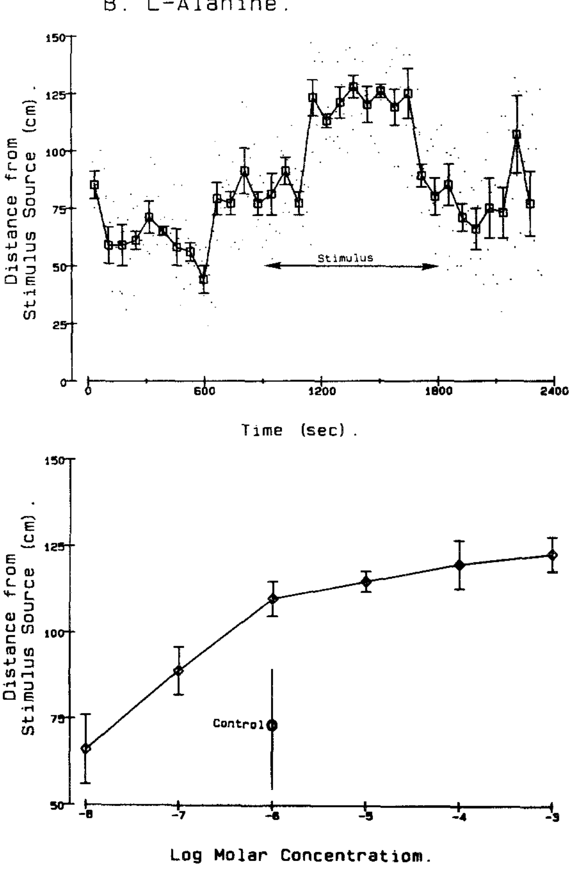

FIG. 2. Continued

under all control conditions, and we randomly introduced stimuli into one of the two input areas (A or B) during our experiments (Figure 1, see stimulus source arrows Figure 4A and 4B, top). In addition, each fish appeared to behave as an individual, rather than as a member of a school. This was apparent from visual inspection of the videotaped experiments, and it was confirmed statistically using a two-way ANOVA analyzing the velocity of all four fish at different pH levels in each pH modulation experiment. While there was no significant variation in velocity for individual fish between pH levels, there were significant ($P \leq 0.05$) differences between velocities of different fish at the same pH level, thus demonstrating that no schooling was occurring.

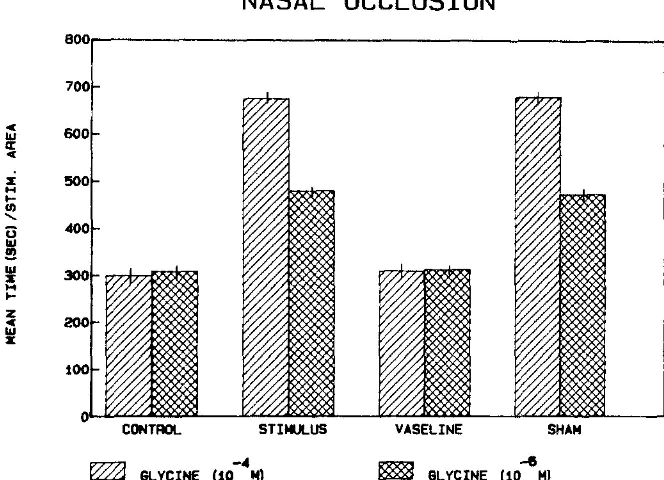

FIG. 3. Response of fish to glycine with and without nares occluded using Vaseline. Under control conditions, with no stimulus present, fish moved randomly about in the test chamber. When glycine was present (stimulus) fish spent more time ($\pm$SEM) in the area of the chamber with the highest concentration of glycine. When those same fish had their nares occluded with Vaseline (Vaseline), they behaved as if no stimulus was present. Sham (sham) control fish, with distilled water injected into their nares rather than vaseline, responded to glycine in a normal manner. At both concentrations of glycine, the responses of the Vaseline occluded and control fish were significantly ($P \leq 0.001$) different from the stimulus and sham control fish ($N = 20$).

DISCUSSION

To our knowledge this is the first demonstration that changes in the pH of surface waters, comparable to those documented in nature, are capable of altering the "normal" behavioral response of fish to olfactory cues. The degree to which our findings pertain to fish migration and behavior in the field remains to be determined. One major problem is that the role of specific substances in fish olfaction and homing is not well understood. Overall, with few exceptions, the specific significance of free amino acids to olfactory-related behavior is not known, and the importance, if any, that amino acids play in salmonid fresh water migration has yet to be demonstrated. The environmental cues which make home stream water unique so that returning adults recognize it and departing smolts imprint to it are at best only vaguely defined. It is considered by many to be heat-labile, volatile, neutral, and dialyzable (Ueda et al., 1967; Hara et al., 1973; Hara, 1976a; Idler et al., 1961; Bodznick, 1978). Others believe a nonvolatile fraction to be important (Atema et al., 1973). Amino acids,

usually in mixtures, can elicit specific behaviors such as exploration and feeding in the goby (*Gobiosoma bosci*) (Hoese and Hoese, 1967) and avoidance by Pacific salmon (Idler et al., 1956, 1961). However, the degree to which amino acids influence the homing behavior of salmonids is not known.

We chose to use amino acids for olfactory stimuli because previous electrophysiological data indicated that the olfactory system responded to certain amino acids in a dose-dependent manner and, in our hands, they gave us reliable, quantitative, avoidance–attraction responses. The dose–response curves that we obtained using our behavioral assay are similar to those determined with the same compounds using electrophysiological techniques (Hara, 1973). Experiments are presently underway to determine how pH affects the behavioral response of salmonids to natural stimuli, such as conspecific odors and home-stream water.

The differential responses of Atlantic salmon to the same compound at two different pH levels is probably the result of a modification of some component of the olfactory system. The level at which this change takes place is not clear, but it may occur at any one, or a combination of levels; receptor site alteration, stimulus molecule charge distribution, or interaction between the two. These receptors are thought to be membrane proteins with anionic and cationic binding sites (Hara, 1982). Hara (1976b) used olfactory bulb recordings to monitor the response of adult rainbow trout (*Salmo gairdneri*) to numerous amino acids at different pHs. He recorded maximal responses to individual amino acids at their isoelectric points (both glycine and L-alanine have isoelectric points of 6.02; Lehninger, 1982), indicating changes in charge may interfere with normal amino acid-receptor interactions. Since the olfactory receptors are exposed to the environment, with limited protection from a permeable mucus layer, they are subject to changes in the external environment (Brown et al., 1982). The fact that our pH effects were reversible suggests that at pH 5.1 the receptors were not permanently damaged. The manner in which one or more of the components of the olfactory system are consistently and reversibly altered by pH, both in electrophysiological (Hara, 1976b) and behavioral studies, remains to be determined.

Our investigations thus far have been limited to juvenile salmonids. We have tested chinook salmon (*Oncorhynchus tshawytscha*) juveniles as well as Atlantic salmon juveniles, and their responses were similar in all cases. Although we do not presently know the extent to which our data on juveniles pertain to adults, it has been shown that salmon parr have the ability to discriminate between odors in much the same way as adult salmon (Stabell, 1982, 1984), and our preliminary data indicate that pH influences the way adult salmon respond to taurocholic acid (Royce-Malmgren and Watson, in preparation). If low pH alters the manner in which adult fish respond to the odor of their home stream water in the same way it modifies the olfactory-related behavior of ju-

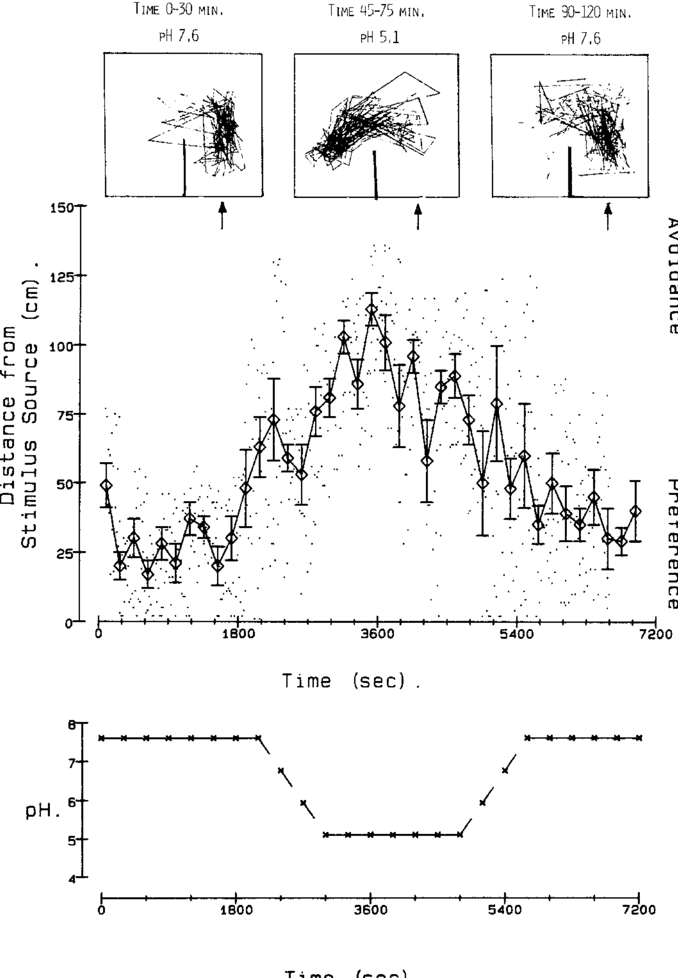

FIG. 4. Modification of the behavioral response of Atlantic salmon to amino acids by pH. (A) Response to 10^{-4} M glycine while the pH of water in the test chamber was changed from 7.6 to 5.1 and then back to 7.6. Top: The track of an individual fish during the course of the experiment. Arrows indicate the source of the glycine. During the initial 30 min of the experiment, the fish spent most of its time in the side of the chamber containing the highest concentration of glycine. However, when the pH was gradually lowered to 5.1 (see bottom graph), the fish moved randomly about the chamber. When the pH was returned to 7.6, the fish was again attracted to the glycine. Center: The aforementioned experiment was repeated with 20 different fish and the results were

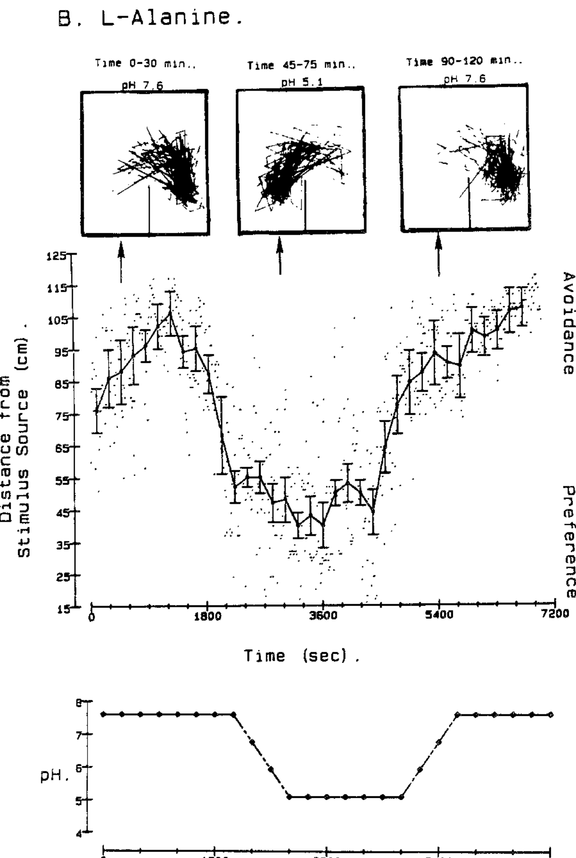

FIG. 4. Continued tabulated and plotted. Diamonds ($\pm$SEM) are means of 20 fish, averaged over 200 sec. Dots are mean values of 20 fish, averaged every 9 sec. (B) The response to L-alanine (10^{-4} M) during pH changes. Center: At a pH of 7.6, the fish avoided the L-alanine, but when the pH (see bottom graph) was lowered to 5.1, they were attracted to the stimulus. Note that in the track of the fish shown, the L-alanine was introduced into the left-hand side of the test chamber (in contrast to the glycine experiment shown in A, top). The side of the chamber used for the stimulus source was varied randomly for each set of experiments. The tracks also show that the activity of the salmon remained fairly constant throughout the course of the experiment.

TABLE 1. COMPARISON OF BEHAVIORAL PARAMETERS OF ATLANTIC SALMON IN ACIDIC (pH 5.1) VS. NEAR-NEUTRAL (pH 7.6) WATER (N = 20)

	pH 5.1	pH 7.6
Mean velocity (cm/sec)	7.5 ± 1.3[a]	7.7 ± 1.1
Mean distance (cm to stimulus source)	75.9 ± 1.5	74.9 ± 1.5
Mean time (sec)/area with stimulus	592.5 ± 13.5	622.0 ± 16.8

[a] Standard error of means.

TABLE 2. RESPONSE OF ATLANTIC SALMON PARR TO ACIDIC (pH 5.1) WATER (N = 20)

Control[a]		Experimental	
Area	Time (sec)/area	Area	Time (sec)/area
A	614 ± 15.8[b]	A (pH 5.1)	592 ± 11.6
B	604 ± 10.4	B (pH 7.6)	589 ± 14.9
Common	580 ± 18.9	Common	619 ± 12.2

[a] The pH was 7.6 throughout the test chamber.
[b] Standard error of means.

venile salmon, then their ability to locate a suitable spawning site may be affected. As a result, fish would become dispersed and spawn in areas unsuitable for the development of eggs, fry, and juveniles. This could eventually lead to a reduction in recruitment and a decline in the salmonid population, even at the nonlethal pH levels now common in many New England and Canadian rivers.

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STEREOISOMERS AND ANALOGS OF 14-METHYL-1-OCTADECENE, SEX PHEROMONE OF PEACH LEAFMINER MOTH, *Lyonetia clerkella*, L.[1]

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Abstract—The synthesis of the enantiomeric 14-methyl-1-octadecenes in >99% EE is described. Enantiomeric 2-methyl-1-hexanols were intermediates in the synthesis. The 1-alkene had been previously identified as the sex pheromone of the peach leafminer moth. Several closely related structures that have Δ12 unsaturation are also described.

Key Words—Chirality, alkenes, synthesis, branched hydrocarbons, diastereomeric amides, resolution, enantiomers, optical purity, olefin inversion.

INTRODUCTION

The peach leafminer, *Lyonetia clerkella* L., is one of Japan's most serious orchard pests (Naruse, 1978). Because larval damage occurs within the leaves, the larvae are not exposed to the usual contact sprays. This results in an emphasis on spray timing, a situation wherein optimal effectiveness of conventional insecticides is compressed into a short time window with attendant un-

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certainties. The situation might be relieved if one could monitor the adult moth population. This could be done by use of a pheromone lure trap. The sex pheromone of the female peach leafminer moth then became the subject of an investigation that led to the isolation and identification of 14-methyl-1-octadecene from female rinses (Sugie et al., 1984). About 100 ng of this compound can be obtained per female moth, and traps baited with 100 μg of the synthetic alkene were as attractive as the crude extract of 40 females.

Assignment of absolute configuration of the pheromone by inference using candidate synthetics remained. This report describes synthetic details for the preparation of the enantiomers of 14-methyl-1-octadecene each $>99.6\%$ pure (99.2% EE). The route chosen also allows generation of analogs that have the same stereocenter with a slight structural alteration adjacent to that center to employ as probes for the effect of selected physicochemical variables on the biological activity. In this initial trial, we opted to place unsaturation at the 12,13-position because we were of the opinion that such an alteration of the pheromone structure would constitute minimal perturbation. Since this research was initiated, a synthesis of 14-methyl-1-octadecene has been reported (Mori and Kato, 1985).

METHODS AND MATERIALS

Gas–liquid chromatography (GLC) was performed with a Varian 2400 instrument (flame ionization detection, He carrier) using the following columns: column A, cholesterol *p*-chlorocinnamate (0.25 mm × 11 m) and column B, Durabond I (0.25 mm × 31 m) operated at temperatures as indicated. Infrared (IR) data were obtained with a Perkin Elmer model 467 spectrophotometer (3% solutions in CCl_4), and nuclear magnetic resonance (NMR) data were obtained with a Nicolet 300-MHz FTNMR spectrometer (1% solutions in $CDCl_3$). Mass spectral data were obtained with a Finnigan model 3200 mass spectrometer in either electron impact or chemical ionization (isobutane) operating mode. The mass spectrometer was equipped with a GLC inlet (Varian model 1400) served by an OV-101 column (0.25 mm × 31 m). Melting points were obtained with a Fisher-Johns hot-stage apparatus and are uncorrected.

2-Methylhexanoic Acid. The acid was synthesized by alkylation of the dilithio anion of propanoic acid with *n*-butyl bromide by the general method of Pfeffer and Silbert (1970) (Figure 1). The yield of the racemic acid from 0.20 mol of propanoic acid was 94.4%: bp 70–73°C (0.05 mm); IR (CCl_4) 1706 cm^{-1}; ($CDCl_3$) 0.89 (bt, 3H, CH_3R); 1.18 (d, 3H, CH_3CH); 1.25 (CH_2 envelope); 10.15 (s, 1H, CO_2H) ppm (prior lit. Creger, 1970).

Preparation and Resolution of Diastereomeric Amides, 2. Both (R)- and (S)-α-methylbenzylamines were purified by recrystallization of monotartrate salts from ethanol and analyzed for configurational purity as the first step of a

resolution procedure that has been previously described (Sonnet, 1984). Acid **1** was converted to its acid halide with $SOCl_2$ and DMF catalyst in anhydrous ether. The acid halide was allowed to react with (R)- or (S)-α-methylbenzylamine in CH_2Cl_2 with 1.1 equivalent of triethylamine. The amides were recrystallized four to five times from aqueous ethanol using charcoal treatment during first recrystallization. The yields of purified R^*S^* diastereomers was 40% of theoretical and each diastereomer was 99.6% pure. Analysis by GLC: col A (120°C) α = 1.098, R^*R^* diastereomer eluted first; col B (150°C) = 1.077, R^*R^* diastereomer eluted first also; mp (R^*S^*): 98–99°C; IR (CCl_4) 3460, 1680 cm^{-1}; NMR ($CDCl_3$) δ0.89 (bt, 3H, CH_3R), 1.11 (d, 3H, J = 6.9 Hz, $C\underline{H}_3CHC{=}0$), 1.49 (d, 3H, J = 6.9 Hz, $C\underline{H}_3CHN$), 2.14 (sextuplet, 1H, J = 6.9 Hz, $CH_3C\underline{H}CH_2$), 5.15 (p, 1H, J = 7.1 Hz, CH_3CHNH), 7.2 (5H, aryl H) ppm; CI-MS (m/e) 234 (M+1).

(R)- and (S)-2-Methyl-1-hexanol, 3. Diastereomerically pure amide **2** (7.1 g, 30.5 mmol) was added via Gooch tubing in portions to a reaction mixture consisting of lithium diisopropylamide (LDA) (45 mmol) in tetrahydrofuran (THF) (75 ml) under nitrogen and cooled to 0°C. The resulting mixture was stirred for ca. 0.25 hr without external cooling. The reaction mixture was then cooled to −30°C, and ethylene oxide (3.0 ml, 60 mmol) was injected. Hexamethylphosphoric triamide (HMPT) (16 ml) was injected, and the resulting mixture was stirred at ambient temperature overnight. The reaction mixture was worked up with 2 N HCl (100 ml) and ether in the usual manner. The crude N-2-hydroxyethylated amide was dissolved in THF (40 ml) containing 2 equiv conc. HCl and heated under reflux for 1.5 hr, at which time GLC analysis indicated absence of the starting material, i.e., transacylation to the aminoester was complete. The mixture was stripped of solvent and heated briefly in benzene to drive off residual water. After the benzene had been removed, the residue was dissolved in dry THF (20 ml) and 2–2.5 g of solid LAH were added. The mixture was stirred under reflux for 1 hr and then worked up with aqueous NaOH and ether. The ethereal layer was suction filtered through Celite accompanied by further ether washes of flask and precipitate. The combined ethereal layer was washed with 2 N HCl, H_2O (2×) and dried ($MgSO_4$). Distillation of the crude product gave 3.1 g of **3** (88%): bp 84–101°C/30 mm; IR (CCl_4) 3560 cm^{-1}; NMR ($CDCl_3$) δ0.90 (m's, 6H, CH_3R and $C\underline{H}_3CH$), 1.1–2.0 (m's, ca. 6H), 3.41 and 3.51 (2d of d's, ABX, 2H, $CHC\underline{H}_2OH$)ppm; (R) $[\alpha]_D^{25}$, −9.3° (c, 8.10, $CHCl_3$); (S) $[\alpha]_D^{25}$, +8.7° (C,8.15,$CHCl_3$).

1,12-Dodecanediol Monotetrahydropyranyl Ether, 4. The diol (7.0 g, 34.6 mmol) was dissolved with warming in 1,2-dichloroethane (DCE) (70 ml). A crystal of p-toluenesulfonic acid was added, and then dihydropyran (3.3 ml) was added dropwise. After 0.25 hr, the mixture was stripped and triturated with hexane. The mixture was suctioned filtered, and the recovered solid diol was treated again with DCE and a proportional amount of dihydropyran. The product mixture was again stripped of DCE, slurried with hexane, and filtered. The

combined filtrate from both reactions was freed of solvent and chromatographed on silica gel (60–120 mesh, 30 g) eluting with 200 ml of 5% ether–hexane [bistetrahydropyranyl ether (THP) and some mono-THP] and 300 ml of 10% ether. GLC analysis: col A (200°C) compound (R_t min), diol (1), mono-THP (2.2), bis-THP (9.5). In this fashion **4** (2.7 g, 27.3%) was obtained containing ca. 3% of diol and no bis-THP; the recovered materials may, of course, be recycled, although no effort was made to optimize yields. Thin-layer chromatography (silica gel, 30% ether-hexane): R_f 0.16 mono-THP, 0.5 bis-THP; IR (CCl_4) 3560, 1050 cm^{-1}; NMR ($CDCl_3$) δ 1.25 (CH_2 envelope), 3.5 (m, 6H, CH_2O), 4.09 (m, 1H, OCHO) ppm.

(R)- and (S)-14-Methyl-12-(Z)-octadecen-1-ol, THF Ether, **5**. The diol-mono-THP, **4,** (4.71 g, 16.5 mmol) was added to a stirred mixture of pyridinium chlorochromate (5.3 g, 24.7 mmol) and NaOAc (5.3 g) in CH_2Cl_2 (30 ml) that was cooled in an ice bath. The resulting mixture was stirred at room temperature for 2 hr. Ether (150 ml) was added, and the mixture was passed through Florisil (10 g). The eluant was concentrated and then chromatographed on silica gel (25 g) eluting with 5% ether–hexane. Concentration of the eluant provided crude aldehyde (GLC 90%, col A 200°C, R_t 1.6 min, principal contaminant was the starting alcohol).

The required phosphonium salt (Figure 1) was prepared from (*R*)- and (*S*)-2-methyl-1-hexanol by first converting the alcohol to a bromide. Triphenylphosphine dibromide (25 mmol) was prepared in the usual way in CH_2Cl_2 adding bromine to triphenylphosphine at 0–25°C. The alcohol (3.0 g, 20.8 mmol) was added with 1–2 ml CH_2Cl_2 rinse, and the mixture was stirred at ambient temperature for 16 hr. After a few drops of methanol had been added to consume unreacted dibromide, the mixture was concentrated on a flash evaporator. The residue was triturated with hexane several times, and the hexane solution was concentrated. The crude bromide was then passed through silica gel (20 g) with hexane. The eluant was concentrated, and the bromide thus obtained was treated with triphenylphosphine (5.7 g, 21.8 mmol) and NaI (8.2 g, 54.6 mmol) in acetonitrile (25 ml) under reflux for 64 hr. The solvent was removed, and the crude product was triturated several times with DCE (phosphonium iodide is soluble). The triturate was concentrated and washed with ether. The residual oil was slurried in heptane and the solvent removed by rotary evaporation (to remove traces of DCE). The yield of crude oily salt was near quantitative.

The oil (ca. 20.8 mmol of phosphonium iodide) was dissolved in dry THF (25 ml) under nitrogen. The mixture was cooled (ca. 0°C) and *n*-butyllithium (1 equiv) was injected to generate the ylid. The mixture was cooled (−78°C) and HMPT (2 equiv) was injected. The aldehyde prepared above from **4** (2.6 g, 9.1 mmol) was added and the mixture was stirred at −78°C for 1 hr and for another 1 hr without external cooling. The product was worked up with water and hexane in the usual manner. The crude product was chromatographed on silica gel (30 g) eluting with 100 ml each of hexane, 1% then 2% ethyl acetate–

hexane. The product, **5**, was obtained: 2.23 g (67% yield from alcohol **4**); GLC Col B (250°C) $k' = 2.27$, containing 8% E isomer $k' = 2.40$, $\alpha = 1.057$; TLC (5% ethyl acetate–hexane $R_f = 0.28$); IR (CCl_4) 1050 cm^{-1}; NMR ($CDCl_3$) δ0.88 (m's, 6H, CH_3R and $C\underline{H}_3CH$), 1.25 (CH_2 envelope), 1.6 (m, ca. 3H, allylic CH), 3.5 (m, ca. 4H, CH_2O), 4.1 (m, ca. 1H, OCHO), 5.34 (m, 2H, HC=CH) ppm.

*(*R*)- and (*S*)-14-Methyl-12-(*Z*)-octadecen-1-yl Bromide,* ***6***. The protected alkenol **5** (1.73 g, 4.7 mmol) was added to a solution of triphenylphosphine dibromide (5.7 mmol) in CH_2Cl_2 (15 ml). After 1 hr, the solvent was removed by rotary evaporation, and the crude product was triturated with hexane several times. The hexane solution was concentrated and filtered through silice gel (15 g) with hexane. The solvent was removed providing 0.80 g (50%) of the (Z)-alkenyl bromide **6**: GLC col A (170°C) $R_t = 5.0$ min, E isomer ca. 6% $R_t = 6.2$ min, $\alpha = 1.03$; NMR ($CDCl_3$) 0.89 (m's, ca. 6H, overlapped CH_3R and $C\underline{H}_3CH$), 1.27 (CH_2 envelope), 1.58 (bs, 3H, allylic CH_2, CH), and 3.41 (t, 2H, $J = 7.9$ Hz, $CH_2C\underline{H}_2Br$) ppm; CI-MS (*m/e*) 345, 347 (M+1).

*(*R*)- and (*S*)-14-Methyl-1-(*Z*)-12-octadecadiene,* ***7***. A solution of LDA was prepared with butyllithium (2.5 mmol) and diisopropylamine (4 mmol) in THF (10 ml) under nitrogen at 0–5°C. The (Z)-alkenyl bromide **6** (3 mmol) was added in ca. 1 ml of hexane. After 10 min, the elimination was complete and the product was worked up with hexane and 2 N HCl. The crude diene was purified by argentation column chromatography. A CH_3CN (50 ml) solution of $AgNO_3$ (2 g) was prepared. Silica gel (60–120 mesh, 10 g) was added and swirled gently for 0.25 hr. The silica gel was collected by filtration and washed with dry benzene. The gel was then dried over P_2O_5 in vacuo. A column of this material was made up in hexane and employed repeatedly in this procedure. Retention volumes and GLC retention data for the various compounds are tabulated below (Table 1). More efficient chromatography is possible, but the required separation was affected in this manner.

Pure (R)- and (S)-Z-diene **7** were obtained: IR (CCl_4) 910 cm^{-1}, NMR ($CDCl_3$) δ 0.90 (m, ca. 6H, overlapped CH_3's), 1.27 (CH_2 envelopes) 2.03 (m, 5H, allylic CH), 4.9–5.8 (5H, vinyl H); CI-MS (*m/e*) 265 (M+1).

*(*R*)- and (*S*)-14-Methyl-1-(*E*)-12-Octadecadiene,* ***8***. Diene **7** (140 mg,

TABLE 1.

Compound	*ks* col B (190°C)	*Rv* (ml)[a]
(E)-Diene, **7**	2.50	70–85
(Z)-Diene, **8**	2.36	80–120
1-Alkene, **10**	3.68	60–110
Alkane, **11**	3.97	20–40

[a] Retention volume using $AgNO_3$ column chromatography and a flow rate of 1 drop/1–2 sec.

0.53 mmol) was added to a solution of metachloroperbenzoic acid (0.25 g, >2 equiv) in CH_2Cl_2 (5 ml). The mixture was allowed to stand at ambient temperature ovenight. The crude bis epoxide was obtained by removing the solvent. A suspension of 3 equiv of triphenylphosphine dibromide in 10 ml of benzene was prepared. The bis epoxide was added thereto with 1–2 ml of benzene, and the resultant mixture was stirred at ambient temperature overnight. The crude tetrabromide was obtained by concentrating the product mixture and triturating the residue with hexane. The hexane was removed, and the product was debrominated by allowing it to react for 2 hr in propanoic acid to which an excess of powdered, activated, zinc had been added (0–5 °C). The mixture was worked up with H_2O and hexane. The organic phase was washed to neutrality with aqueous $NaHCO_3$, and the extract was dried ($MgSO_4$). Removal of the solvent was followed by column chromatography on silica gel (1–2 g) with hexane. The dienes **8** were obtained in ca. 70% yield containing 3–7% *Z* isomer. Passage through the $AgNO_3$ column provided samples containing no more than 3% *Z*: IR (CCl_4) 965, 910 cm^{-1}; CI-MS (*m/e*) 265 (M+1).

(R)- and (S)-14-Methyl-1-Octadecyl Bromide, 9. The (*R*)- and (*S*)-alkenyl bromides **6** were hydrogenated over PtO_2 in propanoic acid and produced the saturated bromdies **9** contaminated with the parent hydrocarbons (ca. 10%). These were not separated at this point: NMR δ 3.40 (t, CH_2Br) ppm and no signals for vinyl H; GLC col A (170°C) for 2.4 min for alkane **11,** 8.0 min for alkyl bromide **9;** CI-MS (*m/e*) 347, 349 (M+1).

(R)- and (S) 14-Methyl-1-Octadecene, 10. The bromides **9** were dehydrobrominated as described above for dienes **7.** The crude product containing the parent alkane was chromatographed on the $AgNO_3$ column to give alkane (eluting first): NMR ($CDCl_3$) δ 0.88 (bt, CH_3R), 1.25 (CH_2 envelope) ppm; EI-MS (*m/e*) 268 (P+1), 267 (P−1), 211 ($C_{15}H_{31}$), and 210 ($C_{15}H_{30}$), and **10:** IR (CCl_4) 910 and 990 (weak) cm^{-1}; NMR ($CDCl_3$) δ 0.89 (m, ca. 6H, overlapped CH_3's), 1.26 (CH_2 envelope), 2.05 (m, ca. 2H, allylic CH_2, and 4.9–5.8 (m's, 3H, vinyl H); CI-MS (*m/e*) 267 (M+1), 266 (P), 265 (M−1).

RESULTS AND DISCUSSION

The asymmetric center was introduced, as shown in Figure 1, by alkylating the dianion of propionic acid with butyl bromide. The original procedure (Creger, 1970) employed the sodium salt of the acid and reported a 54% yield of 2-methylcaproic acid, **1.** We used the general procedure of Pfeffer and Silbert (1970) involving a dilithio anion to obtain a 94% yield. The acid was converted to amide **2** with either (*R*)- or (*S*)-α-methylbenzylamine to produce a mixture of diastereomers that was resolved easily by several-fold recrystallization from ethanol. The process of purification to a nearly pure (>99.6%) diastereomer was monitored by gas chromatography (Methods and Materials). The purified

FIG. 1. Synthesis of configurationally pure phosphonium salts: 1. lithium diisopropylamide, butyl bromide; 2. thionyl chloride, (*R*)- or (*S*)-α-methylbenzyl amine; 3. fractional crystallization, amide reductive cleavage (Sonnet, 1984); 4. triphenylphosphine dibromide, then sodium iodide and triphenylphosphine.

*R*S**- (asterisks indicate relative stereochemistry) diastereomer was then cleaved by (1) *N*-alkylation with ethylene oxide, and (2) transacylation–reduction (Sonnet, 1984) yielding either (*R*)- or (*S*)-2-methyl-1-hexanol, **3**. This procedure has been shown to afford alcohols of configurational purity equal to that of the diastereomeric amide from which it was derived. The alcohols were then transformed to bromides, and these to triphenylphosphonium iodides in preparation for a Wittig condensation reaction.

The other component for this condensation (Figure 2) was obtained by blocking one hydroxyl group of 1,12-dodecanediol and then oxidizing that alcohol, **4**, to the corresponding aldehyde with pyridinium chlorochromate–sodium acetate. The condensation itself was conducted to maximize the *cis*-olefin

FIG. 2. Synthesis of configurationally pure dienes ([a]THP = 2-tetrahydropyranyl): 1. dihydropyran, H^+; 2. pyridinium chlorochromate, sodium acetate; 3. butyllithium (→ ylid), procedure for *cis* (Sonnet, 1974); 4. triphenylphosphine dibromide; 5. lithium diisopropylamide; 6. olefin inversion via bisepoxides (Sonnet and Oliver, 1976).

(R),(S) 6 —1→ [9 (R),(S): (CH₂)₁₃Br] —2→ [10 (R),(S): (CH₂)₁₁CH=CH₂]

FIG. 3. Synthesis of the enantiomers of the peach leafminer moth sex pheromone: 1. H_2/platinum oxide; 2. lithium diisopropylamide.

generated by using hexamethylphosphoric triamide as an additive (Sonnet, 1974). Completion of the pheromone synthesis involved conversion of the tetrahydropyranyl ether of the (*R*)- and (*S*)-alkene **5** to a bromide **6** with triphenylphosphine dibromide (Sonnet, 1976). Catalytic hydrogenation of the double bond (Figure 3) was followed by dehydrobromination with lithium diisopropyl amide. The final product, (*R*)- or (*S*)-14-methyl-1-octadecene, **10,** was purified by argentation column chromatography to separate it from the corresponding alkane that had arisen in the catalytic hydrogenation of the alkenyl bromide **6**.

In order to obtain the several stereoisomers of the desired analog, 14-methyl-1,11-octadecadiene, (*R*)- and (*S*)-**6** was dehydrobrominated to the corresponding (*R*)- and (*S*)- *Z*-isomers, **7** (Figure 2). The *E* isomers were prepared by carrying out an inversion via the bisepoxides (Sonnet and Oliver, 1976). The bisepoxides were allowed to react with triphenylphosphine dibromide in benzene producing tetrabromides that are the result of SN-2 inversions on each oxygenated carbon. Reduction with activated zinc–propionic acid, a *trans*-antiplanar elimination of vicinal halogens, causes net inversion of one of the carbons 12/13 to give the inverted alkene. All of these materials were purified by argentation column chromatography monitored by gas chromatography (Methods and Materials).

CONCLUSION

With these materials in hand we were in a position to measure biological activity versus stereostructure and thereby make the final assignment of pheromone structure. Results of field tests showed that the sex pheromone has the *S* configuration and full details of these studies are published elsewhere (Sugie et al. 1985).

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METHODOLOGICAL APPROACH TO IDENTIFY CHEMICAL OVIPOSITION STIMULANTS FROM MAIZE FOR EUROPEAN CORN BORER

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Abstract—The noncontingent distribution of *O. nubilalis* Hbn. eggs on various corn varieties has allowed their classification as either "favorable" or "nonfavorable" hybrids. This classification is based on a chemical communication system using volatile compounds active at a distance from the insect or active when in contact with the insect as soluble sugar components. Under this hypothesis, gas chromatography is the best way of investigating for the chemical differences between corn silk extracts from the two types of hybrids. A first experiment, using a "desorption-trapping" system on Tenax coupled to a gas chromatograph showed a quantitative difference between two compounds from the two hybrids. A second experiment showed the advantages and disadvantages of this method and allowed the development of two complementary techniques: (1) direct extraction of corn silk with trichlorofluoromethane and direct injection into a capillary column with an apolar stationary phase using an "on-column" system; and (2) extraction under vacuum of volatile compounds from corn silk and their trapping on Tenax, followed by a second desorption. This allows a direct injection on the same capillary column. The first technique allows identification by mass spectrometry of many alkanes with high molecular weights. The second technique seems to confirm the presence of phenylacetaldehyde. Both techniques show quantitative differences in the composition of corn silk extracts from "favorable" and "nonfavorable" hybrids; however, the biologically active chemicals remain unknown.

Key Words—Pyralidae, *Ostrinia nubilalis*, oviposition preference, corn silk volatile chemicals, extraction, desorption, gas chromatography, mass spectrometry alkanes, phenylacetaldehyde.

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INTRODUCTION

Developing a new strategy to protect crops against insect pests by the use of nonpolluting and specific methods requires a basic knowledge of insect–plant relationships. In the sequence of insect reproductive behavior: sex-attraction, mating, insemination, fertilization, and oviposition, the last one is of utmost interest to plant protection specialists because it determines the level of damage. With the European corn borer *Ostrinia nubilalis* Hübn., oviposition behavior and especially the distribution of egg masses in the field were found to differ in various maize varieties. Specific behavioral mechanisms must explain these differences in distribution. Observations under natural conditions (Anglade et al., 1981) enabled us to classify different maize hybrids as "favorable" or "nonfavorable" for the oviposition of *O. nubilalis*. We believe that this choice is mediated by a chemical communication system involving two types of allelochemical substances emitted by the plant and detected by the moth: (1) volatile compounds acting on the insect for long-range identification of plant odors and for locating the host plant, e.g., phenylacetaldehyde by *O. nubilalis* (Cantelo and Jacobson, 1979); and (2) other compounds acting at contact, e.g., soluble sugars (Derridj and Fiala, 1983) which affect the amount of time the insect remains on the plant and stimulate or inhibit oviposition by an unknown mechanism.

This research was designed to identify attractive or repulsive substances and favorable or nonfavorable substances for oviposition of *O. nubilalis*. Similar scientific approaches have been successfully implemented on other models: (1) for attraction of *Delia brassicae* Wied. by natural or synthetic isothiocyanates (Städler, 1978; Finch and Skinner, 1982) or *Acrolepiopsis assectella* Zell. by thiosulfinates and disulfides of leek (Lecomte and Thibout, 1981); and (2) for deterrents or repellents of various species such as *Leptinotarsa decemlineata* Say. or *Mythimna unipuncta* Haw. by the phenylpropanoid extracts from *Bupleurun fructicosum* and *Acaurus calamus* (Muckensturn et al., 1982).

METHODS AND MATERIALS

The purpose of this work was to compare the chromatograms of aromatic extracts of the same organ from two hybrids differing in their effect on *O. nubilalis* and to detect differences which could explain the behavioral choice of females. Fresh corn silk was used because this organ contains many volatile compounds attractive to the European corn borer (Cantelo and Jacobson, 1979). It seemed desirable to use the experimental approach of these authors.

Plant Material. The study was conducted on experimental material developed by Panouillé (Station expérimentale du maïs, St. Martin de Hinx) and recognized as "favorable" or "nonfavorable" to oviposition on the basis of

field and cage trials by Anglade et al., (1981). The experiments were carried out on single hybrids of similar growth habits designated by the following code "favorable" (ABG and AF); "nonfavorable" (LOF and FF). Samples were taken previously during the normal plantings of July 1982 and 1983 and in a greenhouse during April 1983.

To minimize the time between cutting of the corn silk in the field or in the greenhouse and its chromatographic examination in the laboratory (a distance of 3 or 10 km), we transported the whole plant. The plant was removed with about 10 kg of soil, placed in a pail, and transferred to the laboratory in a van to prevent any injury which could influence the natural emission of volatile substances. Under these conditions and with watering, each plant remained apparently normal for at least two weeks in the laboratory and permitted several analyses.

Procedure. The volatile components of the material were analyzed by preliminary concentration in an adsorbent trap before injection and concentration in the column of a gas chromatograph. The plant material was heated in a micro-oven and the volatile compounds were captured by a helium flux and adsorbed in a trap containing Tenax. It could be desorbed later by heat and injected into the column of a gas chromatograph.

For all experiments we used a DCI, Girdel platine (Figure 1). The temperature of the desorption oven can be adjusted from room temperature up to 260°C. The minitrap is a $\frac{1}{8}$-in. inox tube containing about 0.3 ml Tenax. This trap is surrounded by a liquid nitrogen case and a heating device by Joule effect. Valves and transfer lines between oven and trap and between trap and chromatograph are held at 150°C by a heating block regulated by an electronic box.

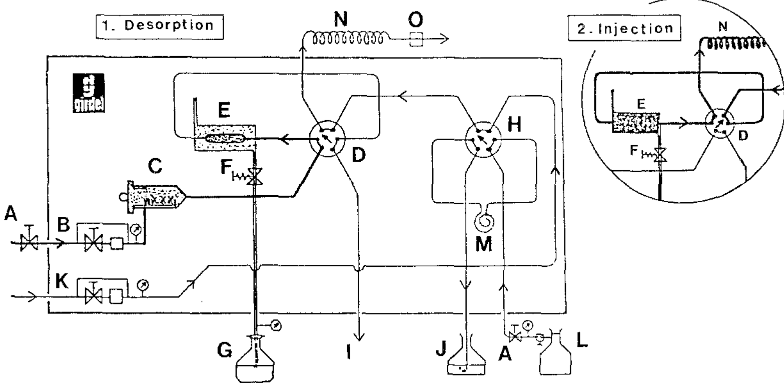


FIG. 1. Gas chromatographic platine DCI Girdel device: A, stopping valve; B, helium scavenging gas; C, desorption oven; D, sampling valve; E, minitrap; F, electrovalve; G, liquid nitrogen; H, valve of standard gas inlet; I, open air; J, ambient pressure system; K, vector gas; L, gaseous standard; M, pattern ring; N, column; O, detector.

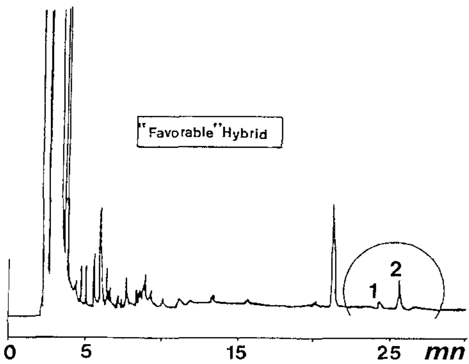
Each step of the analysis is manual. After cooling the trap, the solid sample (corn silk) was placed in a little Pyrex skiff (maximum capacity 1 ml) which was introduced in the micro-oven (oven volume: 7.3 ml). Then, the successive trapping and desorption phases of the volatile chemicals were carried out.

RESULTS AND DISCUSSION

The preliminary 1982 study did not produce spectacular evidence of differences between the two hybrids but a qualitative difference between the two peaks of two unidentified substances (Figure 2). Devising and developing successful improved methods was our main finding; four steps can be distinguished.

Direct Desorption on Corn Silk. In 1982, we used a capillary column (Carbowax 20 M, 50 m) generally suitable for separating aromatic substances which were susceptible to oxidation.

In 1983, the technique was refined to give a better definition of the peaks



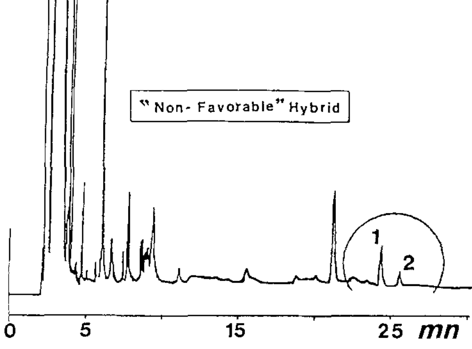


FIG. 2. Corn silk extract chromatograms: preliminary experiment 1982.

as follows: for each experiment, the corn silk mass treated was about 100–300 mg; the desorption oven was heated for 10 min at 50°C under helium scavenging gas (30 ml/mn); during trapping phase, the Tenax trap was not cooled (cooling of the trap at 0°C did not improve the trapping but entailed obstruction of transfer tubes by molding of little blocks of ice); we used a CP cil 5 capillary column (25 m) of 0.31 mm inner diameter. Parameters of analysis were: vector gas pressure 0.6 bar, isotherm 20 min at 80°C, and programming 3°C/min up to 220°C.

The main advantage of this technique is the direct extraction without solvent. According to Visser et al. (1979), the extraction process with organic solvents entails two major disadvantages: (1) impurities from the solvent may accumulate in the aroma concentrate, and (2) nonvolatile components are isolated along with the volatiles. Nevertheless, in our device, the volume of the oven limits the amount of plant material.

The chromatograms obtained were more complicated, but the peaks were well separated. Maximum of retention time reached 70 min. The extractable elements of corn silk seemed to correspond to apolar molecules with high molecular weights.

In the favorable hybrids AF and ABG, we observed the presence of a peak whose retention time might correspond to phenylacetaldehyde. In other respects, the same peaks were found, but the quantitative differences were not consistent and replicability was poor within hybrids. Besides, the blank experiments between each desorption pointed to a recondensation of heavy compounds between the Tenax trap and the column; they were likely to interfere in the following desorptions. These blank experiments were thus necessary and the time of analysis was increased.

Desorption after Extraction by "Coldfinger" Technique. The purpose was to improve the accuracy of the method by indirectly increasing the sample volume. A tube filled with liquid nitrogen (coldfinger) trapped water and some of the aromatic compounds of corn silk treated under vacuum.

By using this procedure, we obtained a very odoriferous watery extract whose organoleptic characteristics were similar to the odors in the open corn field. The Pyrex skiff is filled up with this extract and the desorption is done as previously described. Thus, 10 g of corn silk were extracted instead of 0.3 g by direct desorption, and we expected a significant increase in analytical sensitivity by a factor (10/0.3) = 33.3.

Unfortunately, we did not observe a spectacular increase of the peak size, but rather a decrease. Nevertheless, most of the peaks were recovered. In addition, such watery extracts are extractable by trichlorofluoromethane. After a first desorption during 10 min at 50°C, a strong odor of corn silk remained in the skiff. This odor disappeared after 10 min scavenging by helium vector gas at 150°C. Besides, an increase in chromatogram sensitivity led us to further improve the method using direct extraction of corn silk with trichlorofluoromethane.

Direct Extraction of Corn Silk by Trichlorofluoromethane, Injection on Column, Identification of Compounds. Extractions were done on 20 g of fresh corn silk with 100 ml trichlorofluoromethane for each hybrid. Extracts were concentrated and put in 10 ml hexane before injection on column. Both extracts are very odoriferous. Injections were done as follows: injected volume, 1 μl; vector gas pressure, 0.7 bar; oven temperature, isotherm 40°C during 1 min and programming 4°C/min up to 260°C.

Observation of chromatograms (Figure 3) revealed quantitative rather than qualitative differences between the two hybrids. Thus, peaks 5, 6, and 9 were proportionally more important in hybrid FF than in hybrid ABG, even though peaks 1 and 10 seemed to be constant in both hybrids. Mass spectrometry permitted identification of the first three peaks which were composed of saturated alkanes: 5 corresponds to *N* tricosane, 6 to *N* tetracosane, and 9 to *N* hexacosane. Mass spectra of peaks 7 and 10 presented an analogous structure but were not precisely identified; they might correspond to alcohols (Figure 4). In addi-

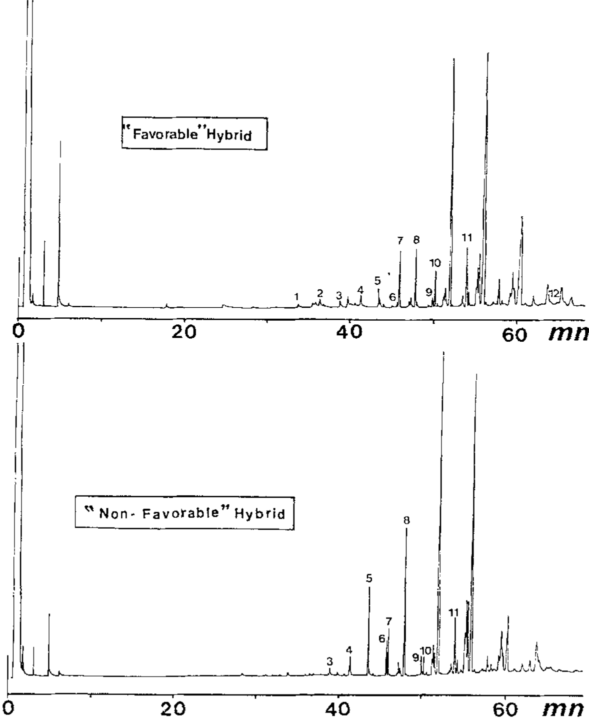


FIG. 3. Gas chromatograms of corn silk extracts with trichlorofluoromethane on-column injection. Peak 1, *N*. nonadecane; 2, *N* eicosane; 3, *N* heneicosane; 4, *N* docosane; 5, *N* tricosane; 6, *N* tetracosane; 7, unidentified; 8, *N* pentacosane; 9, *N* hexacosane; 10, unidentified; 11, *N* octosane; 12, *N* dotriacontane.

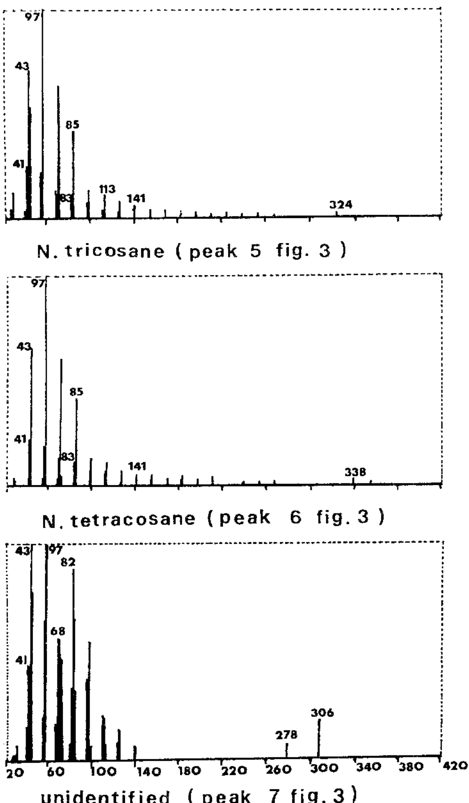


FIG. 4. Mass spectrograms of three compounds obtained by direct extraction of corn silk with trichlorofluoromethane.

tion, there seems to be a relation between the nature of the "favorable" and "nonfavorable" hybrids for insects and the proportions of the peaks which we observed with analogy of structure in mass spectra:

$$\underset{\text{Hybrid FF}}{\frac{\text{height of peak 6 (alkane)}}{\text{height of peak 7 (alcohol ?)}}} > \underset{\text{Hybrid ABG}}{\frac{\text{height of peak 6 (alkane)}}{\text{height of peak 7 (alcohol ?)}}}$$

$$\frac{\text{height of peak 9 (alkane)}}{\text{height of peak 10 (alcohol ?)}} > \frac{\text{height of peak 9 (alkane)}}{\text{height of peak 10 (alcohol ?)}}$$

Addition to FF and ABG hybrid extracts of control alkanes and injection in different polarity columns confirms the above hypothesis. If we hold the mass spectrum on a peak with known molecular weight (mol wt 324 for peak 5, mol wt 338 for peak 6, and mol wt 306 for peak 7), it is possible to record the signal

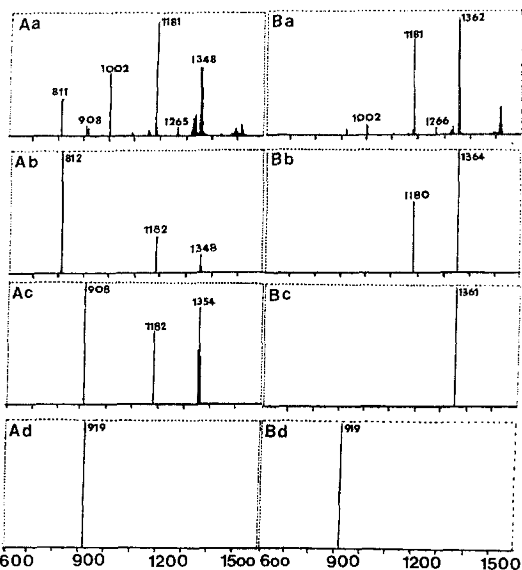


FIG. 5. Mass fragmentometry. Difference between ABG and FF hybrids (A): FF, non-favorable; (B): ABG, favorable. (a) chromatograms; (b) distribution of the mass 324 compound (peak 5); (c) distribution of the mass 338 compound (peak 6); and (d) distribution of the mass 306 compound (peak 7).

given in relation to the time, thus obtaining a "mass fragmentogram." This fragmentogram, similar to a chromatogram, contained only the peaks of compounds having the fragment chosen in their mass spectrum (Figure 5). The preponderance of compounds "5" and "6" in hybrid FF and an identical distribution of compound "7" in both hybrids was then confirmed.

These results should be considered with caution because of the small number of replicates. Also, the concentration phases of trichlorofluoromethane might have induced a loss of the most volatile components. For this reason, we continued our investigations by gathering the volatile compounds.

Extraction under Vacuum and Trapping of Aromatic Compounds with Tenax. As did Buttery and Ling (1984), to identify corn leaf volatiles, we treated corn silk under vacuum and trapped the volatile compounds with Tenax. The advantage of this method was to treat large quantities of corn silk during extended periods of extraction without disturbing the chromatograph examination. Tenax was desorbed in the oven during 30 min at 150°C. Aromatic components which were desorbed from the first Tenax trap were soon readsorbed on the second Tenax trap of the DCI Girdel platine. Otherwise, the other experimental conditions were the same as previously described and the chromatograms obtained were comparable with those obtained after injection on column.

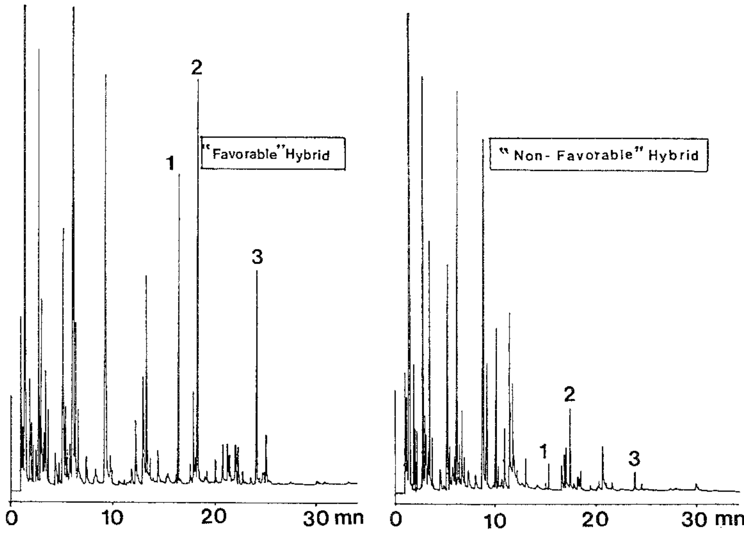


FIG. 6. Gas chromatograms of volatile components: extraction under vacuum and trapping on Tenax. Peaks 1: phenylacetaldehyde?; 2, 3: unidentified.

These chromatograms revealed the presence of numerous light components which did not appear in last method (Figure 6); we could thus observe major differences between the two hybrids studied and especially the greater quantity of three compounds (peaks 1, 2, and 3) in ABG (favorable hybrid) than in FF hybrid. One of these compounds (peak 1) presents a retention time very close to that of the phenylacetaldehyde already observed in the first experimental step.

Flath et al. (1978) did not find phenylacetaldehyde among the volatile components of corn silk by vacuum codistillation with water, followed by ether extraction of the distillate. In contrast, this compound was identified by Cantelo and Jacobson (1979) in the same plant organs by trapping on Porapak-Q and extraction in a Soxhlet apparatus with anhydrous ether.

Most common extraction techniques of chemical insect stimulants entail the use of different organic solvents. Maeshima et al. (1985) thus successively used methanol, *n*-hexane, and a mixture of *n*-hexane–ethyl ether to extract the chemical oviposition stimulants from rice grain for *Sitophilus zeamais*. Woodhead (1983) used two distilled solvents—chloroform and a mixture of *n*-hexane, diethyl ether, and formic acid to identify the components of the surface wax of *Sorghum bicolor*. Last, for isolation and identification of volatiles in the foliage of *Solanum tuberosum*, Visser et al. (1979) only used a mixture of diethyl ether and *n*-pentane in the final extraction phase.

The purpose of this study was to reduce the number and amount of solvent, and this experimental approach enabled us to develop a satisfactory method of

analyzing the aromatic components of fresh maize silk. Comparing the advantages and disadvantages of the techniques will allow future work to progress more quickly. Two complementary methods should be used to analyze the different groups of components: (1) extraction of heavy apolar components with trichlorofluoromethane (third step); and (2) trapping of light components with Tenax (fourth step).

In general, the differences between the two hybrids of maize were quantitative, not qualitative, for both heavy and light aromatic compounds. The heavy components (*N.* tricosane, *N.* tetracosane, *N.* hexacosane) were proportionally more abundant in the silk of FF hybrids (unfavorable) than in the ABG hybrids (favorable). We have not proved that these components diffuse into the atmosphere under natural conditions, but they might play a role either by themselves or as precursors of active components which inhibit oviposition or repel females. This hypothesis agrees with the results of Woodhead (1983). This author also identified in the surface wax of *Sorghum bicolor* the same three alkanes to be deterrent to *Locusta migratoria*. Secondly, the probable presence of phenylacetaldehyde [whose attractive role on *O. nubilalis* was proved by Cantelo and Jacobson (1979)] encourages us to identify the other two compounds which have the same relationship.

CONCLUSION

We have formulated the following hypothesis to explain the oviposition behavior of *O. nubilalis* females: (1) At a remote distance (several meters), the moths would be attracted by the volatile compounds (phenylacetaldehyde type and related compounds) emitted by "favorable" hybrids. (2) On contact, oviposition would be induced preferentially on plants lacking certain heavy components such as alkanes.

To verify this hypothesis, it will be necessary to confirm that the components identified in the two hybrids are responsible for the choice of plants by the females during oviposition. These substances will have to be tested on the insects themselves using olfactometric and neurobiological techniques such as electroantennography in the laboratory and then under natural conditions on plants genetically high or low in these compounds.

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VARIATION IN METHYLGLUCOSINOLATE AND INSECT DAMAGE TO *Cleome serrulata* (CAPPARACEAE) ALONG A NATURAL SOIL MOISTURE GRADIENT

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Abstract—We tested the hypothesis that plant loss to insects, and thus the relative fitness of an annual, was inversely related to spatial variation in the concentration of its characteristic secondary compound, methylglucosinolate, a mustard oil precursor. We found that methylyglucosinolate concentrations decreased significantly and linearly from the dry to the wet end along short soil moisture gradients in dry shortgrass prairie. Both leaf damage and capsule predation increased from the dry to the wet end. Thus, the glucosinolate appears to function defensively. Plant growth and flower production were favored at the wet end of the gradient; yet plants in the wet portion of the gradient were also more vulnerable to significant insect damage. The net result was that seed production by individual plants after predation was actually greater in the drier, harsher half of the gradient. The outcome strongly suggests that environmentally related variation in secondary compound concentration along environmental gradients can mediate and influence host-plant population abundance and distribution.

Key Words—Capparaceae, chemical defense, *Cleome serrulata*, glucocapparin, methylglucosinolate, gradient, herbivory, insect-plant interactions, soil moisture gradient, stress.

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INTRODUCTION

Four lines of evidence suggest that intraspecific variation in plant natural products may mediate insect herbivore impact on host-plant dynamics. First, damage done by phytophagous insects is common and varies tremendously in intensity. Experimental studies show insect herbivory can influence plant seed reproduction, recruitment, or population dynamics (Cantlon, 1969; Janzen, 1971, 1979; Manley et al., 1975; Waloff and Richards, 1977; Rausher and Feeny, 1980; Parker and Root, 1981; Louda, 1982a,b, 1983, 1984, 1986a).

Second, it is evident that secondary compounds, such as glucosinolates, often play a defensive role in insect–plant interactions (Fraenkel, 1959; Ehrlich and Raven, 1965; Whittaker and Feeny, 1971; Feeny, 1975, 1976; Levin, 1976a,b; Rhoades and Cates, 1976; Rosenthal and Janzen, 1979). Consequently, predictable variation in chemical concentrations could determine systematic patterns of insect feeding and impact.

Third, detailed studies of chemical profiles under natural conditions suggest that extensive chemical variation occurs among individuals within populations of a species and represents the usual situation (Dolinger et al., 1973; Lincoln and Langenheim, 1979; Rodman and Chew, 1980; Feeny and Rosenberry, 1982; Louda and Rodman, 1983a,b; Lincoln and Mooney, 1984). This variation is often correlated with changing environmental conditions along spatial gradients (Lincoln and Langenheim, 1979; Cates and Alexander, 1982; Cates et al., 1983; Louda and Rodman, 1983a,b; Redak and Cates, 1984; Louda, 1982b, 1986b).

Fourth, spatial patterns of seed predation and foliage consumption occur, such as between adjacent sun and shade habitats (Huffaker and Kennett, 1959; Lincoln and Langenheim, 1979; Louda and Rodman, 1983a,b; Lincoln and Mooney, 1984; Louda et al., 1986a,b) and between portions of small, local gradients (Handel, 1976; Redak and Cates, 1984; Louda et al., 1986b) or larger, climatic gradients (Janzen, 1975; Louda, 1982b, 1983). Thus, the utilization patterns are also correlated with the spatial variation in physical environmental conditions and in secondary compounds (Redak and Cates, 1984).

Consequently, an interrelated set of three hypotheses can be proposed to explain systematic patterns of variation in plant susceptibility to attack and damage over environmental gradients. These are: (1) often there is ordered spatial variation in the putative defensive chemicals; (2) this variation in secondary compounds reflects variation in physical parameters along environmental gradients; and (3) the underlying variation in defenses mediates loss to insect consumers and thus the spatial distribution of insect influence on host-plant population dynamics (Louda, 1986b).

The purpose of our study was to examine this set of hypotheses for *Cleome serrulata*, an annual species on which insect damage had been noted along a soil moisture gradient during a recent study of population structure (Farris,

1985). An annual plant species provides a particularly interesting model system for this type of work since any reduction in seed production due to herbivory has immediate, demographic consequences (cf., Harper, 1977). Our study has additional relevance since *Cleome* is a glucosinolate-containing member of the caper family (Capparaceae). Glucosinolates are used as a classical example of the qualitative defense strategy in the predominant theories of chemical defense in plant–herbivore interactions (e.g., Whittaker and Feeny, 1971; Feeny, 1976; Rhoades and Cates, 1976). The theory has been developed with data on the interaction of mustards (Cruciferae) and their adapted insect herbivores. Data in other systems, such as the closely related caper–insect interaction, should provide strong corroborative or contradictory evidence on the functional role of glucosinolates in plant defense.

METHODS AND MATERIALS

Site and Gradient Description. Two populations, 250 m apart, were located in the White Rocks Preservation Area, 11 km east of Boulder, CO. Each population was situated on a south-facing, 5–8° hillside (Figure 1). These mild, dry slopes caused slight but significant gradients in soil moisture availability within each population. Gravimetric determinations in 1984 showed that the soils at the "wet" end of the populations had an average of 6.6 ± 0.48% soil moisture from May to September (both sites combined). During the same period, the soils at the "dry" end of the populations had an average of 4.3 ± 0.37% soil moisture. Predawn plant water potentials, as measured with a Scholander-type pressure chamber, varied along the soil moisture gradients; differences of up to 0.9 MPa were found for plants at opposite ends of the soil moisture gradients (Farris, 1985). Plant growth rates, survival, and reproduction also varied considerably along these gradients (Farris, 1985).

Field Assessment of Insect Damage. Estimates of insect damage to developing capsules and their seeds were made in both 1983 and 1984. In August 1983, the proportion of capsules per inflorescence that were chewed and seriously damaged by insects was categorized and recorded for plants in relation to position along the soil moisture gradient at both sites. Plant location was measured as meters from the dry end of each gradient. This location proved to be a reliable predictor of relative soil moisture availability (Farris, 1985). In addition, in September 1983, foliage area removed or damaged at site 2 was scored as light, moderate, or heavy (i.e., 0–5%, 6–25%, or more leaf removal of tissue by insects). The most common insects observed feeding on the foliage were grasshoppers.

In September 1984, seeds that developed on plants growing along the gradient at site 2 were collected. The collection was made early in the month, after the period of most intense capsule and seed predation by lepidopteran larvae

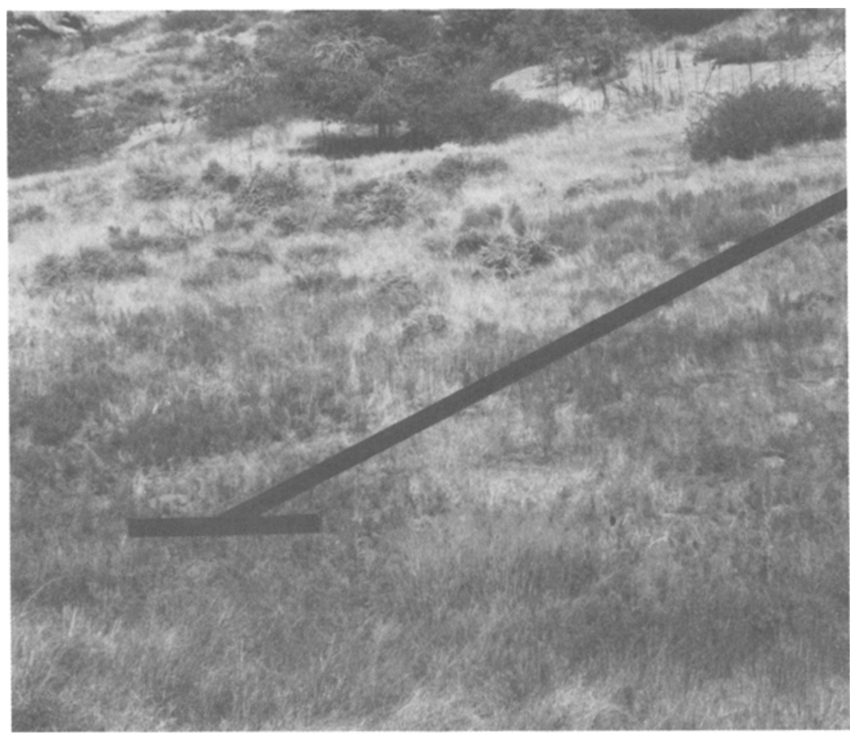

FIG. 1. Location of gradient at site 1 at the White Rocks Preservation Area with the specific microgradient that was sampled marked (transect length-25m).

(Pieridae). Pierids are common herbivores of capers (see Goury and Guignon, 1908). Sixteen plants that spanned the soil moisture gradient had some capsules with viable seeds. Seed production was recorded in the field, and any capsules remaining were collected. Seeds were examined, and the number of viable seeds per plant was determined in relation to plant size and plant position on the gradient.

Collection of Plant Material. On August 14, 1984, leaf material was collected from 22 plants at each site. Capsule and seed material were uncommon. A minimum of 5 g of leaf tissue was removed from each plant, put in a plastic bag, and placed on ice. Plant height, plant diameter, and plant location were recorded. A plant size index was later derived by multiplying height and diameter. The plant samples were returned to the lab and immediately boiled for 10 min in 70% methanol.

Glucosinolate Analysis. Glucosinolate analyses were done according to the methods described by Blua (1984) and Blua and Hanscom (1986). Briefly, the plant tissue in methanol was homogenized at high speed, boiled, condensed to

30 ml, filtered through Whatman No. 2 paper, and condensed to 10 ml by vacuum evaporation. Ten milliliters of 0.05 M sodium phosphate buffer (pH 7.5), 0.10 ml of 4.2% ascorbate in water, 15.0 ml methylene chloride, and 1.0 ml thioglucosidase extract (Rodman, 1978) were added, and the tightly capped Teflon tubes were placed on a shaker for 12 hr at room temperature. The aqueous phase and the methylene chloride phase were then separated by centrifugation (20 min at 1500 rpm).

For chromatographic analysis of nitrogen hydrolytic products, 12 ml of the methylene chloride phase were removed by syringe and concentrated to 1 ml, using an Evapo-Mix vacuum evaporator with an ice-water bath. Undecane was added as an internal standard, making a final concentration of 0.05%. Paper chromatographic analysis for oxazolidinethione-generating glucosinolates was done using published methods (see Rodman, 1978; Louda and Rodman, 1983a). Gas chromatography was done on a Varian Vista 6000 gas chromatograph using a 6-ft, 2 mm ID, glass chromatographic column packed with 10% Carbowax 20 M on 100–120 mesh Chromosorb WAW. Injection and FID temperatures were set at 150°C and 200°C, respectively. Nitrogen flow was 30 ml/min. Oven temperature was maintained at 60°C for 2 min and then raised to a final temperature of 120°C at a rate of 10°C/min. Quantification was done by comparing retention times and area counts to those of known standards (Blua, 1984). Concentrations are given in milligrams methyglucosinolate (glucocapparin) per gram of plant material, dry weight.

RESULTS

Insect Damage. The relative severity of leaf damage increased as soil moisture availability increased. The plants with the heaviest damage were found exclusively in the wettest portion of the site, while those with relatively little damage were generally found toward the drier half of the gradient (Figure 2). The tendency for a relationship between the levels of insect damage and soil moisture was even stronger if plant size was considered as a covariate. Large plants at the wet end of the gradient had lower viable seed production after predation than did much smaller plants at the dry end of the gradient, in spite of having higher flower initiation and potential seed production (Farris, 1985). For example, the largest plant at the wet end of the gradient at site 2 had 900 developing capsules on August 28, 1984, but only four viable seeds by mid-September. At the same time a smaller plant at the dry end of the gradient had one third the number of capsules in August (300) but ten times as many viable seeds (40) in mid-September, after predation by the lepidopteran larvae.

Overall, capsule predation also appeared to increase as soil moisture availability increased. In 1983, the relationship between soil moisture availability and the proportion of capsules damaged was examined on both a per inflores-

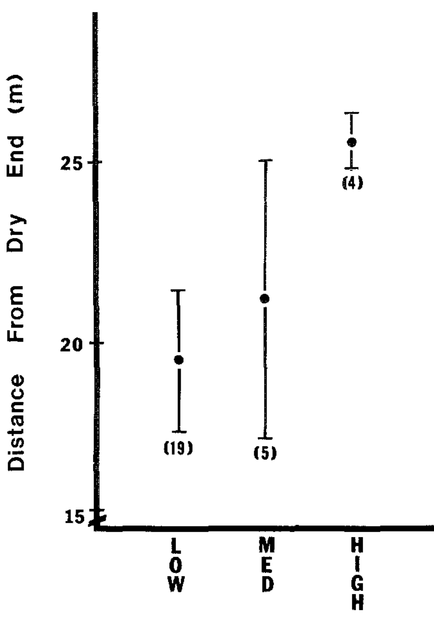

FIG. 2. The relationship between soil moisture availability ($\bar{X} \pm$ SE) and the severity of leaf damage at site 2, September 1983. Plants were scored as having light (0–5%), moderate (6–25%), or heavy (greater than 25%) foliage damage. Sample sizes in parenthesis; groups are not statistically different.

cence and a per plant basis. The Spearman rank-order correlation coefficients (r_s) between distance along the gradient from the dry end and the proportion of capsules damaged per inflorescence were: +0.17 (N = 102) and +0.20 (N = 93) for sites 1 and 2, respectively. The proportion of capsules damaged per plant was also positively associated with increased soil moisture availability [r_s = +0.13 for both sites (N = 11 and 23)]. However, these correlations were not statistically significant. In 1984, the majority of the plants (81.4%, N = 86) had lost all of their viable seeds to the insect predators by early September. However, the number of viable seeds remaining per plant after insect feeding at site 2 was significantly higher at the drier end of the soil moisture gradient (Table 1).

Glucosinolate Concentrations. Most species of *Cleome* that have been studied contain a single isothiocyanate-yielding glucosinolate, methylglucosinolate (glucocapparin), and an oxazolidinethione-generating glucosinolate (2-hydroxy-2-methylbutylglucosinolate, or glucocleomin) (Kjaer and Thomsen, 1963). We found relatively high concentrations of methylglucosinolate in gas chromatographic analysis. In fact, several plants had concentrations which were suprisingly high (46–56 mg/g, dry wt: Blua, personal observation). We found

TABLE 1. SEED PRODUCTION AND METHYLGLUCOSINOLATE CONCENTRATION FOR OPPOSITE SECTIONS OF SOIL MOISTURE GRADIENT AT SITE 2, SEPTEMBER 1984

Distance along gradient	Number of plants	Plants with undamaged capsules (viable seeds/plant)[a,b]				Methylglucosinolate concentration (mg/g, dry wt)[c]		
		N	%	$\bar{X}$	(SE)	N	$\bar{X}$	(SE)
Drier half 0–15 m	37	8	21.6%	22.5	(3.33)	10	41.7	(10.27)
Wetter half 15.1–30	49	8	16.3%	9.0	(2.80)	11	12.0	(3.83)

[a] For $N = 16$ plants with viable seeds, there is a significant negative regression ($r = -0.63$, $P < 0.01$) for the number of viable seeds produced after capsule predation (square-root transformation) and plant position on the gradient [$N = 16$, $Y = 5.5 - 0.105\ X$, where Y = viable seeds per plant for plants with any seeds, X = distance (m) from the dry end of the gradient].

[b] One-way analysis of variance (on square-root transformed number of seeds per plant) shows location effect was highly significant: $F_{1,14} = 12.395$ ($P < 0.01$), with treatment SS = 13.288 (1 df) and error SS = 15.009 (14 df).

[c] One-way analysis of variance (on square-root transformed methylglucosinolate concentration) shows location effect was highly significant: $F_{1,19} = 21.07$ ($P < 0.01$), with treatment SS = 61.606 (1 df) and error SS = 55.562 (19 df).

no indication of the oxazolidinethione-yielding glucocleomin in the paper chromatographic spot test of extracts of *Cleome serrulata.*

Methylglucosinolate concentrations decreased as soil moisture availability increased along the gradient, both at site 1 (Figure 3) and at site 2 (Figure 4). A relatively high proportion of the variance in concentration was explained by position along the gradient: 40% in site 1 and 52% in site 2, semi-log scale.

Concentrations of methylglucosinolate were negatively correlated with both plant height and plant diameter ($r = -0.56$ to -0.77; Table 2). Plant size (as measured by plant height or by plant size index) was also negatively correlated with soil moisture availability, especially in site 2 ($r = -0.71$). A partial correlation analysis showed that when the variation due to plant size was removed, the strong negative relationship between methylglucosinolate concentration and soil moisture availability remained.

DISCUSSION

The results of our study are consistent with the set of hypotheses generated by the converging data from phytochemistry, entomology, and plant ecology. Lower levels of foliage damage (Figure 2), as well as lower levels of capsule damage, with higher production of viable seed after insect predation (Table 1) were associated with higher levels of the characteristic glucosinolate, methylglucosinolate (Figures 3 and 4). The effect of consistent between-year patterns of seed consumption will be cumulative since *Cleome serrulata* is an annual

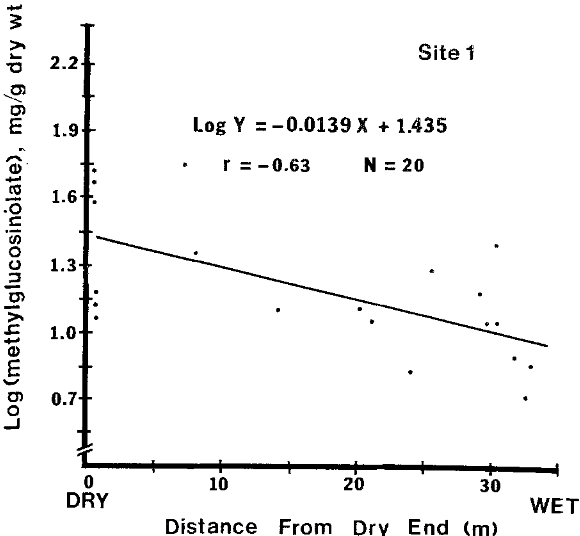

FIG. 3. The relationship between log (methyloglucosinolate) concentration and plant position along the soil moisture gradient at site 1 on August 14, 1984.

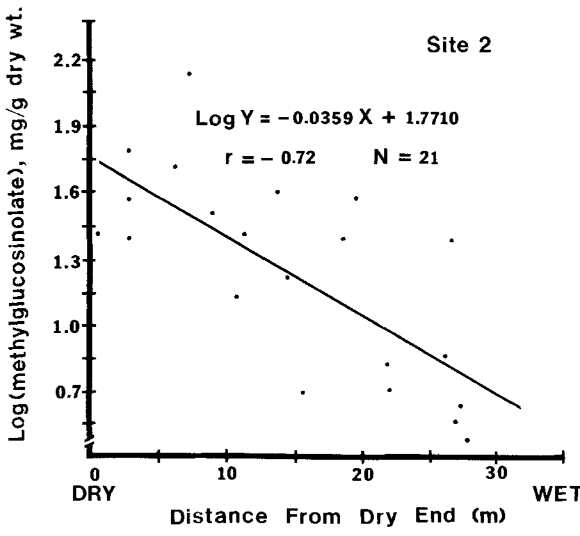

FIG. 4. The relationship between log (methylglucosinolate) concentration and plant position along the soil moisture gradient at site 2 on August 14, 1984.

TABLE 2. PLANT SIZE AND METHYLGLUCOSINOLATE CONCENTRATIONS, $\overline{X}$ (SE), IN RELATION TO PLANT POSITION ALONG SOIL MOISTURE GRADIENT, AUGUST 1984

	Mean distance (m)	N	Plant Size[a]			Methylglucosinolate concentration (mg/g)[c]
			Height (cm)	Diameter (cm)	Index[b] (cm^2)	
Site 1						
Wet	31.0 (0.55)	7	26 (12.8)	44 (7.1)	61 (8.7)	11.8 (2.52)
Mid	22.8 (1.23)	4	91 (15.2)	43 (12.8)	62 (13.6)	12.6 (2.61)
Dry	3.3 (1.75)	6	76 (10.8)	34 (7.4)	46 (8.0)	30.0 (6.20)
Site 2						
Wet	25.6 (0.92)	7	100 (3.5)	52 (6.0)	72 (4.8)	7.6 (2.76)
Mid	15.0 (1.26)	7	80 (9.2)	37 (6.0)	54 (7.3)	23.1 (4.72)
Dry	4.6 (1.21)	7	60 (5.4)	19 (3.1)	33 (4.0)	52.0 (14.77)

[a] Correlations of plant size and soil moisture with $r = -0.42$ to -0.71 ($P < 0.05$ for all cases).
[b] Plant size index = (Ht) × (Diam).
[c] Correlations of plant size (height, diameter in cm) and methylglucosinolate (mglg, dry wt): all with $r = -0.56$ to -0.77 ($P < 0.01$ for all cases).

plant. Consequently, we conclude that methylglucosinolate functions as a defensive compound for this annual caper. In addition, there may be a genetic basis for this variation along the gradient. Farris (1985) has, for example, demonstrated that there is a genetic component to the distribution of drought resistance along this gradient.

Also, greater secondary compound concentrations were observed for smaller plants at the harsher (=drier) end of the soil moisture gradient. Low concentrations of the main qualitative chemical defense compound were observed in plants at the wet portion of the gradient (Figures 3 and 4). In this dry habitat, the wet part of the gradient is the physiologically more benign area for plant growth. Interestingly, our observations support Janzen's (1974) prediction and McKey et al.'s (1978) observation that higher defenses are required, or observed, under harsher or more limiting conditions. They are also consistent with Coley et al.'s (1985) hypothesis that slower-growing plants of resource-limited environments should have a greater investment in chemical defense than fast-growing plants in better environments. However, in our case, the defenses

were qualitative ones (glucosinolates) instead of the quantitative ones discussed by Janzen (1974), McKey et al. (1978), and Coley et al. (1985). Also, our variation was intraspecific, rather than the interspecific variation considered in the other studies.

In some cases carbon and nutrient availability may determine defensive strategy and limit plant production of defensive chemcials (Mooney and Chu, 1974; Bryant et al., 1983; Coley et al., 1985). However, in this case, photosynthetic carbon gain does not appear to limit production of glucosinolates. Carbon gain efficiency should be higher at the wet end of our gradients since this was a very dry environment. Yet concentrations of methylglucosinolate were lower at the wet end. These negative findings are similar to those for: (1) another caper–insect interaction (Blua and Hanscom, unpublished data), (2) Douglas fir–spruce budworm interactions (Cates et al., 1983; Cates and Alexander, 1982; Redak and Cates, 1984), and (3) for lepidopteran herbivory on *Diplacus aurantiacus* (Scrophulariaceae) in central California (Lincoln and Mooney, 1984).

The net effect of plant position on the soil moisture gradient for plant reproductive success was the result of two conflicting sets of pressures: physical and biotic. First, physical conditions for plant growth in this dry habitat were better at the wet end; so, greater plant growth and subsequently larger vegetative size and greater reproductive potential were associated with the higher soil moisture levels (Table 2). Plants at the dry end of the soil moisture gradient were under water deficit stress by the end of the growing season (Farris, 1985). Water stress also increases glucosinolate concentrations in related cases, e.g., in black mustard (*Brassica nigra*) (Wolfson, 1980, 1982) and in bittercress (*Cardamine cordifolia*) (Louda and Rodman, 1983a,b; Louda, 1986a,b). However, water stress can increase insect feeding as well as defensive compound concentrations, e.g., on herbaceous plants by generalized insects such as grasshoppers (Lewis, 1979, 1984) and on Douglas fir by spruce budworm (Cates et al., 1983; Cates and Alexander, 1982; Redak and Cates, 1984). Our results are in contrast with those for another small shrub, *Diplacus aurantiacus* (Lincoln and Mooney, 1984), where no correlation occurred between damage and potential water stress differences between plants in sun and in adjacent shade habitats.

Secondly, however, the biotic set of pressures, such as insect herbivore feeding intensity in our case, were also more intense at the wet, more benign end of the gradient (Figure 2, Table 1). And, as noted above, concentrations of the qualitative defensive compound were higher at the harsh end of the gradient (Figures 3 and 4). Thus loss to insects—of foliage, capsules, and seeds—actually decreased as environmental harshness increased (Figure 2; Tables 1 and 2) and, thus, as potential resource limitation increased. The same pattern, of higher loss of flowers and developing seeds in the more benign portion of more extensive (80 km) environmental gradient, was found for one southern

California shrub, *Haplopappus squarrosus* (Louda, 1982b), but not for its overlapping congener, *H. venetus* (Louda, 1983). Unfortunately, no chemical evidence is available from that system.

In sum, the production of the antiherbivore qualitative defense chemical by *Cleome serrulata* was not proportional to the intensity of insect herbivory but, instead, to environmental harshness. Consequently, and contrary to usual expectation, plants that were in the harsher portion of the environment by physiological criteria were the ones that maximized seed reproduction and, other things being equal, plant fitness. This general result has particularly striking demographic consequences, since *Cleome serrulata* is an annual plant and is thus totally dependent upon seed production for population recruitment and persistence between years.

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HONEYBEE (*Apis mellifera* L.) QUEEN FECES: SOURCE OF A PHEROMONE THAT REPELS WORKER BEES

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Abstract—When placed in a small observation arena with workers, most young virgin honeybee queens released fecal (hindgut) material during agonistic interactions with workers and with each other. On release of this material, workers moved to the sides of the arena and groomed themselves. Bioassays of virgin queen fecal material demonstrated that it contains pheromone that repels workers and stimulates grooming behavior. Pheromone was present only in the feces of virgin queens that were more than 24 hr old and less than 2 weeks old. Feces of 2- to 4-day-old workers and virgin queens more than 2 weeks old did not elicit an avoidance response by workers. Moreover, the feces of young virgin queens had a strong fragrance, while that of older queens had a rancid odor and that of young workers had no detectable odor.

Key Words—Honeybee, *Apis mellifera*, Hymenoptera, Apidae, feces, queen pheromone, queen acceptance, repellent.

INTRODUCTION

Queen honeybees have long been known to produce a variety of pheromones that are involved in integrating colony behavior. The mandibular glands produce substances (primarily 9-oxodec-*trans*-2-enoic acid) that suppress ovariole development and queen cell construction by workers (see reviews by Butler, 1967; Gary, 1974; Michener, 1974). Secondary queen substances are produced

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by epidermal glands located on the abdominal tergites (Velthuis, 1970). In addition, pheromone produced by Koschewnikow's glands located near the sting base (Butler, 1967), as well as the mandibular (Gary, 1961; Butler et al., 1973; Simpson, 1979) and tergal glands (Vierling and Renner, 1977), probably have a role in the attraction of workers to queens and in queen recognition. While the existence and function of pheromones in young virgin queens is not well known, little 9-oxodec-*trans*-2-enoic acid is produced by queens less than 3 days old (Butler and Paton, 1962; Velthuis, 1970), and the chemical composition of their mandibular secretion is very similar to that of workers (Crewe, 1982).

Honeybee colonies raise several new virgin queens in response to stimuli that vary with the age and physiological condition of the extant queen and in response to seasonal stimuli associated with the reproductive cycle. When newly emerged virgin queens encounter each other in the hive, they fight until all except one are stung and killed. By observing interactions between 2- to 4-day-old virgin queens in small arenas containing workers, Page and Erickson (1986) frequently found that during agonistic interactions one or both of the queens defecated. On release of the feces, the workers immediately moved away from the queens and clustered near the top of the arena, suggesting that a volatile substance was released. In this paper we describe laboratory studies of interactions between young virgin queens and workers that are confined in small observation arenas. We also present the results of bioassays of the responses of workers to material contained in the hindgut of queens.

METHODS AND MATERIALS

Rearing of Test Bees. Virgin queens and workers used for observations of virgin queen interactions were obtained from eight different colonies, while virgin queens used for the fecal bioassays came from four of the eight colonies. The queen of each colony was in her first season of egg laying during the virgin queen interaction studies (summer of 1984) and in her second season of egg laying during the bioassays of hindgut material (summer of 1985). Each queen of each colony was mated by instrumental insemination to two different unrelated drones. Thus each queen produced worker and virgin queen daughters belonging to two different subfamilies. Queen mothers were supersisters of each other (coefficient of relationship = 0.75).

Virgin queens were produced by grafting young larvae into queen cell cups that were placed in a queenright cell builder colony that was unrelated to the eight source colonies (see Laidlaw, 1979). Once the queen cells were provisioned and sealed by workers, they were placed into individual glass vials (4 dram) and incubated at 34°C until the queens emerged. The wooden base of each queen cell served as a lid for each vial.

Newly emerged queens were subjected to one of the following four treatments:

1. Provided with royal jelly and sugar candy. A small amount of sugar candy was placed in each glass vial, and the queen cells that contained some residual royal jelly were left in place over the tops of the vials. The vials containing queens were returned to the incubator for an additional 2–4 days. In order to determine when the fecal pheromone is produced by queens, some queens were sacrificed at various ages ranging from emergence to less than 48 hr old.

2. Provided with sugar candy only. Queens were treated as described above, except that queen cells were replaced with wooden tops to eliminate the source of royal jelly.

3. Fed by workers for 3–4 days. Queens were removed from the vials immediately after emergence and placed individually in 8-mesh screen cages, which were then inserted into an unrelated colony. The resident queen in the queen-storage colony was separated from the frames holding the young virgin queens by a queen excluder.

4. Fed by workers for 2–3 weeks. Queens were treated as described in (3) above, except they remained in the queen-storage colony for 2–3 weeks. The queens were then placed in individual glass vials, provisioned with sugar candy, and incubated for an additional two days.

Individual workers to be used as a source of feces were collected as they emerged from the brood comb, placed individually into glass vials provisioned with a small amount of sugar candy, and then subjected to one of the following treatments.

1. Provided with sugar candy and royal jelly. The queen cell cups from which queens emerged in treatments 2–4 above were placed over the top of each vial, providing a source of royal jelly for each worker. Each worker was incubated at 35°C for 2–4 days.

2. Provided with sugar candy only. The workers were treated as described in (1) above, except that vials were covered with wooden caps.

All sugar candy used in provisioning the glass vials was made from confectioner's sugar and came from the same batch.

Observations of Queen Interactions. Groups of ten supersister workers (members of subfamilies were phenotypically marked, see Page and Erickson, 1986) were collected at random from individual colonies by vacuuming them from the top bars of the brood chambers into 135-ml cardboard cups (arenas) with wire screen tops. Ten cups of bees were collected at a time and taken directly into the laboratory where behavioral observations began within 15 min after the collection of the last cup of bees. A single virgin queen from treatment 1 was introduced into each arena (cup of 10 bees) by tilting the arena on its side and inverting the vial containing the virgin queen over a hole in the side. Queens were observed individually for 5 min after introduction. Within 30 min to 6 hr after introduction, a virgin queen was removed from one arena and introduced into another that contained a previously introduced queen. Behav-

ioral interactions between the two queens and the workers were then observed for 5 min. Queens and workers were used just once, then discarded.

Procedures for Bioassays of Feces. Groups of 10 workers were collected in 235-ml cups as described above for the observations of queen interactions. Prior to the collection of bees, a 6.0-cm-diameter piece of filter paper with a 1.8-cm-diameter circle marked in the center was taped to the bottom of each cup for use during the bioassays. Cups were taken into the laboratory, and all bioassays with each group of 10 bees were completed within 1 hr.

Prior to each replicate, the feces of a test bee (queen or worker) was collected by immobilizing the bee by crushing its thorax. The terminal abdominal sternite and tergite were spread apart and a micropipet was placed over the anus. The abdomen was gently squeezed until all of the hindgut contents flowed into the pipet.

For each replicate, a test and a control observational arena (cup) were paired. The behavior of the workers was observed for 60 sec before and then for 60 sec after the introduction of feces in the test arena and for similar time periods before and following the introduction of 10–30 μl of distilled water (about equivalent to the volume of feces) in the control arena. The feces and distilled water were introduced into their respective arenas by using a rubber bulb to squeeze each substance out of a micropipet and into the circle on the floor of each arena. Three behavioral patterns of the workers were quantified during each control and test: (1) the number of workers that walked through the 1.8-cm-diameter circle on the bottom of the arena during each 60-sec observation period, (2) the number of bees that groomed their legs or antennae, and (3) the number of workers on the bottom of the arena at the end of each 60-sec observation period.

The responses of the workers to the tests and controls before and after the introduction of feces or distilled water, respectively, were summed for each treatment and analyzed using a χ^2 test of independence. All replicates in which more than five of the worker bees scent fanned were eliminated from the analysis because scent-fanning behavior (with exposure of the Nasonov gland) was interpreted as a consequence of external disturbances and the new surroundings of the bees.

RESULTS

Observations of Queen Interactions. Upon introduction into an arena of workers, 2- to 3-day-old virgin queens typically extended and bowed their bodies, lifted their mesothoracic pair of legs, and expressed a droplet of clear liquid from between open mandibles (N = 312 replicates). Workers usually imbibed the liquid, groomed the queen with their proboscis, and offered the queen a reciprocal droplet of clear liquid. Occasionally, one or more workers behaved

agonistically and grasped a body part of the queen (usually a leg or a wing) with their mandibles. When this occurred, queens often released a volume of liquid hindgut (fecal) material that was light yellowish-brown in color with a strong floral fragrance. Workers usually moved away from this substance to the top and sides of the arena and groomed themselves. Occasionally, queens released a very minute quantity of this hindgut material. Workers appeared to be highly attracted to small amounts.

When two young virgin queens encountered each other, one or both usually released their hindgut content on each other during biting and stinging behavior ($N = 81$ replicates). Workers responded by moving away from the queens and grooming. Many fights between virgin queens lasted for several minutes to several hours with intermittent short periods of biting and attempted stinging followed by longer periods of reduced activity. During periods of reduced activity, workers groomed and fed queens, although frequently queens with feces on their bodies were not cared for by workers or received less care than uncontaminated queens. Occasionally fights resulted in injury and eventual death to both queens.

Fecal Bioassays. At emergence as an adult, virgin queens did not have any hindgut material ($N = 10$). However, queens less than 24 hr old that emerged in glass vials provisioned with royal jelly and sugar candy had approximately 5–20 μl ($\overline{X} = 12.5$; $N = 8$) of material in their hindgut, while queens 24–48 hr old had about 10–30 μl ($\overline{X} = 22.1$; $N = 7$). All fecal material was light yellowish-brown in color and had a strong floral fragrance.

After the hindgut material of a queen from treatments 1 or 2 was introduced into the arena, worker bees moved away from the bottom, clustered on the side and top, and then groomed themselves. Significantly fewer workers walked through the circle and significantly fewer remained on the bottom of the arena after the introduction of feces from treatment 1, yet grooming was significantly more frequent after introduction of fecal material (Table 1). The results of bioassays using the hindgut content of virgin queens from treatment 2 were similar to those of treatment 1, except the decrease in the number of workers remaining on the bottom after 60 sec was not statistically significant. The cessation of walking and frequent grooming were evident 30 sec after the introduction of feces into the arena, and, generally, the workers turned away from the feces and/or clustered for as long as 2–3 min after its introduction.

The feces from 3- to 4-day-old queens fed by workers (treatment 3) elicited responses from workers similar to that of queens reared in glass vials (treatments 1 and 2) (Table 2). However, the feces of 2- to 3-week-old virgin queens fed by workers (treatment 4; Table 2) and 2- to 4-day-old workers (Table 3) did not elicit grooming behavior or repel the bees. There was no significant difference in the number of workers on the bottom of the arena or walking through the circle before and after the introduction of feces.

The feces of all the queens and workers less than 5 days old had a milky

TABLE 1. RESPONSES OF WORKER HONEYBEES TO FECES OF VIRGIN QUEENS REARED IN GLASS VIALS WITH ROYAL JELLY AND SUGAR CANDY (TREATMENT 1) OR WITH SUGAR CANDY ONLY (TREATMENT 2)[a]

	Before	After	χ^2	*P* value
Numbers of workers walking through circle				
Treatment 1				
Control	357	402	76.31	<0.001
Test	381	152		
Treatment 2				
Control	227	254	41.22	<0.001
Test	227	97		
Numbers of workers on bottom				
Treatment 1				
Control	26	38	6.80	0.009
Test	39	22		
Treatment 2				
Control	23	29	1.17	0.279
Test	17	12		
Numbers of workers grooming				
Treatment 1				
Control	25	34	18.70	<0.001
Test	21	124		
Treatment 2				
Control	13	22	6.76	0.009
Test	12	66		

[a] Data are the total numbers of workers walking through a 1.8-cm-diameter circle on the bottom of the arena during 60 sec before and after the presentation of the control (distilled water) and the test (feces), the total numbers of workers on the bottom of the arena, and the numbers grooming at the end of each 60-sec period. Data for the bioassays in treatment 1 and treatment 2 are based on 40 and 25 replicates, respectively, of each control and test.

yellowish-brown color, although the feces from workers had a slight greenish tint. In addition, the feces from young queens had a strong floral scent, while the feces of workers had no detectable odor. Conversely, the feces from 2- to 3-week-old queens fed by workers was clear with chunks of dark solid matter and had a rancid odor.

DISCUSSION

The rectal content of virgin queens less than 2 weeks old contains a pheromone that, under our test conditions, repels workers and stimulates grooming behavior. Production of this material is independent of the food source of virgin

TABLE 2. RESPONSES OF WORKER HONEYBEES TO FECES OF 3- to 4-DAY-OLD (TREATMENT 3) OR 2- to 3-WEEK-OLD (TREATMENT 4) VIRGIN QUEENS FED BY WORKERS[a]

	Before	After	χ^2	P value
Numbers of workers walking through circle				
Treatment 3				
Control	87	107	41.87	<0.001
Test	110	27		
Treatment 4				
Control	149	132	1.55	0.213
Test	96	107		
Numbers of workers on bottom				
Treatment 3				
Control	9	13	1.23	0.267
Test	10	7		
Treatment 4				
Control	11	12	0.32	0.569
Test	12	18		
Numbers of workers grooming				
Treatment 3				
Control	7	8	6.72	0.009
Test	3	24		
Treatment 4				
Control	1	0		
Test	4	5		

[a] The types of responses recorded are the same as in Table 1. The responses were recorded during 60-sec time periods. Data for treatment 3 and treatment 4 are based on 13 and 14 replicates, respectively, of each control and test.

queens. Although feces from young workers is somewhat similar in appearance to that of young queens and very different from that of old queens, our results show clearly that workers do not have the same material in their feces even when fed the same diet as young virgin queens. These results also corroborate the significance of the odor of the feces; only feces of young queens that had a strong floral fragrance elicited avoidance behavior by workers.

Renner and Baumann (1964) found that young virgin queens have a strong floral odor that apparently originates from tergal glands. This odor is found on the abdomens of virgin queens 4–18 days old, but not on newly emerged or very young laying queens (Boch et al., 1975). Although the function of this odor is not known, it does not appear to have a role in drone attraction during mating flights (Boch et al., 1975). Since our results indicate that a similar odor originates from material in the hindgut, it is possible that the odor detected in previous studies actually originated in the hindgut and spread over the abdomen.

TABLE 3. RESPONSES OF WORKER HONEYBEES TO FECES OF WORKERS REARED IN GLASS VIALS WITH ROYAL JELLY AND SUGAR CANDY (TREATMENT 1) OR WITH SUGAR CANDY ONLY (TREATMENT 2)[a]

	Before	After	χ^2	*P* value
Numbers of workers walking through circle				
Treatment 1				
Control	214	202	1.89	0.169
Test	209	162		
Treatment 2				
Control	274	299	0.11	0.741
Test	259	294		
Numbers of workers on bottom				
Treatment 1				
Control	15	23	1.21	0.271
Test	31	30		
Treatment 2				
Control	21	29	1.40	0.236
Test	19	42		
Numbers of workers grooming				
Treatment 1				
Control	8	9	0.07	0.791
Test	16	21		
Treatment 2				
Control	27	33	0.27	0.605
Test	19	42		

[a]The types of responses recorded are the same as in Table 1. The responses were recorded during 60-sec time periods. Data for treatment 1 and treatment 2 are based on 25 and 38 replicates, respectively, of each control and test.

The adaptive significance (if any) of the fecal pheromone of young virgin honeybee queens remains to be demonstrated. It may be important in inhibiting rejection of virgin queens by disrupting agonistic behavior of workers. In addition, it may provide a ritualistic element to the dominance fight of virgin queens and help ensure that a queen, and thus the colony, survives. Evenly matched virgin queens may inflict reciprocal damage on each other, reducing the potential for the colony to successfully produce a replacement queen. However, if workers use contamination of a queen with feces as a symbolic determinant of subordination or dominance, then they may aid in the final determination of colony succession by not feeding and grooming contaminated queens and thereby placing the contaminated queen at a disadvantage.

Pheromone in the feces may also provide labels used by workers in kin recognition. Page and Erickson (1986) demonstrated that virgin queens have

genetically determined labels and that workers learn these labels and respond differentially on the basis of genetic relationship. The location and nature of these labels are unknown.

Additional behavioral assays under less artificial test conditions are necessary to determine if queens and workers respond to the pheromone in the same way in a natural context and to determine the adaptive significance of the feces pheromone. Further studies also are needed to determine if the odor described by Renner and Baumann (1964) as emanating from the tergites actually originates from the hindgut, and, if it does not, then to determine the functional relationship of these two sources of pheromone. Identification and testing of specific chemical compounds from the fecal material to determine the active components would greatly facilitate investigations of the possible functional significance of this newly discovered honey bee queen pheromone.

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LIGHT-DEPENDENT EFFECTS OF ALPHA-TERTHIENYL IN EGGS, LARVAE, AND PUPAE OF MOSQUITO *Aedes aegypti*

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Abstract—Alpha-terthienyl is toxic to *Aedes aegypti* larvae in the dark, but its activity is much enhanced in the presence of ultraviolet light. The development of first-instar larvae treated with alpha-terthienyl and ultraviolet light was followed until the emergence of adults. The LC_{50} value for first instars was about 0.002 ppm. Practically all the larvae which survived 24 hr reached adulthood. Fourth-instar larvae were also sensitive to photochemical treatment. When their development into adults was followed, the LC_{50} value was 0.45 ppm. Contrary to earlier reports, alpha-terthienyl was also phototoxic in pupae, but not when the adults were about to emerge. The LC_{50} value was ca. 0.06 ppm for pupae which were 1 or 2 days old. This is the first example where the activity of a photoinsecticide has been demonstrated in pupae. Alpha-terthienyl did not affect the hatching of eggs.

Key Words—Alpha-terthienyl, *Aedes aegypti*, Diptera, Culicidae, photosensitization, insecticide.

INTRODUCTION

A recent report described delayed phototoxic effects encountered with three naturally occurring plant products, 8-methoxypsoralen, khellin, and sphondin (Kagan et al., 1986a). In these experiments, first-instar larvae of the mosquito *Aedes aegypti* were treated overnight in the dark with the sensitizer. After a brief irradiation with long-wavelength ultraviolet light, their development in the dark was monitored for the appearance of adults. This approach produced evidence of delayed phototoxic effects by compounds which had shown little or no activ-

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ity during short-term experiments. To date, highly active photosensitizers have not been investigated for delayed phototoxic effects. We therefore selected alpha-terthienyl for this study.

Alpha-terthienyl occurs naturally in the marigold (*Tagetes erecta*) and in many related plants of the family Compositae (Bohlmann and Zdero, 1985). It has a wide range of biocidal activity, particularly when the target organisms are simultaneously exposed to ultraviolet light.

The pronounced phototoxicity of alpha-terthienyl to immature insects was described by Towers (1980), Wat et al. (1981), Arnason et al. (1981a), Kagan et al. (1983, 1984a), Kagan and Chan (1983), Downum et al. (1984), Towers et al. (1984), Champagne et al. (1984), and Philogene et al. (1985). The potential for commercial applications of this natural product as a photoactive insecticide has become apparent, and one patent on this use has already been issued (Towers et al., 1984). However, alpha-terthienyl is also very phototoxic in nontarget aquatic organisms such as tadpoles and fish (Kagan et al. 1984b, 1986b), and this lack of selectivity may create a major obstacle to the use of this chemical.

Our study defines more carefully the response of immature *A. aegypti* to photochemical treatments with alpha-terthienyl under laboratory conditions, starting with eggs, first- and fourth-instar larvae, as well as with pupae.

Since we observed in our earlier screening experiments that resistance to dieldrin (DLS_1 strain) and to DDT (Trinidad strain) conferred no protection to *Aedes aegypti* larvae treated with alpha-terthienyl and light, compared to the wild type (Rock), all work reported herein was performed with the wild-type organism.

METHODS AND MATERIALS

Alpha-terthienyl was synthesized in the laboratory (Kagan and Arora, 1983). Prof. G. B. Craig, Jr., University of Notre Dame, provided the original eggs of the three strains (Rock, DLS_1, and Trinidad) of *A. aegypti*. Except where noted, all the experiments were conducted at room temperature. Fabric supports containing ca. 20 eggs were placed into 3 ml of sensitizer solution (6.7 and 1.1 ppm), and incubated for 30 min in darkness. One set of eggs was irradiated as described for larvae (see below). Hatching at 24 hr was scored by observation of eggs under a microscope and compared to that in dark controls. In the morning, eggs were placed in aged tap water in an incubator at 28°C. First-instar larvae were collected in the afternoon. For each experiment 10 larvae were transferred into a 6-ml vial, almost all the water was removed, and 3 ml of the appropriate solution (Table 1) were added under very dim incandescent light. The number of replicates at each concentration and illumination var-

TABLE 1. SUMMARY OF EXPERIMENTAL CONDITIONS AND RESULTS FOR TREATMENT OF LARVAE WITH ALPHA-TERTHIENYL

Stage	Concentration (ppm)	Treatment	Replicates	Mean % survival (SEM)
1st instar	6.7	Dark	4	0 (0)
	1.1	Dark	3	0 (0)
	0.03	Dark	5	82 (5)
	0.03	Light	4	0 (0)
	0.03	pre-irrad	4	86 (6)
	0.005	Dark	8	82 (6)
	0.005	Light	9	10 (7)
	0.0009	Dark	7	78 (7)
	0.0009	Light	8	78 (9)
	0.0001	Dark	7	72 (7)
	0.0001	Light	8	84 (5)
4th instar	6.7	Dark	2	0 (0)
	1.1	Dark	3	93 (3)
	1.1	Light	3	0 (0)
	0.03	Dark	3	93 (7)
	0.03	Light	3	93 (7)
	0.005	Dark	3	97 (6)
	0.005	Light	3	100 (0)

ied between three and nine (Table 1). All samples were incubated overnight in the dark.

The next morning, one set of larvae was kept as controls, and the other was irradiated for 30 min under a bank of eight lamps (RPR-3500A from Southern New England Ultraviolet Co, Hamden, Connecticut) mounted 5 cm apart. These lamps emit only between 300 and 400 nm, with a maximum at 350 nm. The light intensity, measured with a Yellow Springs Radiometer model 65, was 15 W/m^2. Each set of larvae was transferred into a Petri dish containing 40 ml of water, to which was added 1 ml of a 16-g/liter aqueous suspension of liver powder (ICN Pharmaceuticals, Cleveland, Ohio). The transfers were performed in a darkroom dimly lit under an amber Kodak OC Safelight filter. Controls and irradiated larvae were then kept in the darkroom for the duration of the experiments. Surviving organisms were counted every day afterwards and were fed in the same manner three and six days after irradiations.

Experiments with fourth-instar larvae were conducted identically, except that the incubation period with sensitizer was only 30 min. Food was provided only once, immediately after irradiation.

Once daily on successive days, all pupae which had appeared from the

TABLE 2. SUMMARY OF EXPERIMENTAL CONDITIONS AND RESULTS FOR IRRADIATION OF PUPAE IN PRESENCE OF ALPHA-TERTHIENYL

Concentration (ppm)	Age (days)	Replicates	Mean % survival (SEM)
6.7	1	6	12 (5)
	2	6	13 (5)
	3	4	43 (9)
2.2	1	2	5 (5)
	2	2	5 (5)
1.1	1	15	1.3 (1)
	2	15	3.3 (2)
	3	15	66 (9)
0.4	1	2	5 (5)
	2	2	0 (0)
	3	2	78 (11)
0.2	1	4	25 (9)
	2	6	12 (5)
	3	4	63 (10)
0.06	1	2	20 (20)
	2	2	50 (0)
	3	2	100 (0)
0.03	1	6	88 (8)
	2	6	96 (4)

same pool of fourth-instar larvae were collected and kept separately. On the third day, samples of 10 pupae from each of the three age groups were placed in vials and incubated in the presence of sensitizer for 30 min, as previously described with larvae (Kagan et al., 1986a). After 30 min of irradiation, all pupae were transferred into Petri dishes containing 40 ml of water. Adult emergence was determined six days after collection of the pupae (Table 2). In order to study the sex dependence of phototoxicity in pupae, a large pool of synchronized first-instar larvae was used. Pupae were collected daily and treated with 1.12 ppm of alpha-terthienyl as described above. Sex determination was made six days later.

In all cases, the fate of organisms irradiated in the presence of sensitizer was compared to that of dark controls which had been treated with equal amounts of sensitizer. The fate of organisms placed in sensitizer solutions which had been previously irradiated for 30 min was also determined using the same procedures. LC_{50} estimates were obtained by visual inspection of mortality curves. Differences in mortality data were analyzed by Mann-Whitney or Kruskal-Wallis tests followed by Dunn a posteriori contrasts (Zar, 1984).

RESULTS AND DISCUSSION

Phototoxicity in Eggs. The hatching of eggs treated with 6.7 ppm alpha-terthienyl [mean 81.9%, standard error of mean (SEM) 6.8%] was not significantly different ($P > 0.5$) from dark controls (87.8%, SEM 6%). Similar results were obtained at 1.1 ppm (irradiated 82%, SEM 5.3%; controls 80.2%, SEM 11%; $P > 0.5$). These results confirm the lack of effect on hatching reported by Arnason et al. (1981a). While the concentrations of alpha-terthienyl did not prevent normal hatching, they were fatal for liberated larvae.

Phototoxicity in Larvae. Alpha-terthienyl is toxic to first-instar larvae of *A. aegypti* in the dark (Figure 1). For example, all larvae died within 24 hr at concentrations of 6.7 and 1.12 ppm. At 0.03 ppm, practically all (94%, SEM 3%) the larvae survived 24 hr, and a large proportion of them developed normally into adults (82%, SEM 6%; Figure 2A). At this concentration, however, all larvae were dead after 30 min under ultraviolet light (Figure 2A). The survival of larvae treated in the dark with a 0.03 ppm sensitizer solution which had been previously irradiated for 30 min produced the same percentage of emerging adults (86%, SEM 5%) as dark controls (not shown).

There was no toxicity without irradiation at 0.005 ppm; 86% of the initial larvae produced adults by the end of the experiments, a level comparable to untreated controls (Kagan et al., 1986a). However, phototoxicity at this concentration was still high, with 23% surviving the irradiation itself and only 12% surviving 24 hr later. Almost all of these survivors, however, developed normally into adults (Figure 2B).

Further dilution of the sensitizer, to 0.0009 ppm, removed all visible toxic

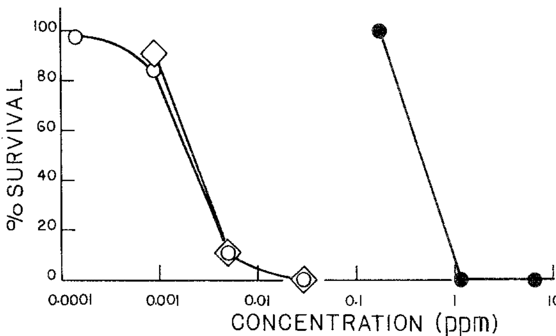

FIG. 1. Survival of *A. aegypti* following the treatment of first-instar larvae as described in text: in the dark (closed circles) and following 30 min of irradiation (open squares: 24-hr survival, open circles: adult survival). The concentration of alpha-terthienyl is shown on a logarithmic scale.

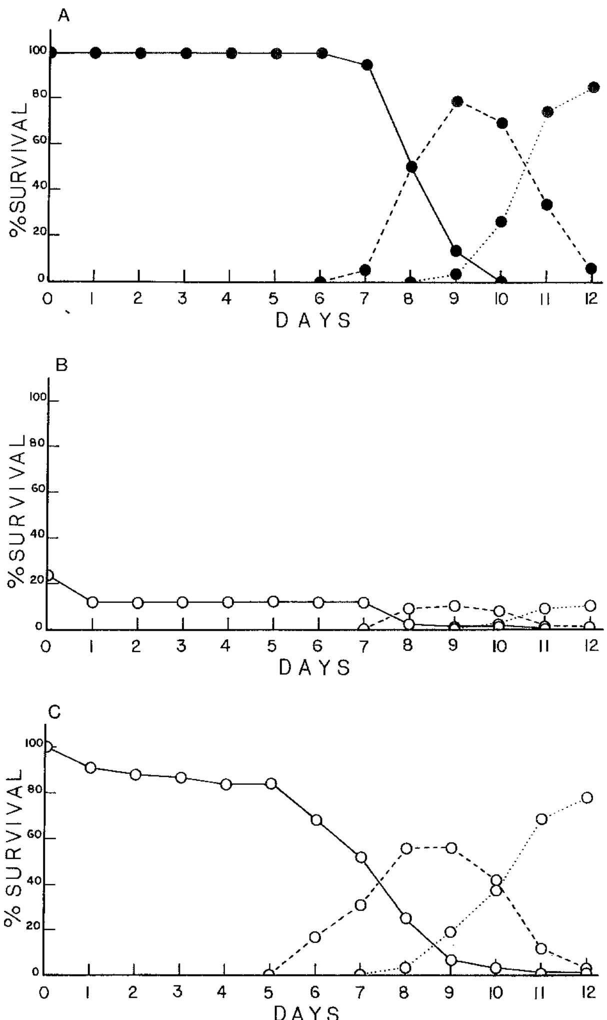

FIG. 2. Surviving larvae (solid lines), pupae (broken lines), and adults (dotted lines) obtained from first-instar larvae in the dark (closed circles) and irradiated (open circles), as a function of time. The alpha-terthienyl concentration was 0.03 ppm (A), 0.005 ppm (B), and 0.0009 ppm (C).

effects, with the same survivorship (78%, SEM 9%) to adults in the irradiated organisms and in the dark controls (78%, SEM 7%; Figure 2C). An LC_{50} value of 0.0018 ppm was measured graphically for the surviving organisms on day 12, the arbitrary end of the experiments determined from survival curves. For comparison, the LC_{50} value determined in the same manner from survival data

determined 24 hr after irradiations was 0.002 ppm (Figure 1). The nearly identical value for survivorship determined 12 days later contrasts with results observed with several phenolic derivatives which displayed significant delayed mortality (Kagan et al., 1986a).

In an earlier work, we demonstrated that the activity of the enzyme acetylcholinesterase was severely reduced in *A. aegypti* larvae during exposure to ultraviolet light in the presence of alpha-terthienyl (Kagan et al., 1984a). Such a change, as well as perhaps the photosensitized inactivation of other enzymes which can be rapidly regenerated by the organisms, could possibly explain the negligible delayed toxicity of alpha-terthienyl following irradiation. If the enzyme(s) activity remains above a critical value and can later be regenerated to optimum level, normal development of the organism is possible. Another reasonable explanation is based on the damage of cell membranes photoinduced by alpha-terthienyl (Downum et al., 1982; Yamamoto et al., 1984). The production of singlet oxygen by alpha-terthienyl has been well characterized by chemical (Bakker et al., 1979; Gommers et al., 1980; Arnason et al., 1981b; Kagan et al., 1984a; McLachlan et al., 1984) and by physical methods (Reyftmann et al., 1985). Damage to membranes by singlet oxygen can possibly disrupt water and ion balance. Anal papillae are delicate structures involved in ion balance in mosquito larvae (Wigglesworth, 1973) and thus may be especially sensitive. Should the damage be limited to parts of the organism which are lost during molting, each surviving larva at its next instar would have shed its damaged components and become able to complete its development unimpaired.

Arnason et al. (1981) reported data for UV-treated first-instar larvae showing 98% survival at 0.1 ppm and 0% at 0.3 ppm. The LC_{50} under these conditions was therefore certainly in excess of 0.1 ppm, perhaps close to 0.2 ppm. We determined the LC_{50} for first-instar larvae to be 0.002 ppm. Differences in light intensity [listed at 6 W/m^2 in an earlier paper by Wat et al. (1981) compared to 15 W/m^2 in ours] and in chemical doses at each concentration level (10 organisms in 3 ml in our work, 25 organisms in 2 ml in theirs, or a 3.75-fold factor) may have contributed to the lack of quantitative agreement between the two studies. Our results for the phototoxicity of alpha-terthienyl in fourth-instar larvae (LC_{50} = 0.5 ppm after 24 hr) are in good agreement with Arnason et al. (1981a), who recorded 52% survival at 1 ppm and 0% at 3 ppm. Their LC_{50} value must therefore have been very close to 1 ppm.

When the development of photosensitized fourth-instar larvae into adults was followed, the LC_{50} value determined visually from the survival curve was 0.45 ppm, compared to 3.5 ppm for the dark controls (Figure 3). The similarity of 24-hr and cumulative-mortality LC_{50}s again underscores the different mode of action of alpha-terthienyl and the phenolic compounds (8-methoxypsoralen, sphondin, and khellin) investigated earlier.

Phototoxicity in Pupae. Arnason et al. (1981a) reported the pupae of *A. aegypti* were not affected 24 hr after a photochemical treatment with alpha-

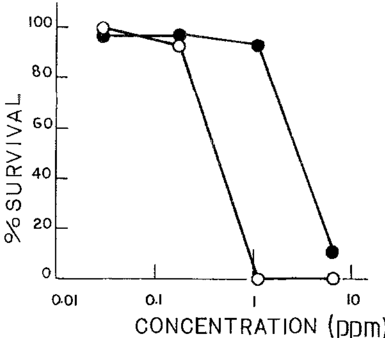

FIG. 3. Surviving organisms following the treatment of fourth-instar larvae with alpha-terthienyl, 24 hr after irradiation (closed circles) and through adult emergence (open circles). The sensitizer concentrations are shown on a logarithmic scale.

terthienyl up to 10 ppm. While we confirmed that no toxicity could be detected over a 24-hr period following irradiations at up to 6.7 ppm, a more careful analysis over the period of time required for adult emergence led to a different conclusion. A systematic study disclosed that the level of phototoxicity for alpha-terthienyl diminished as photochemically treated pupae approached adult emergence.

These experiments indicated that 3-day-old pupae were largely unaffected by the treatment (Figure 4). Adults often emerged during the irradiation of these pupae. Younger pupae were more sensitive to the phototoxic effects of alpha-terthienyl. Practically no difference was found between the survival of 1- and 2-day-old pupae. The survival curves shown in Figure 4 indicate a LC_{50} value of ca. 0.06 ppm for these pupae.

Other experiments were designed to probe whether a sex difference might be responsible for the differential response of pupae according to their age. When series of daily cohorts from a synchronized population were treated with 1.1 ppm of alpha-terthienyl and irradiated, no significant difference in survival was observed for 1-, 2-, or 3-day-old pupae collected over five days (Kruskal-Wallis tests, $Ps > 0.10$). The data from daily collections were pooled and are shown in Table 2. A Kruskal-Wallis test detected a significant difference among the three groups ($P = 0.0001$). It is obvious that the significant difference is due to greater survival of 3-day-old pupae (Table 2), but this could not be statistically demonstrated with a posteriori Dunn contrasts.

Since males emerge slightly ahead of females (Haddow et al., 1959), the age dependence expressed on Figure 4 could have arisen from a depletion of the pool of male organisms during the first two days and be linked to a lesser sensitivity of the remaining females. Actually, a direct analysis of the pupae treated in the experiment described above disclosed that the pupae collected each day essentially had the same sex ratio (range 36.6–54.5% females). The

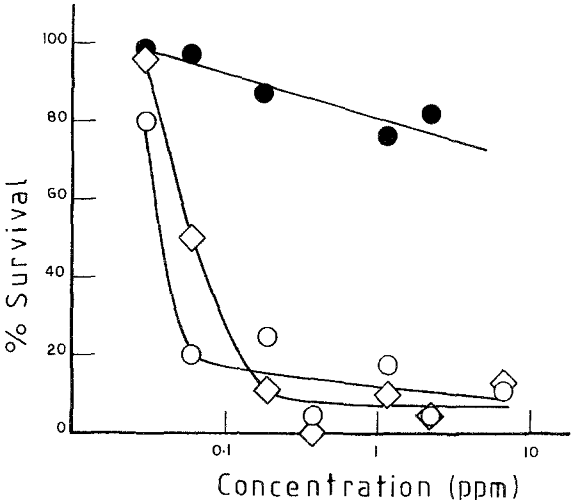

FIG. 4. Survival of pupae through adult emergence following irradiation in the presence of alpha-terthienyl. The age of the pupae at the time of the irradiation was 0–1 day (open circles), 1–2 days (open squares), and 2–3 days (closed circles).

difference in sensitivity to the photosensitized treatment between 3-day-old and younger pupae must therefore be related to developmental changes. For example, decreased sensitivity in 3-day-old pupae is correlated with increased pigmentation of these pupae compared to younger ones: the 3-day-old pupae have complete imaginal pigmentation, and 1- or 2-day-old pupae are mostly unpigmented. It is uncertain whether the amount of pigmentation is directly protective or merely a marker for other coincident phenomena.

CONCLUSION

Alpha-terthienyl was not effective in preventing hatching at the concentration tested. Although some toxicity of alpha-terthienyl to other immature forms of the mosquito *A. aegypti* can be observed in the dark, it is greatly enhanced by exposure to ultraviolet light, even for a short duration. The phototoxicity does not depend on light-mediated synthesis of toxic products, and it is likely to operate because of the extremely favorable photophysical characteristics of the sensitizer, which has a very long-lived triplet excited state, as well as a high quantum yield for energy transfer to oxygen, which produces singlet oxygen (Reyftmann et al., 1985). The detailed mechanism of phototoxicity is still unknown, but the fact that practically all larvae surviving 24 hr emerge as adults suggests that phototoxicity is immediate. Although the sensitizer's potency decreases as larvae mature, it is still appreciable in fourth-instar larvae. Also, the

LC_{50} value for 1- or 2-day-old pupae is one order of magnitude smaller than for fourth-instar larvae.

Our experiments provide the first evidence of phototoxicity to pupae by any sensitizer. Potency appears to be age-dependent since mature pupae (>72 hr) exhibit greatly reduced sensitivity to alpha-terthienyl. Inasmuch as it is not manifested over a 24-hr period, previous investigators (e.g., Arnason et al., 1981a) may have missed the expression of phototoxicity in pupae. Whether the delayed toxicity at this developmental stage indicates a mechanism different from that operating in larvae remains to be established.

Finally, it is instructive to compare the phototoxicity of alpha-terthienyl in *A. aegypti* with that in *A. atropalpus* (Philogene et al., 1985). Sunlight irradiations used in the latter work yielded LC_{50} values between 0.010 and 0.050 ppm, depending on testing conditions. These LC_{50}s are similar to those found in this study. It is clear that, under our experimental conditions, alpha-terthienyl is much more phototoxic to immature *A. aegypti* than the phenolic compounds 8-methoxypsoralen, khellin, and sphondin (see Philogene et al., 1985; Kagan et al., 1986a).

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FAILURE OF TANNIC ACID TO INHIBIT DIGESTION OR REDUCE DIGESTIBILITY OF PLANT PROTEIN IN GUT FLUIDS OF INSECT HERBIVORES:
Implications for Theories of Plant Defense

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Abstract—The rate of hydrolysis of the abundant foliar protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC), in enzymatically active gut fluid of *Manduca sexta* larvae is very rapid and is unaffected by the presence of tannic acid, even when tannic acid is present in the incubation mixture in amounts in excess of the amount of RuBPC. When this protein is dissolved in the denatured gut fluids of *M. sexta* larvae or *Schistocerca gregaria* nymphs, large amounts of tannic acid must be added to bring about the precipitation of significant quantities of protein. The ability of insect gut fluid to prevent the formation of insoluble tannin–protein complexes is due to the presence of surfactants. On the basis of our results and a review of the findings of other investigators, we argue that there is no evidence that tannins reduce the nutritional value of an insect's food by inhibiting digestive enzymes or by reducing the digestibility of ingested proteins and, further, that the failure of tannins to interfere with digestion is readily explained on the basis of well-documented characteristics of the digestive systems of herbivorous insects. In challenging the currently popular notion that tannins are digestibility-reducing substances, we do not challenge the general utility of either the apparency theory or resource availability theory of plant defense. In debating the merits of these two analyses of plant–herbivore interactions, however, the demise of tannins as all-purpose, dose-dependent, digestibility-reducing defensive substances must be taken into account.

Key Words—Tannins, digestibility-reducing substances, surfactants, detergency, herbivory, chemical defense, allelochemics, *Manduca sexta*, *Lepidoptera*, *Sphingidae*, *Schistocerca gregaria*, Orthoptera, Acrididae.

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INTRODUCTION

Ingested tannins interfere with the normal growth and development of many foliage-feeding insects, and this class of allelochemicals has been accorded an important role in protecting vascular plants from herbivory (Feeny, 1976; Rhoades and Cates, 1976; Swain, 1979; Coley et al., 1985). Tannins are known to be protein precipitants (Van Sumere et al., 1975; Hagerman and Butler, 1981; McManus et al., 1983), and it has been proposed that they might interfere with protein digestion in an herbivore's gut by binding proteolytic enzymes or ingested proteins (Feeny, 1976; Rhoades and Cates, 1976). Since the growth of many herbivores is nitrogen limited (Mattson, 1980), any interference with protein digestion could have a severe negative impact on fitness. While there is evidence that tannins act as digestion-inhibiting substances in certain species of vertebrates, possibly by interfering with the growth and metabolism of rumen microorganisms (Waterman et al., 1980, 1984), to date there has been no experimental demonstration that tannins ever act as digestibility-reducing or digestion-inhibiting substances in any herbivorous insect species (Bernays, 1981; Martin and Martin, 1984; Martin et al., 1985). The adverse effects that dietary tannins have on the growth and development of tannin-sensitive insects appear to be due instead to the properties of these polyphenols as feeding deterrents (Klocke and Chan, 1982; Reese et al., 1982; Manuwoto et al., 1985; Manuwoto and Scriber, 1986) and toxins (Berenbaum, 1984; Bernays et al., 1980; Manuwoto et al., 1985; Manuwoto and Scriber, 1986; Steinly and Berenbaum, 1986).

Several characteristics of the digestive systems of insects have been identified which counter the potential anti-digestive properties of tannins. It has been proposed that high gut alkalinity is an antitannin adaptation in lepidopteran larvae (Feeny, 1970; Berenbaum, 1980) and that detergency is a widespread characteristic of insect gut fluids that prevents tannins from precipitating ingested proteins (Martin and Martin, 1984; Martin et al., 1985). In addition, some species of acridids possess digestive β-glucosidases that are able to degrade hydrolyzable tannins and peritrophic membranes that adsorb tannins and remove them from the gut milieu (Bernays, 1981).

In this study we have assessed the capacity of tannic acid to act as a digestion-inhibiting or digestibility-reducing substance in the tobacco hornworm, *Manduca sexta*, and the locust, *Schistocerca gregaria*. *M. sexta* larvae do not normally consume tannin-containing foliage and would not be expected to have evolved specific adaptations to counter the potential adverse effects of tannins, while *S. gregaria* feeds on the foliage of many plant species containing tannins and is quite tolerant of these polyphenols (Bernays, 1981). We have explored the effect of added tannic acid on the rate of hydrolysis of the abundant foliar protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC), in enzy-

matically active gut fluid of *M. sexta* at pH 10.2–10.6 and have measured the amount of RuBPC precipitated from the denatured gut fluids of *M. sexta* and *S. gregaria* at pH values near neutrality by various amounts of tannic acid. We have conducted these experiments at tannin–protein ratios ranging from zero to values in excess of unity, a range which probably includes the values typical of most natural vegetation. Finally, we have performed experiments to establish which components of the gut fluid are responsible for its ability to prevent the formation of insoluble complexes between tannic acid and RuBPC. Our results do not support the idea that tannins are digestion-inhibiting or digestibility-reducing substances in insects.

METHODS AND MATERIALS

Culturing of Insects. Fifth-instar *M. sexta* larvae were reared from eggs on an artificial agar-based diet (Yamamoto, 1969) supplemented with wheat-germ oil (2.99 g/1000 g diet). Alternatively, larvae were reared on artificial diet through the third instar, at which time they were transferred to tomato plants and allowed to feed through the fifth instar on tomato foliage. Late-instar larvae were also collected from tomato plants growing in a tomato field in Washtenaw County, Michigan. Fifth-instar *S. gregaria* nymphs were raised in crowds on bran and wheat seedlings at The Centre for Overseas Pest Research, London, U.K.

Determination of Minimum Gut Passage Time. Ten fifth-instar larvae, weighing between 3.272 and 5.004 g (the size range used as a source of gut fluid), were placed in Petri dishes containing a cube of artificial diet amended with a small amount of finely divided charcoal. After feeding for 30 min, the larvae were transferred back to diet lacking charcoal. Frass pellets were collected every half hour for the next 10 hr and were inspected visually for the presence of charcoal particles. In separate experiments, it was established that the rates of feeding and pellet production by larvae that had ingested charcoal-amended diet were no different from the rates for larvae that had not fed on the charcoal-amended diet.

Collection of Gut Fluid. Gut fluid was collected from actively feeding fifth-instar *M. sexta* larvae. After making an incision encircling the body, the intact midgut was exposed by gently pulling the two halves of the body apart. The exposed midgut was rinsed with distilled water, blotted dry, and slit longitudinally, allowing the contents to flow into a centrifuge tube chilled in an ice bath. Following centrifugation (12,000*g*, 10 min, 5°C), the supernatant fluid was either frozen and stored at −16°C or denatured by heating at 100°C for 10 min, centrifuged as before, lyophilized, and stored dry at −16°C. There was no significant loss of proteolytic activity in active gut fluid stored in a

frozen state at −16°C even after several months. Gut fluid from 3- to 5-day-old (±12 hr) fifth-instar nymphs of *S. gregaria* was obtained both by inducing regurgitation through handling and by expressing gut contents from a small hole cut at the foregut–midgut junction of the excised gut. Gut fluid was removed with 100-μl capillary tubes and collected in 2-ml portions in chilled vials, denatured by heating at 100°C for 15 min, lyophilized, and air-mailed from London, U.K., to Ann Arbor, Michigan. Upon arrival in Ann Arbor, the lyophilized material in each vial was redissolved by adding water (3 ml). The mixture was centrifuged (12,000*g*, 10 min, 20°C), the supernatant solutions from all of the vials were combined, and the pooled preparation was lyophilized and stored dry at −16°C.

Fractionation of Gut Fluid. Nine volumes of 95% ethanol were added to one volume of gut fluid, and the mixture was allowed to stand for 18 hr at 5°C. Following centrifugation (12,000*g*, 10 min, 20°C), the supernatant solution was concentrated almost to dryness on a rotary concentrator at reduced pressure. The concentrated solution was then lyophilized and stored dry at −16°C. This fraction, which contains all solutes not precipitated by ethanol, was designed fraction A. The precipitate from the ethanol treatment was placed under a gentle stream of nitrogen to remove traces of ethanol, dissolved in water (two times the volume of the original gut fluid), and filtered through a YM-10 membrane (Amicon Corp. Danvers, MA). Both filtrate and residue were lyophilized and stored dry at −16°C. The filtrate, which contained solutes precipitated by ethanol having molecular weights less than 10 kD, was designated fraction B. The residue, which contained solutes precipitated by ethanol having molecular weights greater than 10 kD, was designated fraction C.

pH Measurements. Measurements of pH were made on freshly collected *M. sexta* gut fluid or rehydrated *S. gregaria* gut fluid using a combination pH microelectrode (Microelectrodes, Inc.).

Surface Tension. Surface tension was measured using the horizontal thick-walled capillary apparatus of Ferguson (1943). Surfactant concentration, in multiples of the critical micelle concentration (CMC), was determined by measuring the surface tensions of a series of dilutions of the test sample, and noting the dilution at which there was an abrupt increase in surface tension (Martin and Martin, 1984). The CMC is the concentration at which there is a transition between the surfactant in the free, unassociated state and the micellar state. The diluent was 160 mM KCl–3 mM NaCl, chosen to maintain ionic conditions comparable to those typical in insect midgut fluids (Giordana and Sacchi, 1978).

Precipitation of RuBPC by Tannic Acid. Full-strength denatured gut fluid was reconstituted from lyophilized gut fluid powder by adding water (one third of the volume of the gut fluid from which the powder was originally derived) and an equal volume of 0.3 M PIPES buffer [piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid)], pH 6.8, and adjusting the pH of the mixture to 6.8 by the

dropwise addition of either HCl or KOH. Preparations of gut fluid from larvae from the agar-based artificial diet contained suspended lipid-like particles, which were removed by filtration through Celite. Water was added to the solution or filtrate to bring the final volume to that of the gut fluid from which the powder was derived.

Solutions containing the separate gut fluid fractions were prepared from the lyophilized powders by adding water (one sixth of the volume of the gut fluid from which the powder was originally derived) and an equal volume of 0.3 M PIPES buffer, pH 6.8, adjusting the pH to 6.8 by the dropwise addition of either HCl or KOH, and finally adding water to bring the volume to one half the original volume of the gut fluid from which the fraction was derived. This solution, which was twice as concentrated as gut fluid, was then combined with either an equal volume of 0.1 M PIPES, pH 6.8, or with an equal volume of a solution of a different gut fluid fraction prepared in the same manner. A solution containing all three gut fluid fractions was prepared by combining solutions of fractions A and B with a weighed quantity of lyophilized fraction C.

RuBPC (Sigma R-8000, Lot 82F-7340) was added to reconstituted denatured gut fluid or solutions of the gut fluid fractions (2 mg RuBPC/ml), and the mixture was allowed to stand for 18 hr at 5°C, after which it was shaken for 10 min at room temperature and centrifuged (14,500*g*, 10 min, 20°C). The amount of RuBPC in solution was then determined using the assay procedure described below. Virtually all of the RuBPC dissolved in the *M. sexta* gut fluid, while only about three fourths dissolved in the *S. gregaria* gut fluid, perhaps because of the much higher concentrations of solutes already present in the *S. gregaria* fluid (see Table 3). To 250 μl of this solution was added, with vortexing, 25 μl of a freshly prepared solution of tannic acid (Sigma T-0125, lot 40F-0253) in 0.1 M PIPES, pH 6.8. After 10 min, the mixture was centrifuged (14,500*g*, 10 min, 20°C), the supernatant solution was decanted, and the pellet was rinsed carefully with 0.1 M PIPES, pH 6.8. The pellet was dissolved in 0.60 ml of a solution of 1% SDS in 0.05 M Tris, pH 7.5, and protein was precipitated by adding 0.15 ml of 90% trichloroacetic acid. The amount of protein precipitated was determined using the assay described below.

Proteolysis in Active M. sexta *Gut Fluid.* A mixture, prepared by adding 100 μl of a solution of RuBPC (10.8–14.0 mg/ml) in 1.0 M CAPS [3-(cyclohexylamino)propanesulfonic acid], pH 10.5, to 1 ml of active gut fluid, was incubated at 24°C. Duplicate aliquots (25 μl), removed after 5 and 15 min, were added to 0.15 ml of 90% trichloroacetic acid, and the amount of protein precipitated was determined using the assay procedure described below. When tannic acid was included in an incubation, it was added to the gut fluid immediately prior to the addition of the RuBPC solution. When the amount of tannic acid in the incubation mixture was 1.5 mg/ml, it was added as the dry solid; when it was 0.14 mg/ml, it was added in 10 μl of an aqueous solution. In

separate experiments it was established that proteins present originally in the gut fluid sample were stable and did not undergo autolysis or hydrolysis during incubation. Thus, the decrease in protein content during incubation was due to hydrolysis of the added RuBPC.

Protein Assay. Protein was measured using the method of Schaffner and Weissmann (1973) as adapted by Martin et al. (1985). Protein precipitated from a test solution by the addition of trichloroacetic acid was absorbed on a nitrocellulose membrane (0.45 μm) and stained with Amido black 10B. After removing excess unbound Amido black 10B, protein-bound dye was eluted and absorbance at 630 nm was determined. Absorbance at 630 nm resulting from substances other than RuBPC was determined from RuBPC-free blanks, and this value, which was always small, was subtracted from the observed absorbance of the test sample in order to give the absorbance due to RuBPC. Absorbance at 630 nm due to RuBPC was converted into micrograms of protein by the use of a calibration curve constructed from dilutions of a stock solution of RuBPC.

RESULTS

Rate of Passage of Food through Guts of Fifth-Instar M. sexta *Larvae.* When fifth-instar larvae were allowed to feed for 30 minutes on artificial diet to which finely divided charcoal had been added and then transferred back to charcoal-free diet, the average time interval between the initiation of feeding on the charcoal-containing diet and the appearance of charcoal in a fecal pellet was 2.6 hr (standard error 0.16). The last fecal pellet containing detectable charcoal particles was produced after 8.45 hr (standard error 0.31). Although this experiment does not permit an accurate calculation of the average length of time that a food particle is in contact with midgut digestive enzymes, it does clearly indicate that the time is measured in hours, not minutes.

Digestion of RuBPC in Active M. sexta *Gut Fluid.* The hydrolysis of RuBPC in undiluted enzymatically active *M. sexta* gut fluid at pH 10.2–10.6 is very rapid, whether tannic acid is present or not (Table 1). After only 5 min, digestion was 60–87% complete. Our attempts to assess the effects of added tannic acid on the extent of proteolysis after 5 min, when proteolysis was still in progress, were only partially successful, owing to the variability in our measurements of the amount of unhydrolyzed protein present in a solution in which protein content is changing rapidly. In the runs initially containing 1.08 and 1.18 mg/ml of RuBPC, the inclusion of tannic acid in the incubation mixture did not result in a reduction in the amount of hydrolysis after 5 min, while in the run initially containing 1.36 mg/ml of RuBPC, the inclusion of 1.50 mg/ml of tannic acid resulted in a 30% decrease in the amount of RuBPC hydro-

TABLE 1. EFFECT OF TANNIC ACID (TA) ON HYDROLYSIS OF RuBPC IN ENZYMATICALLY ACTIVE GUT FLUID FROM FIFTH INSTAR *M. sexta* LARVAE FED TOMATO LEAVES DURING LAST TWO INSTARS[a]

Incubation mixture		RuBPC digested [mg/ml (% of original)]	
RuBPC (mg/ml)	TA (mg/ml)	5 min	15 min
1.08	0.00	0.86 (80)	1.07 (99)
1.08	0.14	0.90 (83)	1.11 (103)
1.18	0.00	0.80 (68)	1.22 (103)
1.18	1.50	0.83 (70)	1.16 (98)
1.36	0.00	1.18 (87)	1.30 (96)
1.36	1.50	0.82 (60)	1.30 (96)

[a]The incubation mixture had a volume of 1.1 ml and a pH of 10.2–10.6. Values are the averages of duplicate determinations.

lyzed after 5 min. After 15 min, however, digestion was complete in all cases, even when tannic acid was present in the incubation mixture in excess of the RuBPC present. Since food is in contact with midgut digestive enzymes for a period far in excess of 15 min, it is clear that there is no significant interference with the digestion of RuBPC by tannic acid in the undiluted active gut fluid of *M. sexta* larvae.

Precipitation of RuBPC from Denatured M. sexta *Gut Fluid or Solutions of Gut Fluid Components by Tannic Acid.* Much larger quantities of tannic acid are required to bring about precipitation of an insoluble RuBPC–tannic acid complex from denatured *M. sexta* gut fluid adjusted to a pH of 6.7–6.9 than are required to precipitate this protein from an aqueous salt solution buffered at the same pH (Table 2). While 50 μg of tannic acid precipitates 465 out of 525 μg of RuBPC from a salt solution at pH 6.8, this amount of tannic acid precipitates only 7 out of 500 μg of this protein when it is dissolved in the gut fluid of larvae reared on an artificial diet. The effectiveness of the gut fluid as a solvent for RuBPC in the presence of tannic acid depends upon the dietary history of the larvae. Much more tannic acid is required to precipitate significant amounts of RuBPC from the gut fluids of larvae switched from artificial diet to tomato leaves during their final two instars or larvae which had been collected from tomato plants growing in a cultivated field and which had presumably fed on tomato foliage during their entire larval stage (Table 2).

Using a straightforward procedure involving precipitation with ethanol and ultrafiltration through a membrane with a nominal molecular weight cutoff of 10 kD, the gut fluid was separated into three fractions (Table 3). Fraction A contains all solutes not precipitated by ethanol, fraction B contains solutes pre-

TABLE 2. AMOUNTS OF RuBPC PRECIPITATED BY TANNIC ACID (TA) FROM AQUEOUS BUFFER AND DENATURED GUT FLUIDS FROM FIFTH-INSTAR *M. sexta* LARVAE AND *S. gregaria* NYMPHS[a]

	RuBPC in incubation mixture (μg)	RuBPC precipitated (μg) by			
		50 μg TA	75 μg TA	200 μg TA	300 μg TA
0.1 M PIPES buffer, pH 6.8, 160 mM KCl, 3 mM NaCl	525	465 ± 3 [3]	ND	ND	ND
Gut fluid from *M. sexta* larvae reared entirely on artificial diet	500	7 ± 2 [3]	35 ± 9 [4]	414 ± 4 [3]	480 ± 6 [3]
Gut fluid from *M. sexta* larvae switched from artificial diet to tomato leaves for last two instars	500	ND	ND	11 ± 2 [3]	92 ± 8 [3]
Gut fluid from *M. sexta* larvae collected from a tomato field	500	ND	ND	ND	8 ± 0 [2]
Gut fluid from *S. gregaria* nymphs reared on wheat and bran seedlings	360	ND	ND	11 ± 5 [3]	87 ± 4 [5]

[a]The incubation mixture had a volume of 275 μl and a pH of 6.7–6.9. Values are $\bar{X}$ ± SEM for the number of replicates given in brackets. The small number of replicates precludes the calculation of statistical significance. Values of SEM are included only to provide an indication of the low variance between replicates. ND, not determined.

cipitated by ethanol with molecular weights less than 10 kD, and fraction C contains solutes precipitated by ethanol with molecular weights greater than 10 kD. Experiments testing the effects of fractions A, B, and C on the precipitation of RuBPC by tannic acid implicate surfactants as the components responsible for the effectiveness of gut fluid in preventing the precipitation of protein by tannic acid.

Fraction B contains low-molecular-weight solutes, including inorganic salts, which favor the formation of insoluble tannin–protein complexes (Martin et al., 1985). It is to be expected, therefore, that relatively small quantities of tannic acid would be effective in precipitating protein from a solution of fraction B, and indeed, most of the RuBPC is precipitated by only 50 or 75 μg of tannic acid when the source of fraction B is gut fluid from diet-fed larvae (Table 4). Thus a solution of fraction B from diet-fed animals differs little from a simple buffered salt solution in terms of its properties as a solvent for tannin–protein complexes.

It is interesting to note that more tannic acid is required to precipitate the RuBPC dissolved in a solution containing fraction B from *M. sexta* larvae fed leaves during their last two instars than one containing fraction B from larvae reared exclusively on artificial diet (Table 4), suggesting that the composition of this fraction depends upon the larval diet. Insufficient material was available for a test of the properties of fraction B from field-collected larvae.

TABLE 3. COMPARISON OF pH, SURFACE TENSION (ST), SURFACTANT CONCENTRATION AND SOLUTE COMPOSITION OF GUT FLUIDS OF FIFTH-INSTAR *M. sexta* LARVAE AND *S. gregaria* NYMPHS[a]

Species	Food	pH	ST, dynes/cm (surfactant conc.)	Concentrations of components (mg/ml)			ST, dynes/cm (surfactant conc.)		
				A	B	C	A	B	C
M. sexta	Artificial diet[b]	9.5	33 (10 × CMC)	22.3	12.6	2.8	42 (5 × CMC)	72	69
M. sexta	Diet/tomato leaves[c]	9.7	33 (10 × CMC)	26.1	15.0	2.4	31 (5 × CMC)	71	72
S. gregaria	Wheat/bran seedlings	5.3	37 (10 × CMC)	78.3	57.4	28.4	37 (5 × CMC)	55 (30 × CMC)	53 (20 × CMC)

[a] CMC is critical micelle concentration. Fraction A contains solutes not precipitated by ethanol; B contains solutes precipitated by ethanol with molecular weights less than 10 kD; C contains solutes precipitated by ethanol with molecular weights greater than 10 kD.

[b] Reared through the entire larval stage on artificial diet.

[c] Switched from artificial diet to tomato leaves for the last two instars.

TABLE 4. AMOUNTS OF RuBPC PRECIPITATED BY TANNIC ACID (TA) FROM SOLUTIONS CONTAINING VARIOUS COMPONENTS OF GUT FLUIDS FROM FIFTH-INSTAR *M. sexta* LARVAE AND *S. gregaria* NYMPHS[a]

Species	Food	Components present in incubation mixture			RuBPC precipitated (μg) by			
		A	B	C	50 μg TA	75 μg TA	200 μg TA	300 μg TA
M. sexta	Artificial diet[a]	−	+	−	314 ± 8	451 ± 38	ND	ND
M. sexta	Artificial diet[a]	+	+	−	26 ± 1	52 ± 7	ND	ND
M. sexta	Artificial diet[a]	+	+	+	10 ± 1	35 ± 4	ND	ND
M. sexta	Diet/tomato leaves[b]	−	+	−	ND	75 ± 12	434 ± 35	493 ± 7
M. sexta	Diet/tomato leaves[b]	+	+	−	ND	5 ± 1	13 ± 1	81 ± 27
M. sexta	Tomato leaves[c]	+	+	−	ND	ND	ND	8 ± 2
S. gregaria	Wheat/bran seedlings	−	+	−	ND	37 ± 5	293 ± 34	447 ± 55
S. gregaria	Wheat/bran seedlings	+	+	−	ND	12 ± 4	133 ± 6	309 ± 9
S. gregaria	Wheat/bran seedlings	+	+	+	ND	ND	24 ± 5	122 ± 8

[a] The incubation mixture had a volume of 275 μl, a pH of 6.8–7.0, and contained 500 μg of RuBPC (except in the experiment in which all three components from *S. gregaria* gut fluid were recombined, in which case 300 μg of RuBPC were present). Values are $\bar{X} \pm$ SEM for three replicates. The small number of replicates precludes the calculation of statistical significance. Values of SEM are included only to provide an indication of the low variance between replicates. The contents of fractions A, B and C are described in the heading for Table 3. ND, not determined.
[b] Reared through the entire larval stage on artificial diet.
[c] Switched from artificial diet to tomato leaves for the last two instars.
[d] Collected from tomato plants in a tomato field.

Fraction A is the only fraction from either diet- or leaf-fed *M. sexta* larvae which contains surfactants, as indicated by surface-tension measurements (Table 3). Since water has a surface tension of 72 dynes/cm, the values of 69–72 dynes/cm for solutions of fractions B and C indicate that these fractions do not contain significant concentrations of surface-active materials. However, the values of 31 and 42 dynes/cm for solutions of fraction A from diet- and leaf-fed larvae, respectively, indicate the presence of surfactants in those fractions. The amount of RuBPC precipitated by 50 and 75 μg of tannic acid is reduced to 26 and 52 μg, respectively, when the solution contains fraction A, the surface-active fraction, in addition to fraction B. In fact, the combination of fractions A and B is nearly as effective as the original gut fluid or reconstituted gut fluid (fractions A + B + C) from the diet-fed insects in preventing the precipitation of dissolved RuBPC by tannic acid (Tables 2 and 4). Likewise, when RuBPC is dissolved in a solution containing both fractions A and B from the gut fluid and leaf-fed larvae, as much tannic acid must be added to precipitate this protein as is required to effect precipitation from the original gut fluid.

Precipitation of RuBPC from Denatured S. gregaria *Gut Fluid or Solutions of Gut Fluid Components by Tannic Acid.* The gut fluid of *S. gregaria* nymphs reared on bran and wheat seedlings, adjusted to pH 6.7–6.9, is as effective at preventing the precipitation of RuBPC by tannic acid as the gut fluid of *M. sexta* larvae fed tomato leaves during their last two instars (Table 2). However, the results of experiments testing the effects of the individual fractions from the locust gut fluid were somewhat different from those using the fractions from the hornworm gut fluid (Table 4). With materials from both *M. sexta* and *S. gregaria*, the precipitation of RuBPC was brought about by a relatively small amount of tannic acid when it was dissolved in a solution of fraction B, the salt-containing fraction. However, in contrast to the results obtained with the *M. sexta* materials, the combination of fractions A and B from *S. gregaria* did not give a solution as effective at preventing the precipitation of protein by tannic acid as the original gut fluid. Only when all three of the gut fluid fractions from *S. gregaria* were combined was the resulting solution as effective as the original gut fluid.

In marked contrast to the results obtained when *M. sexta* gut fluid was subjected to fractionation, the surface-active components of *S. gregaria* gut fluid were not cleanly separated into a single fraction (Table 3). Solutions of all three fractions had surface tensions well below the value for water, indicating that all three contained surface-active constituents. It is also evident from the concentrations of dissolved solids in the various fractions that locust gut fluid is a much more concentrated solution than hornworm gut fluid. Perhaps the failure of the fractionation procedure to result in a clean separation of surfactants into a single fraction is simply a consequence of the much higher solute content of *S. gregaria* gut fluid (164 mg/ml) than *M. sexta* gut fluid (37 and 44 mg/ml). In any event, the observation that all fractions containing surfactants must be recombined in order to obtain a solution with properties comparable to those of the original gut fluid is consistent with the suggestion that surfactants are responsible for the ability of the gut fluid to prevent the precipitation of RuBPC by tannic acid.

DISCUSSION

Three mechanisms have been proposed by which tannins might interfere with digestion in an insect herbivore: (1) by inhibiting the herbivore's digestive enzymes, (2) by inhibiting the metabolic activities of symbiotic microorganisms that contribute to digestion, and (3) by reducing the digestibility of ingested nutrients.

The suggestion that tannins might inhibit an herbivore's digestive proteinases is a very plausible one, supported by well-documented examples of the inhibition of a variety of enzymes by tannins. Tannins have been shown to

inhibit α-amylase (Gadal and Boudet, 1965), pectinase (Bell et al., 1962), cellulase (Bell et al., 1962; Mandels and Reese, 1965), and β-glucosidase (Goldstein and Swain, 1965; Goldstein and Spencer, 1985). The inhibition of proteolysis by tannins has also been observed, but it has not been possible to determine whether this effect is a consequence of inhibition of the proteinases or of binding to substrates (Feeny, 1969; Rhoades, 1977). It is interesting that Mole and Waterman (1985) have recently demonstrated that under certain conditions tannins can stimulate the tryptic hydrolysis of proteins. In all of the studies in which tannins have been reported to inhibit enzyme-catalyzed processes, very dilute preparations of the enzymes have been used, and conditions have been employed which are very different from those that prevail in an insect's gut. In this study we have shown that in undiluted gut fluid from *M. sexta* larvae, the hydrolysis of RuBPC occurs with extraordinary rapidity and is not inhibited by tannic acid, even when the incubation mixture contains as much or more tannic acid as it contains RuBPC. Of course, it is still possible that tannins might inhibit the proteinases of insect species with less active enzymes, less alkaline midgut, or lower concentrations of detergents in their gut fluids, but to date there is no evidence that they do. Until direct evidence for such inhibition is provided, we maintain that there is no justification for believing that tannins interfere with protein digestion by inhibiting digestive proteinases in any insect herbivore.

Tannins have been observed to inhibit rumen microbes (Waterman et al., 1980, 1984), suggesting the possibility that polyphenols might interfere with digestive processes mediated by symbiotic microorganisms. While this mode of action is a possibility in insects, to date it has not been demonstrated, and there is no basis for believing that it is of widespread importance in herbivorous species. It is most unlikely to be a factor in lepidopteran or acridid herbivores, since gut microbes are not thought to play a significant role in digestion in these groups.

The suggestion that tannins might interfere with protein utilization by forming indigestible complexes with ingested protein is also a very plausible one, considering the extensively documented capacity of tannins to act as protein precipitants. However, in this study we have shown that the abundant leaf protein, RuBPC, is not readily precipitated from the gut fluids of *M. sexta* and *S. gregaria*. We conducted our experiments at pH 6.7–6.9, a pH which is very favorable for the formation of an insoluble complex between tannic acid and this protein (Martin and Martin, 1983). Only when enough tannic acid is added to the mixture to produce a ratio of tannic acid to protein close to unity is a significant portion of the protein precipitated from the gut fluids of hornworm larvae that had been feeding on tomato foliage or locust nymphs that had been feeding on bran and wheat seedlings.

In this study we have shown that the capacity of gut fluid to counter the

effects of tannic acid on the digestion and precipitation of RuBPC manifests itself at tannic acid–RuBPC ratios of 1.3 : 1.0 and 0.4–0.6 : 1.0, respectively. Unfortunately, tannin–protein ratios in foliage are known accurately for very few plant species. In the mature foliage of the pedunculate oak, *Quercus robur*, tannins were quantified accurately by actual isolation, and the tannin–protein ratio was found to be 0.34 (Feeny, 1970). The more common practice in assessing tannin content in foliage is to use functional group assays, such as the Folin-Denis assay, the proanthocyanidin assay, and the catechin assay, and translate the data into "tannin equivalents." The accuracy and utility of such estimates of tannin content are highly suspect (Martin and Martin, 1982). While the proanthocyanidin and catechin assays may provide rough indications of the levels of condensed tannins, the Folin-Denis assay provides no measure whatsoever of total tannin content. Indeed, there is no satisfactory functional group assay for "total tannin," and none of the functional group assays correlate with protein-binding capacity. With these caveats in mind, we note that in only 15 of the 102 species of forest vegetation surveyed by Oates et al. (1980), Waterman et al. (1980), and Coley (1983) did assays for proanthocyanidins or catechins suggest that the condensed tannin–protein ratio might be greater than unity.

In this study and our earlier ones (Martin and Martin, 1984; Martin et al., 1985), we implicated surfactants as the constituents of insect gut fluids that prevent the precipitation of proteins by tannic acid or pin oak tannins. Mole and Waterman (1985) have also demonstrated that surfactants can prevent tannic acid or quebracho tannin from inhibiting the tryptic hydrolysis of bovine serum albumin. In our earlier study (Martin and Martin, 1984), we restricted our attention to a fraction of *M. sexta* gut fluid corresponding to fraction A of this study. Since the preparation of fraction A results in the separation of surface-active substances from the inorganic salts present in the gut fluid, and since inorganic salts enhance the precipitation of proteins by tannins (Martin et al., 1985), it was possible that our earlier study overemphasized the potential for surfactants to interfere with the precipitation of proteins by tannins. The present study has ruled out that possibility. We have found that unfractionated gut fluid is extremely effective in preventing the precipitation of RuBPC by tannic acid and that a solution prepared by recombining gut fluid surfactants and gut fluid salts is as effective in this capacity as the unfractionated gut fluid.

We were surprised to find that the capacity of the gut fluid of *M. sexta* larvae to counter the protein-precipitating capacity of tannins is affected by the nature of the larval diet. The most effective gut fluid was from larvae that had consumed tomato foliage throughout larval life, while the least effective was from larvae that had been reared exclusively on an artificial diet. Gut fluid from larvae that had been switched from an artificial diet to tomato foliage for their last two instars was intermediate in its properties. These differences cannot be attributed to differences in detergency of the gut fluids of larvae with different

dietary histories. Surface-tension measurements of the unfractionated gut fluid and fraction A from diet-fed and leaf-fed larvae indicate no differences in surfactant concentrations (Table 3). Rather, the differences seem to be due to differences in the capacity of fraction B to cause precipitation of RuBPC by tannic acid. Perhaps the gut fluids of diet-fed insects have higher concentrations of inorganic salts, especially alkali metal salts, which are particularly effective in bringing about the precipitation of tannin–protein complexes (Martin et al., 1985).

In *M. sexta* midgut fluid, which normally has a pH of 9.5 or higher, both high alkalinity and detergency interfere with the formation of insoluble tannin–protein complexes. In *S. gregaria*, however, high alkalinity cannot be a factor. Evans and Payne (1964) reported a pH of 5.5 in the crop of this species, where a significant amount of digestion occurs, and a pH range of 6.2–7.0 in the midgut. The *S. gregaria* digestive fluid used in this study, which was primarily crop fluid mixed with some midgut fluid, had a pH of 5.3. RuBPC is readily precipitated from an aqueous salt solution in the pH range 5.5–7.0 (Martin and Martin, 1983). The observation that *S. gregaria* gut fluid interferes with the formation of tannin–protein complexes at pH 6.7–6.9 emphasizes the significance of surfactants in countering the protein-precipitating capacity of tannins in insect herbivores with gut fluids which are neutral or slightly acidic.

This study, in conjunction with other studies which have failed to produce any evidence that dietary tannins reduce digestive efficiency in any insect (Bernays et al., 1980; Klocke and Chan, 1982; Reese et al., 1982; Manuwoto et al., 1985; Manuwoto and Scriber, 1986), adds further support to our contention that tannins do not deserve the status they have been accorded as general, all-purpose, digestibility-reducing substances. It is now abundantly clear that the digestive systems of insects possess a number of attributes that effectively counter the potential protein-precipitating capacity of tannins. At least two of these attributes, alkalinity and detergency, occur widely in insects, in species both with and without an evolutionary history of exposure to tannins, indicating that these characteristics have not necessarily evolved specificially as antitannin adaptations (Martin and Martin, 1984; Martin et al., 1985). In order to ensure that our position not be misinterpreted, we wish to emphasize that we are not proposing that tannins are innocuous chemicals that pose no problems to insect herbivores. There is no question that tannins can act as toxins (Berenbaum, 1984; Bernays et al., 1980; Manuwoto et al., 1985; Manuwoto and Scriber, 1986; Steinly and Berenbaum, 1986) and feeding deterrents (Bernays, 1981; Klocke and Chan, 1982; Reese et al., 1982; Manuwoto et al., 1985) to non-adapted insects. We are only arguing that there is no evidence that tannins reduce the nutritional value of an insect's food by inhibiting digestive enzymes or by reducing the digestibility of ingested proteins, and further that the failure of tannins to interfere with digestion is readily explained on the basis of well-documented characteristics of the digestive systems of herbivorous insects.

Furthermore, we emphasize that in challenging the currently popular notion that tannins are digestibility-reducing substances, we are not challenging the general utility of either the apparency theory (Feeny, 1976; Rhoades and Cates, 1976) or the resource availability theory (Coley et al., 1985) of plant–herbivore interactions. According to both of these theories, low nutrient quality can reduce herbivory by imposing slower growth rates on herbivores, thereby subjecting them to higher mortality from parasitism, predation, and other factors during an extended juvenile period. The only points that we dispute are the assertions that polyphenols make a crucial contribution to low nutritive quality by interfering with digestion and that counteradaptation to the presence of polyphenols is unusually difficult. The demise of tannins as all-purpose digestibility-reducing substances requires that the advocates of these theories of plant defense find other explanations for the reduced nutrient quality of the foliage of "apparent" or inherently slow-growing species.

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SEX PHEROMONE DIFFERENCES IN POPULATIONS OF THE BROWNHEADED LEAFROLLER, *Ctenopseustis obliquana*[1]

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Abstract—*Ctenopseustis obliquana* females collected from Christchurch were found to produce a mixture of (*Z*)-5-tetradecenyl acetate and tetradecyl acetate in their sex pheromone gland, in contrast to *C. obliquana* from Auckland which produce an 80:20 mixture of (*Z*)-8-tetradecenyl acetate and (*Z*)-5-tetradecenyl acetate. This identification has been made on the basis of instrumental and chemical analyses. Antennae of male *C. obliquana* from Christchurch gave a maximal electroantennogram response to (*Z*)-5-tetradecenyl acetate. A field trapping program in Christchurch using combinations of synthetic (*Z*)-5-tetradecenyl acetate and tetradecyl acetate caught *Ctenopseustis* males equally well if the latter chemical was present or absent. No males were caught in traps baited with the Auckland-type pheromone. In Alexandra, *Ctenopseustis* males were caught in traps baited with Auckland-type pheromone and not in traps baited with Christchurch-type pheromone. This phenomenon is ascribed to the existence of sibling species within the described *C. obliquana*.

Key Words—Sex pheromone, Lepidoptera, Tortricidae, (*Z*)-5-tetradecenyl acetate, *Ctenopseustis obliquana*, brownheaded leafroller, sibling species.

INTRODUCTION

The endemic New Zealand leafroller complexes in *Ctenopseustis obliquana* (Walker), *Planotortrix excessana* (Walker), and the Australian immigrant *Epiphyas postvittana* (Walker) form the most significant group of insect pests

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of New Zealand's export fruit crops. In particular, *C. obliquana* is probably the most serious pest of New Zealand's most economically important horticultural crop—kiwifruit—causing typical leafroller damage to the leaves and fruit (Sale, 1982).

We have been studying the sex pheromones of these three leafrollers, with a view to monitoring population levels and hence timing pesticide applications more efficiently. The sex pheromone components of *E. postvittana* (Bellas et al., 1983), *P. excessana* (Galbreath et al., 1985), and *C. obliquana* (Young et al., 1985) have been determined, but the routine use of pheromones throughout orchards in New Zealand has been hindered not only by poor trap catches of the New Zealand native leafrollers (Steven, personal communication) but also by the discovery of sibling species within *P. excessana* (sensu Dugdale, 1966), detected by sex pheromone differences (Galbreath et al., 1985).

The sex pheromone identification of *C. obliquana* (Young et al., 1985) was conducted on a population originally collected from the type locality of *Teras obliquana* Walker (Green and Dugdale, 1982) in Auckland (upper North Island). Two compounds were identified as sex pheromone components, (*Z*)-8- and (*Z*)-5-tetradecenyl acetates (Z8-14:OAc and Z5-14:OAc), in a ratio of approximately 80:20. However, when this blend was used in Christchurch (midcoastal South Island)—a locality of *Cacoecia inana* Butler, regarded by Green and Dugdale as a synonym of *Ctenopseustis obliquana*—it failed to catch *Ctenopseustis* males (Thomas, personal communication). We report here the identification of a single sex pheromone component of *a Ctenopseustis* population collected from Christchurch.

METHODS AND MATERIALS

Insects were collected as larvae from Christchurch and reared on a semi-synthetic diet containing dried *Acmena smithii* (Poiret) leaf (Galbreath et al., 1985). Pupae were sexed, and males separated from females prior to emergence. Adults were maintained on a reverse light-dark cycle (16L:8D).

Crude sex pheromone extract was prepared from stripped glands rather than excised abdominal tips. Pheromone glands of 3- to 4-day-old female moths were stripped with fine forceps under a binocular microscope and allowed to soak in 10 μl of distilled analytical grade *n*-pentane for 3–12 hr prior to gas-liquid chromatographic (GLC) analysis.

Capillary columns (50 m BP20, 50 m CPS-1 and 20 m BP5), packed column (2 m, 2 mm OD, 3% OV-1 on Chromosorb W) and chromatographic procedures are described elsewhere (Foster et al., 1986). Ozonolysis (Beroza and Bierl, 1967) was used to determine the position of unsaturation, comparing capillary GLC retention times of ozonolysis products with those from ozonolysis of synthetic compound and with synthetic aldehyde. The technique as de-

scribed by Foster et al. (1986) involves collection of the natural compounds from the packed column in a 30-cm capillary tube, followed by ozonolysis of the compounds in the tube and the GLC analysis of the products on a capillary column.

The BP20 column resolved positional isomers of long-chain unsaturated acetates more efficiently than the corresponding alcohols; therefore, the collected alcohol fraction was acetylated by allowing it to react with ca. 10 μg of acetyl chloride in 40 μl of pentane for 1–2 hr. The reaction was worked up by allowing a gentle stream of nitrogen to slowly evaporate the mixture to apparent dryness. The residue was dissolved in pentane and the resulting acetates analyzed by capillary GLC.

Synthetic chemicals were assayed by capillary GLC using a 50-m Quadrex Corporation CPS-1 column, and the isomeric purity of Z5–14:OAc was determined to be greater than 97%.

Electroantennograms of responses of excised male antennae were recorded by the method of Roelofs (1984).

Field tests were conducted at and near Christchurch (midcoastal South Island) in home gardens using Sectar 1 traps (3M Co., St Paul, Minnesota) and at Alexandra (central South Island) in an orchard using Pherocon 1C® traps (Zoecon Company, Palo Alto, California), baited with synthetic chemical absorbed on rubber septa (A.H. Thomas Co., Philadelphia, Pennsylvania). Traps were placed on trees 2 m above the ground and at least 10 m away from each other.

Data from field tests were analyzed by the use of log linear models (McCullagh, 1983).

RESULTS

A number of pheromone gland extracts (1–4 FE) were analyzed on the BP20 column, with a consistent pattern for each chromatogram. Unlike gland analyses of *C. obliquana* from the North Island, there was no peak for Z8–14:OAc but two prominent peaks at the retention times of Z5–14:OAc and tetradecyl acetate (14:OAc). Trace components were found at the retention times of dodecyl acetate (3% of Z5–14:OAc), dodecanol (<1%), tetradecanol (14:OH, 2.6%), an unsaturated tetradecenol (3%), and hexadecyl acetate (2%). While baseline separation of the *E* and *Z* isomers of Δ-5–14:OAc could not be achieved using the BP20 column, these chemicals were easily resolved by the CPS-1 column. No (*E*)-5-tetradecenyl acetate (above 1% of the other isomer) was detected in the female gland extracts. Further analysis using the CPS-1 and BP5 columns was consistent with the above identifications.

The 14-carbon acetate and alcohol fractions from crude extract of 30 females were collected from the packed GLC column and the 14-carbon acetate

fraction was ozonized. Capillary GLC analysis showed three major peaks, with retention times corresponding to the ozonolysis products of synthetic *Z*5-14 : OAc (5-acetoxypentanal and *n*-nonanal) and 14 : OAc.

These data confirm the presence of both unsaturated *Z*5-14 : OAc and saturated 14 : OAc in the female pheromone gland. Quantification by addition of 16.4 ng of tridecanyl acetate internal standard to an extract of five female pheromone glands showed that there was an average of 3.3 ng of *Z*5-14 : OAc and 1.7 ng of 14 : OAc per female.

The collected 14-carbon alcohol fraction was shown to consist of 14 : OH and (*Z*)-5-tetradecanol by acetylation and subsequent analysis of the products by GLC on the BP20 column.

EAG profiles of responses of male antennae to series of 12, 14, and 16 monounsaturated acetates and alcohols showed generally a higher response to 14-carbon acetates, with a maximum response within this series to *Z*5-14 : OAc (3.5 $\pm$ 0.3 mV). The response to this isomer was significantly higher than that to *E*5-14 : OAc (2.6 $\pm$ 0.3 mV). The response to *Z*8-14 : OAc (2.0 $\pm$ 0.3 mV) was barely above background. In the 16-carbon acetate series, responses were generally low except to (*Z*)-5-hexadecenyl acetate. Responses to alcohols were low.

A field trapping study testing the effectiveness of *Z*5-14 : OAc and 14 : OAc as attractants showed the attractiveness of *Z*5-14 : OAc to male *Ctenopseustis* moths at Christchurch (Table 1). A comparison of the cross-attractancy of lures baited with *Z*5-14 : OAc and 14 : OAc and with *Z*8-14 : OAc, *Z*5-14 : OAc, and *Z*8-14 : OH (at the time of these trials *Z*8-14 : OH was thought to be synergistic at this level; subsequent work has shown it to give no detectable increase in trap capture of male *C. obliquana*; Clearwater and Foster, unpublished) conducted at Christchurch and Alexandra showed the specificity of the two types of lure to the respective *Ctenopseustis* populations (Table 2).

DISCUSSION

A population of *C. obliquana* in the midcoastal region of the South Island of New Zealand near Christchurch and previously synonymized on morphological criteria with Auckland *C. obliquana* was found to use *Z*5-14 : OAc as its main sex pheromone component. This pheromone is quite distinct from the sex pheromone blend used by *C. obliquana* on the North Island and several other regions on the South Island. Single female gland analyses by capillary GLC easily distinguish the populations, not only by the different components, but also because the Christchurch population generally has a larger quantity of pheromone (particularly *Z*5-14 : OAc) stored in the gland (Young et al., 1985). This is consistent with male trapping tests in which Christchurch males were trapped with much higher release rates of their pheromone than were males from the North Island population with their pheromone (Table 1).

TABLE 1. TRAP CATCHES OF MALE *Ctenopseustis* MOTHS NEAR CHRISTCHURCH TO BLENDS OF (*Z*)-5-TETRADECENYL ACETATE AND TETRADECYL ACETATE

	Dosage (μg) on rubber septa		
	Z5-14:OAc	14:OAc	Mean no. male moths/trap[a]
Trial 1[b]	100	100	11.8a
	300	300	9.8a
	1000	1000	16.5a
			0.4b
Trial 2[c]	300	300	14.3a
		300	0.0b
	300		11.7a
			0.7b

[a]Means followed by the same letters are not significantly different at the 5% level.
[b]Conducted March 22-Arpil 13, 1984. Five replicates were counted three times.
[c]Conducted November 8-December 17, 1984. Three replicates were counted six times, each time rerandomized.

The comparative field catches at two geographically distant locations, Christchurch and Alexandra (Table 2) are clear evidence of attraction of male *Ctenopseustis* moths from the different populations to a specific synthetic pheromone source. The lack of cross-attractancy of the two pheromone sources clearly suggests that there is unlikely to be gene flow between the two pheromone strains, and hence they are probably distinct (sibling) species (Dobzhansky, 1970). Unfortunately, we have no direct evidence to support this as in neither Christchurch nor Alexandra have we found the two pheromone strains

TABLE 2. CROSS-ATTRACTANCY TEST FOR *Ctenopseustis* MALES AT CHRISTCHURCH AND ALEXANDRA

	Mean No. male moths caputred at each site	
Lures (μg on rubber septum)	Alexandra[a]	Christchurch[b]
Z5-14:OAc (300) 14:OAc (300)	0.0	17.4
Z8-14:OAc (24) Z5-14:OAc (6) Z8-14:OH (12)	48.0	0.6
Blank	—	0.8

[a]Trial at Alexandra conducted February 10-April 19, 1984, using four replicates.
[b]Trial at Christchurch conducted March 2-March 23, 1984, using seven replicates.

in sympatry. It is worth noting that we are (so far) unable to distinguish males of the two populations.

Sex pheromone analyses also have been used in New Zealand to differentiate at least three sibling species in the Tortricinae genus *Planotortrix* (Galbreath et al., 1985; Foster et al., 1986). One of these species, which is host-specific to mangrove (*Avicennia resinifera*), apparently uses the same two chemicals as those produced in the female sex pheromone gland of the *Ctenopseustis* species at Christchurch. The wide geographical separation of these two species, however, precludes speculation on cross attraction. In New Zealand, mangrove bushes are found only in the semitropical upper North Island and not below latitude 38° South (Allan, 1961). Pheromone analyses of female moths within this area (Foster, unpublished) have failed to detect any *Ctenopseustis* individuals without Z8-14:OAc. Additionally, field tests conducted for the mangrove-specific *Planotortrix* species showed that no *Ctenopseutis* males in the mangrove area were captured using the two-component blend (J.R. Clearwater, unpublished).

Our pheromone analysis suggests that it is unlikely that there is cross-attraction between these two populations supporting their distinction as sibling species. Further work will be aimed at more precisely determining this phenomenon.

Further similar sex pheromone studies are underway in New Zealand to detect additional sibling species in the genera *Ctenopseustis* and *Planotortrix*. In-depth analyses of pheromone biosynthetic pathways, morphological characters, and genetics should help to clarify species status of these populations and to formulate evolutionary relationships.

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SEX PHEROMONE OF *Planotortrix*[1] SPECIES FOUND ON MANGROVE

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Abstract—(*Z*)-5-Tetradecenyl acetate and tetradecyl acetate were identified as sex pheromone components of an unnamed *Planotortrix* leafroller moth species found on *Avicennia resinifera* (mangrove). An equal mixture of the two compounds used as bait gave field trap catches at least as good as those baited with caged virgin females. Traps baited with the two chemicals caught male *Planotortrix* moths in a mangrove swamp not previously found to host the unnamed *Planotortrix* species. Adults of the unnamed *Planotortrix* species and of the greenheaded leafroller, *Planotortrix excessana* are morphologically indistinguishable. The sex pheromone of *P. excessana* has been found previously to be a mixture of (*Z*)-8-tetradecenyl acetate and tetradecyl acetate, and this means that the two species may now be distinguished by sex pheromone differences.

Key Words—Sex pheromone, Lepidoptera, Tortricidae, (*Z*)-5-tetradecenyl acetate, *Planotortrix*, mangrove, sibling species.

INTRODUCTION

Dugdale (1966) described the genus *Planotortrix* since certain New Zealand Tortricidae appeared to have been incorrectly classified in the genus *Tortrix* Linnaeus. Eleven species were assigned to the new genus, although it was noted that more remain to be described. Two species of this genus are major pests; *P. excessana* (Walker) the greenheaded leafroller, is a pest of most fruit crops while *P. notophaea* (Turner) can cause considerable damage to young conifers.

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The sex pheromone of *P. excessana* (collected from Christchurch in the South Island) was found to be a mixture of (*Z*)-8-tetradecenyl acetate (*Z*8–14 : OAc) and tetradecyl acetate (14 : OAc) (Galbreath et al., 1985). Galbreath et al. (1985) also reported that a population of this species collected in the central North Island utilized a different pheromone, possibly involving a mixture of (*Z*)-5- and (*Z*)-7-tetradecenyl acetates (*Z*5–14 : OAc and *Z*7–14 : OAc). These data underscored the importance of defining pheromone blends for various *Planotortrix* populations throughout New Zealand. The results would be of value in reexamining the systematics of the genus and in determining the pheromone to be used in monitoring traps in each geographic area.

We initiated our study with a population of *Planotortrix* that was known to exist in the Firth of Thames (North Island) and whose larvae feed on foliage of mangrove (*Avicennia resinifera* Forst.f.) (Dugdale, personal communication). While the larvae can be distinguished from the larvae of *P. excessana*, the adults are morphologically indistinguishable from *P. excessana* populations that feed on other plants (Dugdale, personal communication). Although adults from the mangrove species are generally darker than those of *P. excessana*, they cannot be definitively distinguished due to the large variation in color of *P. excessana* adults (Dugdale, personal communication). This paper details the identification of two sex pheromone components of the unnamed *Planotortrix* species that feeds on mangrove.

METHODS AND MATERIALS

Insects were initially collected from mangrove bushes as larvae and reared on a semisynthetic diet containing dried acmena leaf (Galbreath et al., 1985). Further larval generations were reared on the semisynthetic diet containing approximately 17% (by weight) ground mangrove leaf.

Adults were maintained on a reverse light–dark cycle (16L : 8D).

Gland extracts were taken by the following method. One hour into the scotophase, the ovipositor of a 3- to 4-day-old female moth was extruded under a binocular microscope and the pheromone gland excised using fine forceps. The gland was placed in 10 μl of distilled analytical grade *n*-pentane in a flamed 4-mm-OD glass tube drawn to a point. This was stored 3–12 hr in a 4-ml glass vial prior to gas–liquid chromatographic (GLC) analysis.

Capillary GLC analysis of gland extracts was performed with splitless injection and flame ionization detection. Capillary columns were a 50-m SGE BP20 (stabilized Carbowax 20 M), capable of resolving most straight-chain 14-carbon acetate isomers, a 50-m Quadrex Corporation CPS-1 (bonded SP2340 equivalent), capable of resolving not only most straight-chain but also most geometric 14-carbon acetate isomers, and a 20-m BP5 (SE54 equivalent). A

program of 100–200°C at 4°C/min, following an initial delay of 1 min was generally used. For analysis of the ozonolysis products, the initial temperature was changed to 40°C. For the CPS-1 column, a program of 100–180°C at 3°C/min was used. Carrier gas was nitrogen at a linear velocity of 35 cm/sec. Samples were quantified by electronic integration of peak areas, relative to 16.4 ng of added tridecanyl acetate (13:OAc) internal standard.

A 2-m 3% OV-1 on Chromosorb W packed column was used for fraction separations prior to collection. Fractions were collected in 30-cm glass capillaries cooled by an aluminium block partially immersed in liquid nitrogen. Following collections of the separated fractions, positional isomers were determined by ozonolysis (Beroza and Bierl, 1967) and capillary GLC identification of the products.

Ozonolysis was performed by allowing a steady stream of ozone (ca. 2–3 ml/min) to pass through the capillary tube for 30 sec at ambient temperature. The tube was rinsed with 30–40 μl of pentane: to the solution was added ca. 1 μg of solid triphenylphosphine. The retention times of the resultant acetoxy aldehyde and aldehyde were determined by capillary GLC and compared with those of the corresponding products obtained from ozonolysis of synthetic compound and with synthethic aldehyde.

Synthetic chemicals used were assayed by capillary GLC, and their isomeric purity was determined to be greater than 97%.

Electroantennograms (EAGs) from excised male antennae were recorded by the method of Roelofs (1984).

Four field trials were conducted in the mangrove swamp from which the larvae were collected, and in an Auckland mangrove swamp using Pherocon 1C® traps (Zoecon Company, Palo Alto, California) and Sectar 1 traps (3M Company, St. Paul, Minnesota), baited with synthetic chemicals absorbed on rubber septa (A.H. Thomas Co., Philadelphia, Pennsylvania). Virgin females (used in trial 2) were from the laboratory-reared colony originally collected from the mangrove swamp in Thames. Three females were enclosed in each wire-mesh cage (5 × 5 × 5 cm), suspended in Pherocon 1C® traps. Traps were randomized each time they were counted.

Data from the field tests were analyzed by the use of log linear models (McCullagh, 1983).

RESULTS

Capillary GLC analysis of a single female pheromone gland extract revealed two major components, which had retention times identical to 14:OAc and *Z*5–14:OAc on the polar BP20 and CPS-1 and the nonpolar BP5 columns. The retention time of *Z*5–14:OAc on the BP20 column (38.08 min) was not

separable from that of the $Z6$ isomer (38.11 min), but was separable from the $Z7$ (38.37 min) and $Z8$ (38.63 min) isomers. Baseline separation of the two geometric isomers of $\Delta5$–14:OAc could easily be achieved, using the CPS-1 column. No (E)-5-tetradecenyl acetate was detected in the female pheromone gland extracts.

Ozonolysis of the two compounds collected from an OV-1 column confirmed their identification as 14:OAc and a $\Delta5$–14:OAc. The compound with a retention time corresponding to 14:OAc had the same retention time after ozonolysis, and the other compound was ozonized to give peaks at the same retention times as 5-acetoxypentanal and *n*-nonanal (16.23 min and 21.41 min, respectively) on the BP20 column (revised temperature program).

EAG profiles showed a consistent maximum response to $Z5$–14:OAc. The average response of 17 antennae (22 replicates) to $Z5$–14:OAc was 5.5 ± 0.6 mV compared to 3.8 ± 0.6 mV for $E5$–14:OAc.

Quantification using pheromone extracted from five females and added internal standard (13:OAc) gave an average of 1.3 ng of $Z5$–14:OAc and 0.6 ng 14:OAc per female. Analysis of further female extracts showed the ratio of $Z5$–14:OAc to 14:OAc to vary from this 2.2:1 ratio to 0.45:1, with an average of approximately 1:1.

Field trials were carried out to determine the attractiveness of these chemicals to male moths. Traps baited with lures known to be attractive to *P. excessana* (Galbreath et al., 1985) were also used. The results (Table 1) clearly show the attractiveness of equal quantities of 14:OAc and $Z5$–14:OAc to male moths of this species, even though catches of males generally were low. In trial 1, the catch to 100 μg of both chemicals was significantly higher than that to a lower concentration of each or to the *P. excessana* lure ($Z8$–14:OAc and 14:OAc). Some 96% of males in the traps were caught in the final two weeks of the trial. This is due probably to an initiation of a moth flight period rather than to the decay of pheromone components in the lures to a more optimal concentration, since a similar phenomenon was observed in the lower concentration in the trial.

Trial 2 confirmed the efficacy of the synthetic lure in comparison with virgin females and trial 3 the necessity for the two-component mix for attraction. Trial 4 was conducted at a site where the moth has not previously been found and confirms the lure's efficacy in what is probably a low-density population (previous searches for this moth in this area failed to detect its presence, Dugdale, personal communication). The traps baited with *P. excessana* lures caught few moths. The morphological similarity between *P. excessana* and the mangrove-feeding *Planotortrix* males precluded absolute identification of the moths in the trap, and so it could not be determined if any *P. excessana* were captured in these tests.

TABLE 1. TRAP CATCHES OF MALE *Planotortrix* MOTHS TO COMBINATIONS OF (Z)-5-TETRADECENYL ACETATE, TETRADECYL ACETATE, AND (Z)-8-TETRADECENYL ACETATE

	Z5-14:OAc (μg)	14:OAc (μg)	Z8-14:OAc (μg)	Mean No. of male moths per trap[a]
Trial 1	10	10		5.0[a]
	100	100		22.7[b]
		100	100	0.3[c]
Trial 2	10	10		3.0[a]
	100	100		6.3[a]
	300	300		3.3[a]
	1000	1000		5.7[a]
	Virgin females			4.7[a]
		100	100	0.7[b]
Trial 3	0	0		0.0[b]
	100	0		0.2[b]
	100	100		5.6[a]
Trial 4	100	100		5.0[a]
		100	100	1.0[b]

[a] Means not followed by the same letter are significantly different, $P < 0.05$, ANOVA. Trial 1 conducted January 27–February 20, 1984, at Thames, three replicates, Pherocon 1C traps. Catches recorded three times. Trial 2 conducted February 20–March 1, 1984, at Thames, three replicates, Pherocon 1C traps. Trial 3 conducted February 27–March 14, 1984, at Thames, five replicates, Sectar 1 traps. Trial 4 conducted February 21–March 23, 1984, at Avondale, Auckland, five replicates, Sectar 1 traps.

DISCUSSION

The sex pheromone, Z5-14:OAc + 14:OAc, of the mangrove-feeding species of *Planotortrix* was found to be quite different from the populations of *P. excessana* which had previously been reported to be Z8-14:OAc + 14:OAc in one location and possibly Z5-14:OAc + Z7-14:OAc in another location (Galbreath et al., 1985). Although all three populations are morphologically indistinguishable, the significant difference in mate recognition systems should be sufficient to classify these populations as sibling species. Sibling or cryptic species have been defined as "species that morphologically resemble each other so closely that they can be recognized only after careful study of biochemical, cytological, or behavioral traits." (Diehl and Bush, 1984). The host preference of the mangrove-feeding *Planotortrix* species is also very different from the polyphagous *P. excessana* (Dugdale, 1966).

In Tortricidae, Z5-14 : OAc has been found as a sex pheromone component only twice—both times in New Zealand species. *Ctenopseustis obliquana* (Walker), the brownheaded leafroller, was found to use a combination of Z5-14:OAc and Z8-14:OAc (Young et al., 1985), and the *P. excessana* "species" from the central North Island apparently uses a combination of Z5-14:OAc and Z7-14:OAc (Galbreath et al., 1985). Z5-14:OAc also has been reported to be a sex pheromone component of a cossid moth (Capizzi et al., 1983) and a noctuid moth (Bestmann et al., 1980), and as part of an attractant blend for several other noctuid moth species (Steck et al., 1982). Since the compounds found in the New Zealand leafroller species are uncommon in other Tortricidae (Roelofs and Brown, 1982), further studies on the sex pheromone of other related New Zealand leafroller populations and the biosynthetic pathways used in these primitive species should provide valuable information on evolutionary relationships within the genus *Planotortrix* and between other genera in the tribe Archipini.

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VARIATION IN RELATIVE QUANTITIES OF AIRBORNE SEX PHEROMONE COMPONENTS FROM INDIVIDUAL FEMALE *Ephestia cautella* (LEPIDOPTERA: PYRALIDAE)

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Abstract—The airborne sex pheromone components (*Z,E*)-9,12-tetradecadien-1-yl acetate and (*Z*)-9-tetradecen-1-yl acetate from single calling females of *Ephestia cautella* (Walker) were trapped within glass capillary tubes and were measured by gas chromatography–mass spectrometry. Broad and similar distributions of relative quantities were found for a laboratory strain and three Australian field strains, and means differed strongly from those reported previously for this species. The overall mean proportion of the two components found for Australian females was 88 : 12. The composition in individuals ranged from 63 : 27 to 97 : 3. The proportions for individuals appeared to vary slightly in a random fashion from day to day, and proportions for first-generation progeny were influenced by the maternal blend.

Key Words—Effluvium, variation, *Ephestia cautella*, Lepidoptera, Pyralidae, single insect, pheromone composition, behaviour, GC-MS, (*Z,E*)-9,12-tetradecadien-1-yl acetate, (*Z*)-9-tetradecen-1-yl acetate.

INTRODUCTION

The relative quantities of the components of airborne sex pheromone blends are likely to be important at a number of evolutionary levels: in sexual selection, stabilizing selection, allopatric speciation, and reproductive isolation between species (Cardé and Baker, 1984). In spite of this pervasive influence, however, information on variation in proportions of emitted pheromone components is

[4] Author order in this paper is alphabetical.

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available for only a few species such as *Spodoptera littoralis* (Campion et al., 1980), *Heliothis virescens* (Pope et al., 1982) and *Pectinophora gossypiella* (Haynes et al., 1984).

This dearth of information is partly because techniques for collecting and measuring the effluvia of single insects have become available only recently. Thus, Baker et al. (1981) and subsequently Pope et al. (1982) showed how to collect and quantify the pheromone blend released aerially from the forcibly extruded ovipositor of a female moth. Shani and Lacey (1984) later described an efficient procedure for collecting and measuring airborne sex pheromones from naturally calling female moths. The present paper reports results of a study of the sex pheromone blend of the moth *Ephestia cautella* (Walker) using the latter method.

E. cautella is one of several closely related phyticine pests of stored products. Two sex pheromone components have been identified from the effluvia of calling females, namely, (*Z,E*)-9,12-tetradecadien-1-yl acetate (*Z,E*9,12–14:Ac) (Brady et al., 1971b; Kuwahara et al., 1971b) and (*Z*)-9-tetradecen-1-yl acetate (*Z*9–14:Ac) (Brady, 1973) and have been shown to be active in attracting the male to the calling female (Brady, 1973; Read and Haines, 1976, 1979). These two compounds have been reported in somewhat differing ratios (Table 1), and total quantities recovered in various ways from the pheromone gland have been estimated as between 1.5 and 50 ng per female (Sower et al., 1973; Kuwahara and Casida, 1973; Read and Beevor, 1976; Coffelt et al., 1978).

(*Z,E*)-9,12-Tetradecadien-1-ol (*Z,E*9, 12–14:OH) has been reported from extracts of the sex pheromone gland of *E. cautella* (Kuwahara and Casida, 1973; Read and Beevor, 1976). Although looked for in effluvia, the compound was not found in the present study. Sower et al. (1974) similarly were unable to find *Z,E*9,12–14:OH in the effluvia of calling females and showed that this com-

TABLE 1. MEAN RATIOS OF (*Z,E*)-9,12-TETRADECADIEN-1-YL ACETATE (*Z,E*9,12–14:Ac) TO (*Z*)-9-TETRADECEN-1-YL ACETATE (*Z*9–14:Ac) OBSERVED IN DIFFERENT STUDIES OF *Ephestia cautella*

Ratio of *Z,E*9,12–14:Ac to *Z*9–14:Ac	Source	Study
1.8:1	extracted glands	Brady (1973)
2.2:1	effluvium	
2:1	extracted glands	Read and Beevor (1976)
3.6:1	rinsed glands (scotophase)	Coffelt et al. (1978)
4.9:1	rinsed glands (photophase)	
7.5:1	effluvium	present study

pound significantly inhibited male responses to the female's sex pheromone blend. Thus, in this paper we report only the relative contributions of *Z,E*9,12–14 : Ac and *Z*9–14 : Ac to the effluvia of individual calling *E. cautella* females. For preliminary reports, see Barrer et al. (1984) and Shani et al. (1985).

METHODS AND MATERIALS

Ephestia cautella (Walker) was reared as described by Barrer (1976). Pharate adults were collected as they emerged from the culture and were held individually until required for experiments.

Four strains were investigated. The laboratory strain (L) had been in culture for 11 years and had been combined periodically with other strains, the last time three years previously. Each of three field strains had been started from the eggs of between 10 and 20 field-collected females. Field strains were used in the present study when they had passed two or three generations under culture. Strains were collected from Pinkenba Grain Export Terminal, Brisbane, Queensland (P strain); the Peanut Marketing Board premises, Tolga, Queensland (T strain); and Scott Creek, a pastoral and farming property near Katherine, Northern Territory (S strain).

Apparatus for making collections of airborne pheromones from individual free-moving insects is described in full by Shani and Lacey (1984). Briefly, a gentle air stream passes over each unrestrained insect in its own glass cage and the effluent flows through the capillary section of a clean Pasteur pipet. Typical pheromone molecules adhere to the walls of the capillary and are readily recovered at the end of the collection period with microliter quantities of solvent. The sealed capillary sections containing the trapped pheromone solutions are then stored at $-5°C$ until required for measurement.

The efficiency of the collection device is difficult to assess for a free-moving moth, but it may be high for the following reasons. There is no breakthrough of the pheromones released from synthetic sources for low nanogram/subnanogram quantities (Shani and Lacey, 1984). The cage design and the airflow rate were selected to minimize entrainment of emitted pheromone by the cage walls. Any pheromone entrained by the cage walls would be fixed irreversibly (Shani and Lacey, 1984) and would not contribute further to the blend in the trap. Because the mean quantities of C-14 acetates in the pheromone gland are no more than one order of magnitude greater than our yields of trapped pheromone, measured quantities of emitted pheromone are not unexpected and are probably close to the natural rates of emission.

To detect the possible presence of *Z,E*9,12–14 : OH in the effluvia from *E. cautella*, the contents of capillaries collected from 14 females during the same collection period (2 hr) were amalgamated. This was effected by washing the capillaries sequentially with the same limited volume of solvent, using cen-

trifugation to facilitate recovery of the pooled solution. Since the alcohol was not detectable in this experiment ($<0.2\%$ of the amount of Z,E9,12–14 : Ac), nor in individual aerial collections, comparative analyses of the effluvia were directed to the known pheromone components Z9–14 : Ac and Z,E9,12–14 : Ac. It seems that cuticular enzymes that hydrolyze *Choristoneura pinus* pheromone acetates to alcohols (Silk et al., 1985) are not a problem for effluvia collected from individual *E. cautella* by the current method.

In earlier experiments, Dry Ice was used to cool the pipet traps as a precaution against possible breakthrough of pheromone components. However, cooling of the glass walls proved to be unnecessary to trap such small quantities (less than 2 ng/hr) released from calling female *E. cautella*, and later experiments were performed with the traps at ambient temperature ($22 \pm 1\,°C$). Pheromone was collected from calling virgin females (0, 1, or 2 days old) in the 1–2 hr following scotophase. Subdued lighting (approximately 1.5 lux) was used for the period of collection and the calling behavior (Barrer and Hill, 1977) of females was monitored at 15- to 20-min intervals.

Standard solutions of Z9–14 : Ac and Z,E9,12–14 : Ac were prepared from HPLC purified material ($>99\%$) in hexane (HPLC grade, Waters Associates) and kept at $-5\,°C$ when not in use. Mixtures of Z,E9,12–14 : Ac and Z9–14 : Ac in blend proportions of 85 : 15, 91 : 9, and 96 : 4 were prepared in duplicate. Separate syringes were used for standards and unknowns to obviate any possibility of cross-contamination.

Blend proportions and quantities of the trapped effluvia from individual female moths were assessed by gas chromatography–mass spectrometry (GC-MS). The gas chromatograph was a Varian 1400 Series interfaced to a VG Micromass 70-70 mass spectrometer through a single-stage glass jet separator. The conductance of the vacuum jacket of the separator was increased to improve the efficiency of molecular transfer to the mass spectrometer. Injections were made on-column to a packed glass column (10% Silar 10C, 1.8 m length $\times$ 3 mm ID) under conditions indicated previously (Shani and Lacey, 1984). The column was conditioned immediately before use with nanogram injections of Z9–14 : Ac and Z,E9,12–14 : Ac and was used exclusively for aerial collections.

Most of the MS measurements were carried out under electron ionization conditions at 70 eV, but some later measurements were made at 20 eV to improve the signal-to-noise ratio. The selected $(M - HOAc)^{+\bullet}$ ions for Z9–14 : Ac and Z,E9,12–14 : Ac were monitored using a VG multiple ion detection (MID) module in its electric field switching mode, using the column bleed peak of m/z 207 for mass locking. The analog output from the MID was directed to a Servogor 460 multichannel analyzer, and results were assessed from peak heights.

The GC-MS strategy for quantitation capitalized upon the constancy of

response of the mass spectrometer to small quantities over a short time interval. For instance, seven successive injections at 5-min intervals from a standard pheromone blend containing quantities similar to aliquots from individual moths (35–55 pg Z9–14 : Ac and 190–300 pg *Z,E*9,12–14 : Ac) gave a coefficient of variation of 3.9% for the mean ratio of mass channel responses. The responses of the selected mass channels were essentially linear with quantity over the range 40–210 pg Z9–14 : Ac ($r = 0.990$) and 230–1150 pg *Z,E*9,12–14 : Ac ($r = 0.994$), for 13 successive injections at 5-min intervals.

The solution of the trapped effluvium from each individual moth was divided to allow duplicate measurement of its relative quantities of components by GC-MS. The two injections of the unknown were interposed by one for a standard solution, and blend proportions and quantities for the duplicates were assessed by interpolation. The blend proportion of the particular standard for each assessment was chosen to be close to the apparent blend proportion for the first of the duplicates to increase the reliability of the interpolation. Successive injections of unknowns and standards could be carried out at 5-min intervals because the selectivity of the GC-MS procedure nullified interference from any coeluting impurities from the effluvia.

Typically, the measured difference between the duplicate blend proportions was <2% of the total blend for the aerial collection from a strongly calling female, and duplicate measurements for individual *E. cautella* were rejected if they differed by more than an arbitrary 3.5%. Not all females called, and not all calling females released sufficient quantities of pheromone for accurate measurement. About 50% of all females released sufficient pheromone during their bouts of calling to meet criteria for acceptance of the duplicates. Of the 122 aerial collections that were accepted, duplicate blend proportions varied by 0–0.5% in 27%; 0.5–1.5% in 47%; 1.5–2.5% in 18%; and 2.5–3.5% in 8%.

With a multicomponent system, relative quantities can be expressed either as ratios or as proportions. Ratios and proportions are each transformations of the raw data, each with their own particular properties. Where the relative quantities of components are very different, the use of proportions compresses distributions and the use of ratios expands them. Because of this and because our results show relative quantities far removed from equimolar, we report values both as proportions and as ratios. Calculations were performed on arcsine transformed proportions (Zar, 1974). Mean quantities are reported here with the standard error (i.e., ±SE).

RESULTS

Relationship between Calling Behavior and Pheromone Production. There was an approximate relationship between the calling behavior of a female and

the amount of pheromone later detected. When an insect exhibited no overt calling behavior, the pheromone produced and trapped was either barely detectable or, more often, undetectable. Conversely, pheromone was usually trapped and readily detected from insects that were observed to call. Moreover, a sample of 28 females (L strain, no cooling of traps) that called strongly (Barrer and Hill, 1977) and continuously for 2 hr produced a measured 1285 ± (SE) 193 pg per female, whereas a sample of 24 otherwise similar females calling less strongly and/or less frequently produced only 563 ± 102 pg over the same period. Nevertheless, calling strength and frequency did not provide accurate predictions of pheromone yield, where a range from 78 to 4503 pg was detected among strongly and continuously calling females, and an overlapping range from undetectable to 1890 pg was obtained among females that called less readily.

Relative Quantities of Acetates among L Strain Females. A sample of 38 calling females produced the distribution of pheromone proportions summarized in Figure 1A. The mean value for this population was 89.1 ± 0.8% *Z,E*9,12-14:Ac, equivalent to 8.2 parts *Z,E*9,12-14:Ac to 1 part Z9-14:Ac (8.2:1). The range for the sample population was 75.9-95.7% *Z,E*9,12-14:Ac (3.1:1-29:1, almost an order of magnitude).

Both the mean value and the total range of the individual aerial collections were unexpected. The mean differed markedly from previously published data (Table 1) for this species, while the distribution of blend ratios was two or more times broader than the differing blend ratios reported, for example, for the pher-

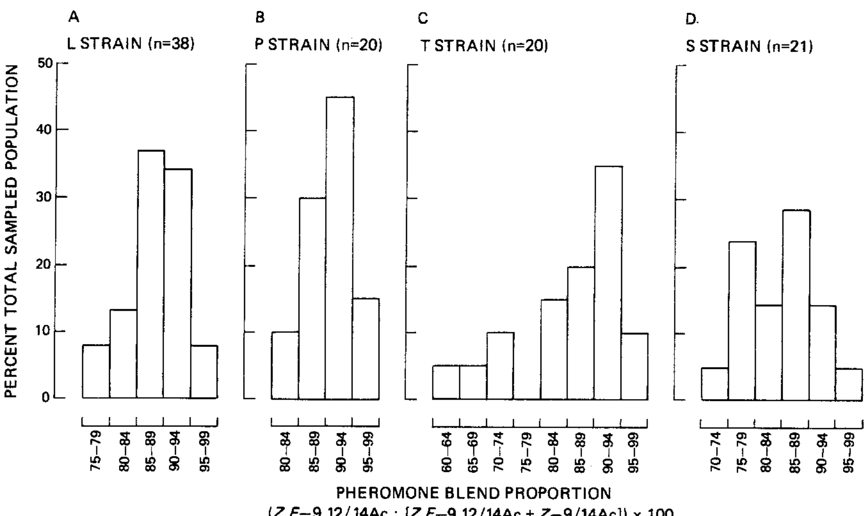


FIG. 1. Distributions of pheromone blend proportions in samples from each of one laboratory strain and three field strains of *Ephestia cautella*.

omone blend components of *Argyrotaenia velutinana* (Miller and Roelofs, 1980) or *Pectinophora gossypiella* (Collins and Cardé, 1985).

The laboratory history of the L strain may have rendered it atypical of *E. cautella* in the field. To assess this possibility, samples were taken from the three field strains (P, T, and S) obtained from widely separate sources in Australia.

Relative Quantities of Acetates among Field-Strain Females. Samples of 20 (P), 20 (T), and 21 (S) females produced the distribution of pheromone proportions summarized in Figure 1B–D. The mean of the P strain was 91.0 ± 1.0% *Z,E*9,12–14:Ac (10.1:1), that of the T strain was 86.8 ± 1.7% *Z,E*9,12–14:Ac (6.2:1), and that of the S strain was 85.7 ± 1.6% *Z,E*9,12–14:Ac (6.0:1). The three ranges were 80.8–97.3% (4.2:1–36:1), 62.7–96.2% (1.7:1–31:1), and 71.7–95.7% (3.2:1–22:1), respectively. Within these samples, 13 (P), 10 (T), and 10 (S) females called strongly and continuously during the 2-hr period of collection. These females produced 1202 ± 162 pg, 831 ± 60 pg, and 636 ± 83 pg, respectively.

Given the considerable variation in relative quantities within the samples, the means and ranges of pheromone proportions for laboratory and field strains appear quite similar. A Kruskal-Wallis test with tied ranks (Zar, 1974) gave a χ^2 of 7.62 with 3 df and $P > 0.05$, suggesting similarly that the four strains should not be considered samples from pheromonally distinct populations.

Results from the laboratory population were representative of those from the sampled field strains, so that results from all four strains could be amalgamated. Figure 2 shows the distribution arising from amalgamation of individual pheromone blends from all four strains. The mean of the amalgamated population was 88.3 ± 0.7% *Z,E*9,12–14:Ac (7.5:1) and the range, 62.7–97.3% *Z,E*9,12–14:Ac (1.7:1–36:1).

Pheromone Blend of Same Individual from Day to Day. To determine whether the pheromone blend of an individual varied from day to day, pheromone was collected from 10 L strain females over the first 2 hr of scotophase on the day of emergence and on the two days following.

Two females produced pheromone sufficient to be reliably assessed on only two of the three days. The remaining results are summarized in Figure 3. Pheromone blends appeared to change by small amounts and in an apparently random fashion from one day to the next. The mean change per day for a female was plus or minus 3.2 ± 0.5% *Z,E*9,12–14:Ac. The change was considerably greater than the measuring error, where the mean difference between duplicate measurements was 1.3 ± 0.2% *Z,E*9,12–14:Ac. It is not clear whether or how much other small errors could have contributed to this apparent change. In any case, there was little or no evidence of a constant trend in any one direction, where the mean change over two days was plus or minus 3.8 ± 1.0% *Z,E*9,12–14:Ac, hardly different from the change observed over one day only. Evidently, the measured pheromone blend of an individual changed relatively little

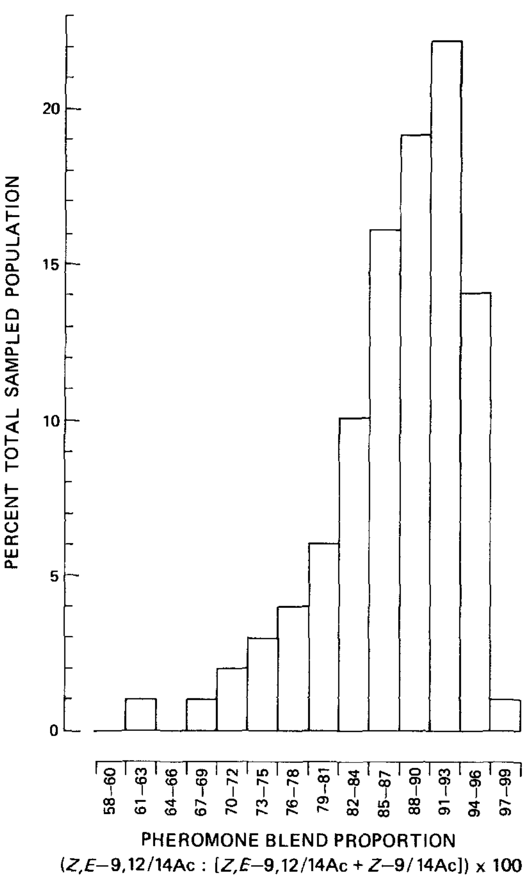


FIG. 2. Distribution of pheromone blend proportions in combined samples from one laboratory strain and three field strains of *Ephestia cautella*.

or not at all, and where it did vary, it tended to do so randomly from day to day around a mean value.

Relationships between Pheromone Blends of Two Consecutive Generations. As has been shown, relative quantities of *Z*,*E*9, 12–14 : Ac and *Z*9–14 : Ac varied within a population but individual females tended to maintain approximately the same pheromone blend composition from day to day. A preliminary experiment was set up, therefore, to determine whether there was evidence of an inherited component to an individual's pheromone blend composition.

Pheromone was collected from each of four virgin L strain females which were then mated with siblings, allowed to oviposit, and their progeny reared to

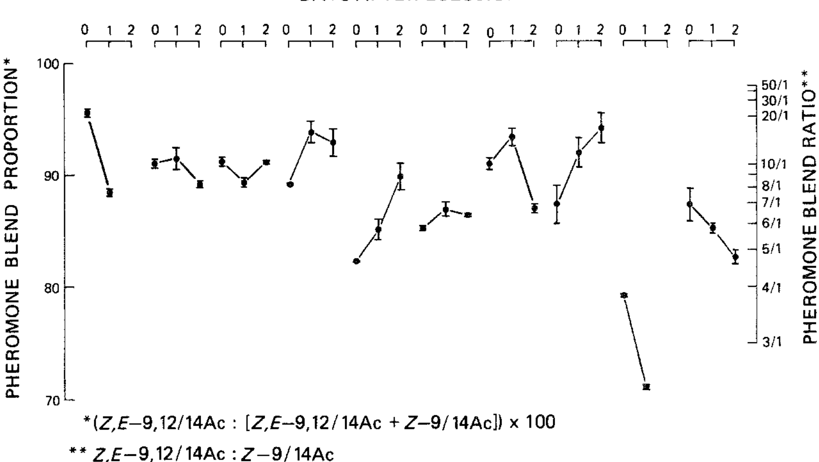


FIG. 3. Pheromone blend ratios and proportions of ten individual *Ephestia cautella* females over two or three days (bars represent differences between duplicated measurements).

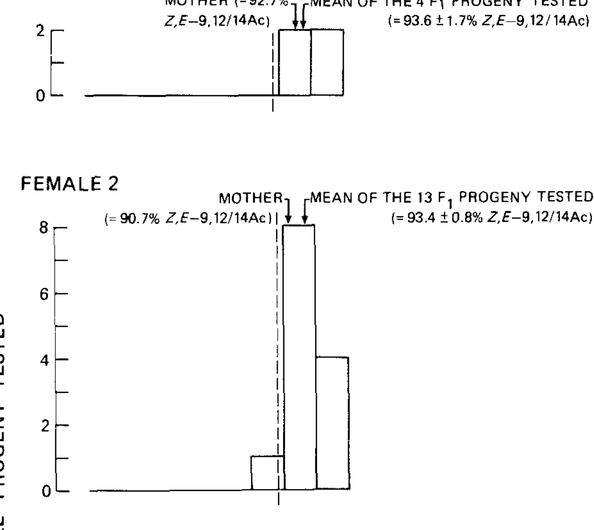
F_1 adults. Pheromone samples were collected in turn from several randomly selected F_1 female progeny of each of the original females.

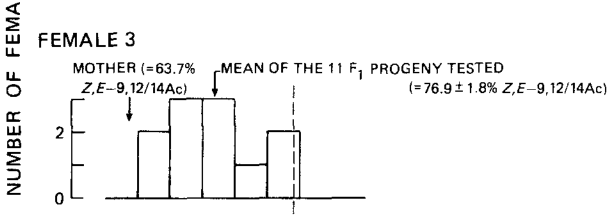
Results are summarized in Figure 4. The mean blend composition and range of each F_1 population sample were influenced by the mother's blend composition, although a moderate range of F_1 blends was evident even for small population samples. More detailed study is required to clarify the genetic mechanisms involved and to determine whether populations selected for extreme pheromone blends will "breed true" over a number of generations.

DISCUSSION

The mean composition of the sex pheromone blend of Australian strains of *E. cautella* (Figure 2) differs from compositions reported previously for strains from outside Australia (Table 1), and the reported compositions differ also between themselves. However, the range of the reported blend proportions falls within the range of individual values obtained in the present study of Australian strains (Figure 2). There is evidence, therefore, of an unexpectedly wide and more or less continuous range of blend proportions within and between populations of this species.

The differences between blend proportions for *E. cautella* are less than those reported between the so-called *Z* and *E* strains of *Ostrinia nubilalis* (Klun et al., 1973; Klun and cooperators, 1975), but the two *O. nubilalis* strains are





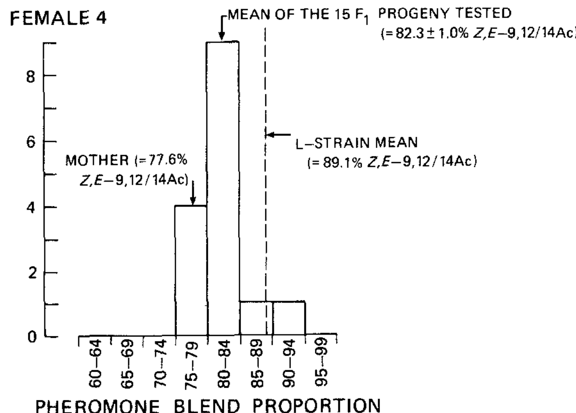


FIG. 4. Pheromone blend proportions of four *Ephestia cautella* females and samples of their female progeny.

genetically differentiated to an extent that may indicate semi- or sibling species status (Cardé et al., 1978). There is no reason to believe that *E. cautella* populations from different parts of the world are genetically differentiated to a similar extent, although it is perhaps surprising that the means of the four sampled Australian strains (Figure 1) are all so similar to each other but unlike those reported from North America and Europe. It is appropriate to consider what factors could account for this apparent pattern of similarities and differences in pheromone blend proportions.

Blend proportions obtained from pheromone gland extracts need not reflect the composition of the effluvium, but Brady (1973) measured both the gland contents and the effluvium from *E. cautella* and found similar blend ratios (Table 1). Thus the different methodologies are unlikely to have contributed much to the dissimilarities in mean blend composition.

Evidence of the relationships between the pheromone blend of parents and progeny (Figure 4) suggests that environmental factors in the laboratory, such as variation of the quality of larval rearing medium, could not account for the large differences between individuals in Australian strains of *E. cautella*, nor between mean blend proportions of strains around the world. Furthermore, the pheromone blend of an individual tends to remain relatively constant from day to day (Figure 3). It does seem necessary, therefore, to assume moderately precise genetic control over pheromone blend.

Do strains of *E. cautella* from various parts of the world differ in mean blend composition as a result of selection pressure or genetic drift? The similarity between the four sampled Australian strains of presumably heterogeneous origin suggests the former, although it is not immediately apparent what external factors can mediate a population mean typical of Australia, with its wide range of conditions, and atypical of North America and Europe. It is possible that selection pressures are active in some parts of the world only, pushing pheromone blend proportions towards some extreme that is not maintained in the absence of such pressures.

Competition for sex communication channels between *E. cautella* and other cosmopolitan phyticine moth pests of stored produce is likely to be limited, since *Plodia interpunctella* (Hübner) is the only species with broadly similar temperature and food preferences. Other species are likely to compete less frequently with *E. cautella*, either because of different temperature requirements (*E. kuehniella*, *E. elutella*) or because of more specific food requirements (*E. figulilella*, *E. calidella*). Although most of these species are known to share $Z,E9,12$–14 : Ac as the sole or major identified component of their sex pheromone blends, *E. cautella* and *P. interpunctella* possess qualitatively different blends (Table 2) and appear not to rely on different ratios of common components to distinguish between the species.

Behavioral evidence confirms that *E. cautella* readily distinguishes be-

TABLE 2. PHEROMONE BLEND COMPONENTS RECORDED FOR *Ephestia* AND *Plodia* SPECIES

Species	Source	Pheromone[a] A	B	C	Study
Ephestia cautella					
	effluvium	+			Brady et al. (1971b)
	whole insect	+			Kuwahara et al. (1971b)
	gland extract }	+	+		Brady (1973)
	effluvium }				
	gland extract	+		+	Kuwahara and Casida (1973)
	effluvium			–	Sower et al. (1974)
	gland extract	+	+	+	Read and Beevor (1976)
	gland rinse	+	+		Coffelt et al. (1978)
Plodia interpunctella					
	effluvium	+			Brady et al. (1971b)
	whole insect	+			Kuwahara et al. (1971b)
	gland extract	+		+	Kuwahara and Casida (1973)
	effluvium	+		+	Sower et al. (1974)
	gland rinse	+		+	Coffelt et al. (1978)
Ephestia elutella					
	gland extract	+			Brady and Nordlund (1971)
Ephestia figulilella					
	gland extract	+			Brady and Daley (1972)
Ephestia kuehniella					
	whole insect	+			Kuwahara et al. (1971a)
	effluvium	+			Brady et al. (1971a)

[a] A, (*Z*,*E*)-9,12-tetradecadien-1-yl acetate; B, (*Z*)-9-tetradecen-1-yl acetate; and C, (*Z*,*E*)-9,12-tetradecadien-1-ol. + , present; –, absent.

tween its own and *P. interpunctella* sex pheromone (Ganyard and Brady, 1971; Sower et al., 1974) and that the converse is true, although to a lesser extent. Under these conditions, there would be little selective pressure for *E. cautella* to maintain precise control over the relative quantities of *Z*9–14 : Ac to *Z*,*E*9, 12–14 : Ac, and the mean and variance would be limited only by other, possibly intraspecific, factors. If, however, *E. cautella* was sometimes sympatric with other species that shared the same two blend components, then such populations of *E. cautella* could be expected to maintain blend proportions much more precisely and possibly centered on a different mean. In Australia, *P. interpunctella* is the only phyticine species to compete at all commonly with *E. cautella*. However, *E. cautella* does occur together with *E. figulilella* in the dried-fruit growing areas of Australia, and samples of *E. cautella* from these localities could be instructive. A similar situation appears to influence the responsiveness of male *Archips argyrospilus* from different parts of North America (Roelofs et al., 1974; Cardé et al., 1977).

We have not studied the responses of males to the range of pheromone blend ratios found within Australian populations of *E. cautella*. Because of the wide and continuous distribution of ratios found within all tested populations, however, it seems quite possible that the majority of males will prove to be broadly tuned, responding almost equally to the different proportions. In a population where this was not so, relatively large numbers of females from each generation would attract finely tuned males too infrequently for significant reproductive success. In the absence of other selective pressures against wide distributions of blend proportions in the population, extreme ratio females that did mate would tend to propagate or transmit those genes that made it possible for them to reproduce. An effective way of doing this would be to produce male progeny with a broad, rather than a narrow, responsiveness to blend proportions.

Also requiring comment is the wide range of pheromone quantities obtained from otherwise similar strongly calling females. Some of this variation could have been methodological (e.g., in airflow rates around pheromone glands), but some may be a reflection of basically different release rates. Greenfield (1981) points out that sexual selection by female moths would be facilitated where the female emitted a very weak signal and selected "superior" males with better searching abilities. Given that release rates above and below the norm represent two quite distinct reproductive strategies, then it seems possible that variation in release rates might characterize populations where sexual selection is mediated in this way. Moreover, in the crowded conditions of laboratory cultures of *E. cautella*, background pheromone levels tend to be high and sexually excited males attempt to mate indiscriminately with any insect they encounter. As a consequence, the occasional receptive female that releases little

or no pheromone should be able to "parasitize" her siblings' pheromone production with no reproductive penalty.

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PARTIAL CHEMICAL CHARACTERIZATION OF URINARY SIGNALING PHEROMONE IN TREE SHREWS (*Tupaia belangeri*)

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Abstract—Tree shrews of both sexes exhibit marking behavior ("chinning") in response to scent marks made by the urine of fertile male conspecifics. To isolate the effective odor components, the urine was fractionated by liquid–liquid extraction and TLC, and the fractions were tested by bioassay. The results show that chinning is elicited by several lipophilic urine fractions, which are more effective in combination than alone. To characterize the complex scent signal, the lipophilic extracts from urine of the two sexes were analyzed by GC-MS and compared. The GC profile of the males is distinguished by pyrazine compounds not detected in the profile of the females. The profiles of the sexes also differ with regard to several volatile monocarboxylic acids, which are present at higher concentrations in male than in female urine. More than 30 urine components have been identified. Synthetic equivalents of these urine components were bioassayed for effectiveness in eliciting chinning and compared with one another as well as with scent substances not normally present in tree-shrew urine. Strong chinning responses were elicited by (1) certain pyrazine compounds and (2) some monocarboxylic acids, when presented at the high concentrations specific to male urine. Marking behavior is usually not elicited by scent substances not contained in tree-shrew urine or by urine components common to mammals in general. The data so far available indicate that the male-specific scent signal of tree shrews is based less on a single unique component than on the qualitative and quantitative characteristics of a multicomponent mixture. In the bioassay, tree shrews of both sexes respond equally to the male specific substances by chinning. As the scent signal represented by these substances has a different meaning to males and females (rival or potential mate, respectively), chinning probably serves several different functions.

Key Words—Chinning response, pyrazines, scent marking, signaling pheromone, tree shrew, *Tupaia belangeri*, urinary chemosignal, volatile carboxylic acids.

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INTRODUCTION

Tree shrews mark the area in which they live with urine (Martin, 1968), as many other mammals do (Coblentz, 1976; Hopp and Timberlake, 1983; Johnson, 1973; Whitten et al., 1980). When an adult tree shrew of either sex encounters the urine mark left by a strange, fertile, adult male conspecific, it immediately overmarks it with secretion from its sternal glands (Holst, 1985) by a movement called "chinning." The urine of juvenile or castrated male conspecifics does not elicit chinning, nor does that of male mice (Stralendorff, 1986). The implication is that the urine of male tree shrew contains a characteristic, androgen-dependent signal that produces a specific behavioral reaction. In the experiments described here, this signal was characterized chemically by a bioassay procedure.

METHODS AND MATERIALS

Animals. A total of 30 adult tree shrews (15 male and 15 female *Tupaia belangeri*) were used, some as urine donors and some as test animals in the bioassay. The animals were kept singly in cages; their living conditions and food and the method by which urine was obtained are described elsewhere (Stralendorff, 1986). The concentration of the urine samples was estimated prior to chemical analysis by creatinine measurement (Haeckel, 1980), and only samples approximately equal in concentration were used in the experiments.

Bioassay. Ten animals of each sex were used for bioassay. In each trial, 25 μl of a test solution (urine, a particular urine fraction, or a solution of a synthetic substance) was applied to the front (wire mesh) of the animal's home cage (for details see Stralendorff, 1986), and the number of times the sample was overmarked with sternal-gland secretion within 2 min after sample application was recorded as the "chinning score." At 30 min before or after this test, the same animal was presented with a control solution—for example, the corresponding fraction of female urine or another fraction of male urine. The chinning score in response to the control solution was also recorded. Each combination of sample and control solution was tested on all of the animals, five times (on different days) in each case.

From the results of the five tests on a single animal (and the control responses), the median was found and recorded as the individual chinning score. As prior experiments have shown (Stralendorff, 1986), the individual chinning scores of the experimental animals in response to a given test solution differ considerably. In order to reduce the individual values to a common denominator, an additional term has been introduced: the average chinning score. The average chinning score for a given sample (whole urine, a particular urine frac-

tion, or a synthetic scent substance) was obtained by taking the median of the 20 individual chinning scores.

Since responses of males and females to the urine of fertile male conspecifics cannot be distinguished, the data obtained from the two sexes are not treated separately.

Statistical Analysis. The individual chinning scores served as the basic data for statistical evaluation. Responses to samples to be compared were first tested for difference by the Friedman two-way analysis of variance by ranks (Siegel, 1959). The significance of the differences between the responses to sample and control solutions was evaluated with the Wilcoxon matched-pairs signed-ranks test. Differences were considered significant when the two-tailed test gave $P < 0.05$.

Urine Fractionation. Urine from male and female tree shrews was treated separately. The first step was liquid–liquid extraction to give three fractions: lipophilic acidic extract, lipophilic basic extract, and aqueous residue. Fractionation was done by the method of Wimmer et al. (1978); 20 ml of urine was buffered, acidified, and applied to a ready-to-use Extrelut® column (Merck, Darmstadt). After separation, the lipophilic acidic and neutral components were extracted with 40 ml diethyl ether. The eluate was washed three times with 5 ml of 1 N hydrochloric acid and dried over anhydrous sodium sulfate. The dried extract was concentrated to 20 ml under nitrogen, to return the components to the concentration they had had in the original urine sample. Of this extract, 5 ml was divided into 25-μl samples and deep-frozen ($-40°C$) for later use in bioassay. The remaining 15 ml of acidic lipophilic urine extract was concentrated further and analyzed by thin-layer chromatography (TLC) and gas chromatography (GC).

The residue on the Extrelut column was basified by ammonia-saturated air and extracted with 40 ml methylene chloride. The eluate was washed three times with 5 ml of 1 N potassium hydroxide, dried, and concentrated to 20 ml. As above, 5 ml of this lipophilic basic extract was used for the bioassay and the rest concentrated further for chemical analysis.

After extraction of the lipophilic components of the urine, the solvent remaining on the column was removed in a weak vacuum, and the remaining hydrophilic components were eluted by application of 40 ml saturated sodium chloride solution. This aqueous urine residue (ca. 20 ml) was neutralized, divided into samples for bioassay, and deep-frozen ($-40°C$).

Thin-Layer Chromatography (TLC). TLC was performed using precoated plates of silica gel 60 F (Merck, Darmstadt). Chromatograms were run with chloroform–methanol (9 : 1) and visualized by exposure to UV light and staining with vanillic–sulfuric acid (LeRosen et al., 1952) or Tillman's reagent (Passera et al., 1964).

Subfractions for bioassay were obtained with the same precoated plates

and the same carrier solvent used for analytical TLC. The chromatograms were developed and dried three times and subsequently divided into four sections; the adsorbed material in each section was scraped from the plate. Each of these four fractions was extracted with diethyl ether for 3 hr in the Soxhlet; the extracts were concentrated to 20 ml and divided into samples for bioassay.

Gas Chromatography (GC). A Siemens gas chromatograph (Sicromat 1) with flame ionization detector (FID) was used. It was equipped with glass capillaries (length 25 m, inside diameter 0.32 mm) lined with Carbowax 20 M, OV-101, or OV-17 (Machery and Nagel, Düren). The carrier-gas flow rate was about 1 ml nitrogen per minute. The analysis was temperature programmed from 60°C to 220°C with 5°C/min. The gas chromatograms were evaluated by means of a Hewlett Packard 3380 A Integrator.

The concentrated lipophilic basic extracts were gas-chromatographed directly, with no additional treatment. Because the components were present in such low concentrations, the splitless injection technique (Grob and Grob, 1978) was used.

The free carboxylic acids in the lipophilic acidic extracts were converted to methyl esters by treatment with boron fluoride–methanol (Morrison and Smith, 1964; Edwards et al., 1975). The carboxylic acid methyl esters were applied to the GC capillary by inlet splitter (split ratio 1 : 100).

Coupled Gas Chromatography–Mass Spectrometry (GC-MS). An LKB-2091 device equipped with a Pye-Unicam gas chromatograph was used; the carrier gas was helium. The columns and the operating conditions were as described above; the temperature between this device and the mass spectrometer was 250°C, electron energy 70 eV.

The scent substances were identified by comparing the mass spectra of the samples with those of authentic substances (where available) and with various published mass spectra (Bondarovich et al., 1967; Buttery et al., 1971; Maga and Sizer, 1973; Stenhagen et al., 1974).

Reagents. All the solvents used were of analytical grade; they were obtained from EGA-Chemie (Steinheim), Fluka (Neu-Ulm), Merck (Darmstadt), Serva (Heidelberg), and Woelm-Pharma (Eschwege). Diethyl ether was further purified on a column with basic aluminum oxide and active silica gel.

RESULTS AND DISCUSSION

Prior to analysis, untreated samples of the urine from males and females were tested by bioassay (Table 1). Male urine was overmarked by both males and females; the average chinning score was 12. As observed in previous experiments (Stralendorff, 1986), female urine did not elicit chinning in most animals. The urine samples from the two sexes did not differ appreciably in concentration (creatinine: males 9.6 μmol/ml, females 9.7 μmol/ml) or in pH.

TABLE 1. COMPARISON OF AVERAGE CHINNING SCORES[a] OF 20 TREE SHREWS IN RESPONSE TO DIFFERENT FRACTIONS OF URINE[b] FROM MALE AND FEMALE CONSPECIFICS

	Test series		Male urine		Female urine		Significance of difference	
			Median	[25–75%]	Median	[25–75%]	T^c	N^d
1	Untreated whole urine		12	4–13	0	0–0	0	20
2	Urine fractions	aqueous urine residue (A) vs.	0	0–0			A:B = 0	20
	after liquid–	lipophilic acidic extract (B) vs.	5	2–7			B:C = 18.5	20
	liquid extract.	lipophilic basic extract (C)	10	8–15			C:A = 0	20
3		lipophilic basic extracts	12	6–15	1.5	0–2	0	20
4		lipophilic acidic extracts	5	3–9	2	0–2	0	20
5	Subfractions of	fraction I	2	0–3	0	0–1	0	11
6	lipophilic	fraction II	4.5	2–11	2	1–3	2	13
7	basic extract	fraction III	1	1–3	0	0–0	0	15
8	after TLC separ.	fraction IV	0	0–1	0	0–1	3*	4
9		fractions I–IV, recombined	6.5	4–8	1.5	0–3	13.5	17

[a] Each value is the median of the individual chinning scores of all animals tested; 25–75 percentiles in brackets; the individual chinning score is the median of the scores obtained in five tests of a given sample.

[b] In each bioassay 25-μl aliquots were presented; the concentration of each fraction was adjusted to approximate the natural concentration of the components in the urine.

[c] Wilcoxon matched-pairs signed rank test; all differences are significant except as marked with asterisk.

[d] N = number of matched pairs with $d \neq 0$.

Hence the difference in chinning scores probably results from differences in composition of the urine.

Response of Animals to Various Urine Fractions. Male and female urine samples were each separated into two lipophilic fractions (acidic and basic extract) and one hydrophilic fraction (aqueous residue).

The aqueous residue of male urine does not elicit chinning in the bioassay (Table 1) and was therefore discarded. The lipophilic acidic extract of male urine was overmarked five times on average, and the lipophilic basic extract was overmarked 10–12 times. The responses to the two lipophilic extracts differ significantly from one another ($P < 0.005$). From these results we conclude that chinning is elicited by various lipid components in the male urine, and that the lipids in the basic extract are the most effective.

As a control, both lipophilic extracts were tested against the corresponding extracts from female urine (Table 1: test series 3 and 4). Surprisingly, both the basic and the acidic extracts from females elicited chinning in most animals tested. The average chinning scores are considerably lower than those in response to the corresponding extracts from males; the differences between them are significant ($P < 0.005$).

The responses to lipophilic extracts from female urine raise the question: Why, when less than a quarter of the animals respond to whole female urine, do extracts from it elicit chinning in more than half of those tested (see medians and percentiles, Table 1)?

Probably the whole urine of females contains a female-specific component that the animals can detect. Its presence is indicated by the following response of two males in the bioassay. Both males were clearly sexually excited, sniffing and licking the female urine and giving "rhythmic clicking" calls. The latter are produced only by males in pursuit of an estrous female (Sprankel, 1961; Martin, 1968). Samples obtained by fractionation of the urine no longer caused either male to give rhythmic clicking calls. Evidently the female-specific material was either not present or not detectable in these samples. In whole urine, this characteristic substance could act antagonistically to the substances that elicit chinning and thus inhibit the marking response to female urine. This "inhibition" could be eliminated by fractionation of the urine.

On the other hand, the different responses to whole urine and the lipophilic extracts may be due to a solvent-dependent concentration effect. In aqueous solutions, as whole urine is, physical and/or chemical interactions between volatile components and nonvolatile material such as salts and proteins may result in retention of volatile components (Maier, 1970). The lipophilic organic extracts of the urine lack these proteins and may therefore retain volatile components to a lesser degree. Consequently the headspace over the untreated female urine and those over their organic extracts may differ in their content of chinning-eliciting components. This would result in different chinning responses

even though these components are contained in nearly equal concentrations in both whole urine and the organic extract.

The lipophilic extracts of both sexes were analyzed by thin-layer chromatography to find out whether the chinning response to the extracts from females is elicited by the same components that are responsible for the response to the extracts from males. The basic extracts, because their concentration in the original urine is so low, had to be brought to a concentration ten times that of the acidic extracts for this purpose.

Comparative TLC of Lipophilic Extracts from Male and Female Urine. The thin-layer chromatograms characterize the lipophilic extracts from tree-shrew urine as complex lipid mixtures (Figures 1 and 2). No qualitative differences between the acidic extracts of the two sexes could be detected (Figure 1), although the staining intensities of comparable zones (dotted areas) indicate quantitative differences. One's subjective impression of the smell of the extracts corroborates these quantitative differences; that is, the acidic extract from males smells more strongly of meat broth than does that from females.

The differences between the chromatograms of the two basic urine extracts are more pronounced (Figure 2) and are qualitative as well as quantitative. That is, there are several zones in which spraying with vanillic-sulfuric acid produces a yellow spot in the chromatogram for male urine (hatched areas) but does not stain that for female urine. This qualitative difference between the basic extracts from the two sexes can also be detected by the human nose: the extract from

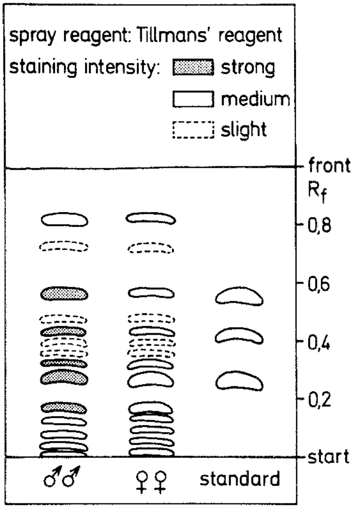

FIG. 1. Thin-layer chromatogram of lipophilic acidic extracts from the urine of male and female tree shrews. Standard: a mixture of 2-methyl-butyric acid (top), phenylacetic acid (middle), and 3-methyl-2-oxo-valeric acid (bottom).

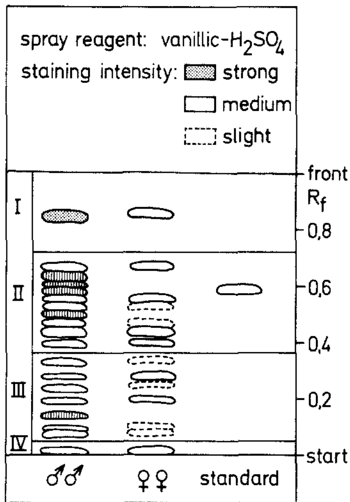

FIG. 2. Thin-layer chromatogram of lipophilic basic extracts from the urine of male and female tree shrews. Standard: a mixture of three isomeric dimethylpyrazines. The hatched areas are demonstrable only with male urine. The numbers I–IV designate subfractions that were analyzed further by bioassay.

males has a strong smell of roasted nuts, and that from females has only a weak mousy odor.

In order to isolate the characteristic male components, the lipophilic basic extracts from both sexes were separated into four subfractions by preparative TLC (Figure 2).

Behavioral Effect and Odor Characteristics of Subfractions of Basic Extracts. The boundaries of the subfractions were positioned so as to separate the male-specific components (in fractions II and III) from some of the components common to both sexes (fractions I and IV).

Fraction IV, whether of male or female urine, did not elicit chinning in most animals tested (Table 1) and was therefore discarded.

Fraction I of male urine is overmarked by nearly three quarters of the animals, whereas the corresponding fraction of female urine usually does not elicit chinning. The TLC analysis (Figure 2) and the subjective impression of odor intensity both indicate that the difference between the two fractions is quantitative; that is, presumably the concentration of the component(s) in fraction I from females is too low to elicit chinning in most animals.

Fractions II and III also include some components common to both sexes, but only in fraction II is the concentration of these components in female urine high enough to elicit chinning in most animals tested. Fractions II and III from males contain additional, male-specific substances, which make both fractions

smell different (to humans) from the corresponding fractions of female urine. In fraction II from males, after the solvent has evaporated, one can detect the roasted-nut odor that characterized the basic lipophilic extract before TLC fractionation. A few seconds later, however, this roasted smell disappears and is replaced by an intense pyrazine odor, which also characterized fraction III from males. Fractions II and III from females have neither a roasted nor a pyrazine smell; they tend more to smell mousy to urinous, like the basic extract from female urine before fractionation. As would be expected from the results of TLC analysis and of organoleptic evaluation, fractions II and III from males are overmarked by nearly all animals. The strongest chinning response is elicited by fraction II (average chinning score 4.5). This reaction is significantly different ($P < 0.005$) from that to fraction II from females.

The bioassay results show that several different fractions of the basic urine extract are effective in eliciting chinning. No differences have been observed between the chinning responses of both sexes. The various fractions of male urine are nearly additive in effectiveness; that is, the mixture of given amounts of all components elicits more chinning than does the same amount of any of the components alone (Table 1, test series 9).

The basic extract before fractionation gives a higher chinning score than does a mixture of fractions I to IV (test series 3 and 9 in Table 1). This loss of biological activity (or behavioral effectiveness) is accompanied by a subjectively discernible reduction in the intensity of the roasted odor. It may be that during fractionation, the male odor substances are partially lost by evaporation, oxidation, and so on.

Comparative GC-MS Analysis of Volatile Components of Lipophilic Acidic Urine Extracts. The GC-MS analysis was undertaken in order to identify the odor substances present in male but not in female urine. The GC profiles also provide a means of characterizing sex-specific proportions of the various components in the mixture.

The acid profiles of the urine samples from males and females differ sharply in quantitative composition (Figure 3), but reveal no qualitative differences. (Some peaks in the acid profile for females are not labeled by capital letters although they were identified by GC-MS.) Some peaks (e.g., X, Y, and Z) are of almost equal height in both profiles, while others (e.g., G, H, and L) are considerably higher in the profile for males; that is, these components are present in higher concentration in male than in female urine.

On the basis of mass spectrometry, the main components of these profiles can be divided into three groups (Table 2): aliphatic monocarboxylic acids (peaks A–K, V, and Z), aromatic monocarboxylic acids (peaks N, R, and U), and aliphatic dicarboxylic acids (the other peaks labeled with letters).

Component G, identified as 3-methyl-2-oxo-valeric acid, is responsible for the meat-broth odor of the lipophilic acidic extracts, and the difference in height

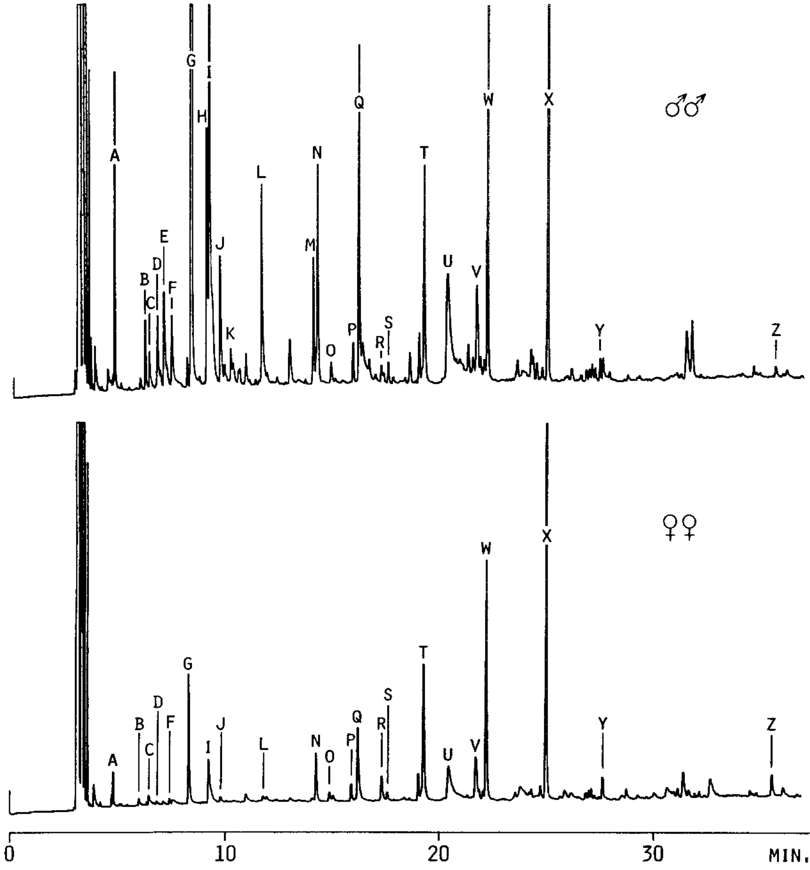

FIG. 3. Comparison of the carboxylic acid profiles obtained by gas chromatography of the urine of male and female *Tupaia belangeri*. The free carboxylic acids were methylated prior to analysis. The capital letters above the peaks label compounds analyzed by mass spectrometry; not all of the peaks so identified in the female profile are labeled. Analysis conditions for GC: 2.0-μl sample injected; split ratio 1:100; phase OV-101.

of this peak in the profiles corresponds to the difference in odor of the male and female extracts. (Male urine, depending on its concentration and the individual producing it, contains 40–250 μg of 3-methyl-2-oxo-valeric acid per milliliter; female urine on average contains only 1/20 as much.) Probably the lower concentration of this component in female urine is one reason for the relatively low chinning scores obtained with the acidic extracts from female urine.

Some of the identified carboxylic acids have been found also in the urine or glandular secretions of other mammals (Albone et al., 1974; Bakke and Figenschou, 1983; Berüter et al., 1974; Brundin et al., 1978; Holland et al.,

TABLE 2. CARBOXYLIC ACIDS[a] FROM PROFILES OF LIPOPHILIC ACID EXTRACTS FROM URINE OF *Tupaia belangeri*

Peak[b]	Mol. weight	Elemental formula	Chemical name	Identification criteria[c]
A	116	$C_6H_{12}O_2$	2-Methyl-butyric acid methyl ester (m.e.)	II
B	132	$C_6H_{12}O_3$	2-Hydroxy-2-methylbutyric acid m.e.	I
C	132	$C_6H_{12}O_3$	3-Hydroxy-3-methylbutyric acid m.e.	I
D	132	$C_6H_{12}O_3$	2-Hydroxy-3-methylbutyric acid m.e.	I
E	130	$C_6H_{10}O_3$	3-Oxo-2-methylbutyric acid m.e.	I
F	132	$C_6H_{12}O_3$	3-Hydroxy-2-methylbutyric acid m.e.	I
G	144	$C_7H_{12}O_3$	2-Oxo-3-methylvaleric acid m.e.	II
H	146	$C_7H_{14}O_4$	2-Hydroxy-4-methylvaleric acid m.e.	I
I	146	$C_7H_{14}O_4$	2-Hydroxy-3-methylvaleric acid m.e.	I
J	134	$C_5H_{10}O_2S$	3-Methyl-thiopropionic acid m.e.	II
K	174	$C_9H_{18}O_3$	3-Hydroxycaprylic acid m.e.	I
L	172	?	?	III
M	172	?	?	III
N	150	$C_9H_{10}O_2$	Phenylacetic acid m.e.	II
O	172	$C_8H_{12}O_4$	2-Methyleneglutaric acid dimethyl ester	I
P	172	$C_8H_{12}O_4$	isomer of O	I
Q	174	$C_8H_{14}O_4$	Adipic acid dimethyl ester	II
R	164	$C_{10}H_{12}O_2$	Phenylpropionic acid m.e.	I
S	188	$C_9H_{16}O_4$	3-Methyladipic acid dimethyl ester	I
T	188	$C_9H_{16}O_4$	Pimelic acid dimethyl ester	II
U	180	$C_{10}H_{12}O_3$	α-Hydroxyphenylpropionic acid m.e.	I
V	200	?	?	III
W	202	$C_{10}H_{18}O_4$	Suberic acid dimethyl ester	II
X	216	$C_{11}H_{20}O_4$	Acelaic acid dimethyl ester	II
Y	230	$C_{12}H_{22}O_4$	Sebacic acid dimethyl ester	II
Z	270	$C_{17}H_{34}O_2$	14-Methylpentadecanoic acid m.e.	I

[a] The free acids were methylated for GC analysis.
[b] The peaks are labeled as in the profile of Figure 3.
[c] Identification criteria: I, mass spectra comparison: II, reference spectra comparison with authentic substances and their coinjection on three different capillaries; III, not identified.

1983; Kaji and Saito, 1983; Lefebvre et al., 1982; Martin, 1973; Mueller-Schwarze et al., 1974; Spiteller and Spiteller, 1979; Suemitsu et al., 1968; Thiessen et al., 1974; Yasuhara and Fuwa, 1979). It is, nevertheless, possible that the particular mixture of carboxylic acids found in tree-shrew urine gives a profile characteristic for *Tupaia belangeri*. To test this hypothesis, we compared the urine profiles of our tree shrews with those of Mongolian gerbils. (To exclude the possibility of diet-related differences, the gerbils were given the same food as the tree shrews for several months before their urine was sampled.) As expected, the acid profiles of tree shrews and gerbils differ by the qualitative and quantitative composition of their components. For example, no keto acids were detectable, and phenylacetic acid was the main component of the acid profile of the gerbils (unpublished results).

Comparative GC-MS Analysis of Volatile Components of Lipophilic Basic Urine Extracts. The profiles of the basic urine extracts of the two sexes (Figure 4) differ primarily in the peaks H, J, K, M, N, O and U. The urine components corresponding to these peaks were identified by their mass spectra as alkylpyrazines (Table 3); none of them are traceable in the urine from females. These findings are consistent with the result of TLC analysis (R_f values and staining of reference substances).

To evaluate the odor properties of components H–U, the GC analysis was repeated without FID (flame extinguished) and the column effluent was evaluated organoleptically. About 10 min after injection of the sample (i.e., at the time when peak H appears in the chromatogram when the FID is in operation), the male-specific roasted odor can be detected in the column effluent, closely followed by the typical odors of 2,5-dimethylpyrazine, mentioned above in the context of TLC analysis. Hence peak H includes two different components, which are not separated from one another under these conditions of analysis. The two components can be separated by using another capillary (phase OV-101 instead of CW-20 M), which gives the profile shown in Figure 5. Here the roasted odor (peak W) does not appear until about 2 min after the pyrazine odor (peak H). The mass spectrum of peak W with the fragments *m/e*: 42 (100), 69 (65), 108 (54), 41 (40), 110 (27), 54 (14), 109 (11), 81 (8), 68 (7), 95 (5) closely resembles the spectrum of 5,6-dihydro-2,3-dimethylpyrazine (Flament and Stoll, 1967).

According to Bondarovich et al. (1967), certain dihydropyrazines have an intense roasted odor. Some of these are unstable and difficult to isolate; for example, when 3,6-dihydro-2,5-dimethylpyrazine is heated, it readily oxidizes to 2,5-dimethylpyrazine, giving the same mass spectrum as the latter (Wilen, 1970). Accordingly, the characteristic male odor substances could be dihydropyrazine compounds which, at least in part, are oxidized to the identified pyrazines during collection or analysis of the urine. This possibility will be tested in future studies.

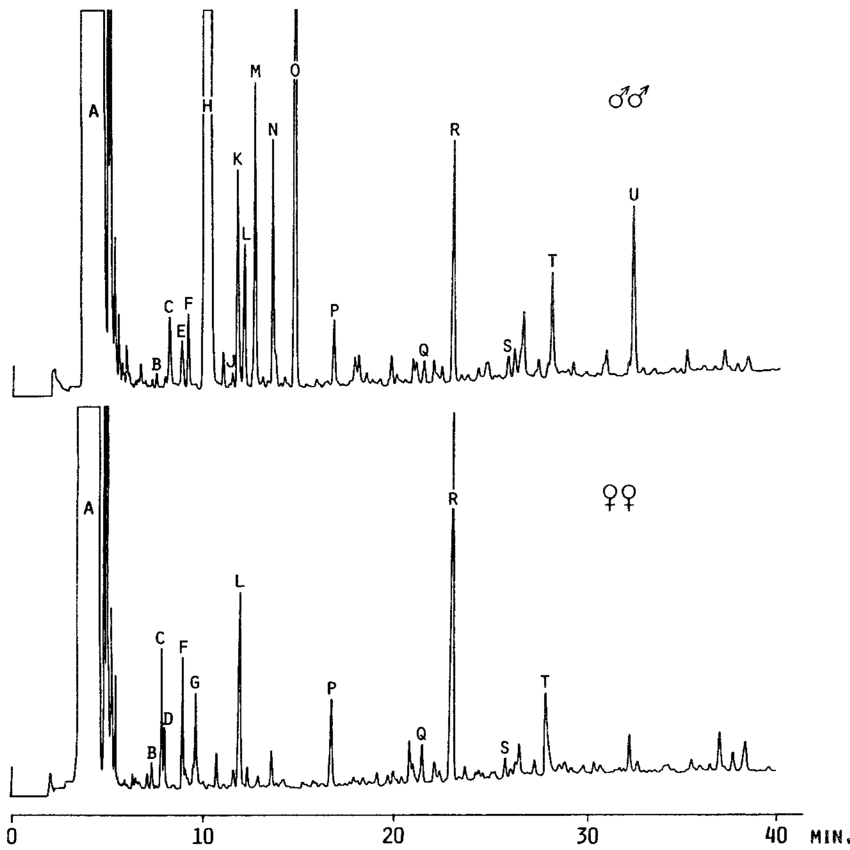

FIG. 4. Comparison of the profiles of the lipophilic basic extract from urine of male and female *Tupaia belangeri*. The capital letters over the peaks label compounds analyzed by mass spectrometry. Analysis conditions for GC: 3.0-μl sample injected; splitless injection; phase CW-20 M.

Regardless of whether the pryrazine compounds or their metabolites are the signal substances, they are male-specific urine components not previously found at this concentration (50–400 μg 2,5-dimethylpyrazine per milliliter urine from a male in the intermediate weight class) and in this combination in any other mammalian species (Table 4). Pyrazines are naturally produced by the metabolism of various bacteria (Hayward et al., 1977; Labows et al., 1980). In insects, they play a number of important roles; to list only a few examples; they serve as marking substances (Borg-Karlson and Tengö, 1980), alarm pheromones (Wheeler and Blum, 1973), trail pheromones (Evershed et al. 1981), and warning odor components (Rothschild et al., 1984).

TABLE 3. VOLATILE COMPOUNDS FROM PROFILES OF LIPOPHILIC BASIC EXTRACTS OF URINE FROM *Tupaia belangeri*

Peak[a]	Mol. weight	Elemental formula	Chemical name	Identification criteria[b]
A		CH_2Cl_2	Solvent	
B	94	$C_2H_6S_2$	Dimethyl disulfide	II
C	170	$C_{12}H_{26}$	*n*-Dodecane	II
D	87	C_4H_9NO ?	?	III
E	113	?	?	III
F	120	$C_4H_8O_2S$	?	III
G	80		Solvent impurity?	
H	108	$C_6H_8N_2$	2,5-Dimethylpyrazine	II
J	122	$C_7H_{10}N_2$	2-Methyl-5-ethylpyrazine	I
K	122	$C_7H_{10}N_2$	2,3,5-Trimethylpyrazine	II
L	198	$C_{14}H_{30}$	*n*-Tetradecane	II
M	136	$C_8H_{12}N_2$	3-Ethyl-2,5-dimethylpyrazine	II
N	120	$C_7H_8N_2$	2-Methyl-5-vinylpyrazine	I
O	134	$C_8H_{10}N_2$	3-vinyl-2,5-dimethylpyrazine	I
P	226	$C_{16}H_{34}$	*n*-Hexadecane	II
Q	254	$C_{18}H_{38}$	*n*-Octadecane	II
R	94	$C_2H_6O_2S$	Dimethylsulfone	II
S	282	$C_{20}H_{42}$	Eicosane	II
T	222	$C_{15}H_{26}O$	?	III
U	164	$C_{10}H_{16}N_2$	*R*-Pyrazine compound	I
V	114	C_8H_{18}	*n*-Octane	II
W	110	$C_6H_{10}N_2$	Dihydrodimethylpyrazine?	I
X	142	$C_{10}H_{22}$	*n*-Decane	II
Y	162	$C_{10}H_{14}N_2$	?	III
Z	222	$C_{12}H_{14}O_4$	Diethyl-*o*-phthalate	I

[a] Peaks labeled as in the profiles of Figures 4 and 5.
[b] Identification criteria: I, mass spectra comparison; II, reference spectra comparison with authentic substances and their coinjection on three different capillaries; III, not identified.

Pyrazines have also been identified as volatile constituents of various foods (Goldman et al., 1967; van Praag et al., 1968; Mason et al., 1966; Buttery et al., 1971) and are used in the food industry as food flavorings (Maga and Sizer, 1973). They are naturally formed during the roasting process from amino acids and carbohydrates by a Maillard reaction (Koehler et. al., 1969; Rizzi, 1972).

Therefore the tree-shrew pyrazines could, in principle, also be artificially produced from urine components during heating (e.g., in the injector of the gas chromatograph). Evidence that they are not is provided by the following findings: (1) The male-specific roasted odor is detectable in the headspace of urine samples taken directly from the urinary bladder of fertile males. (2) The pres-

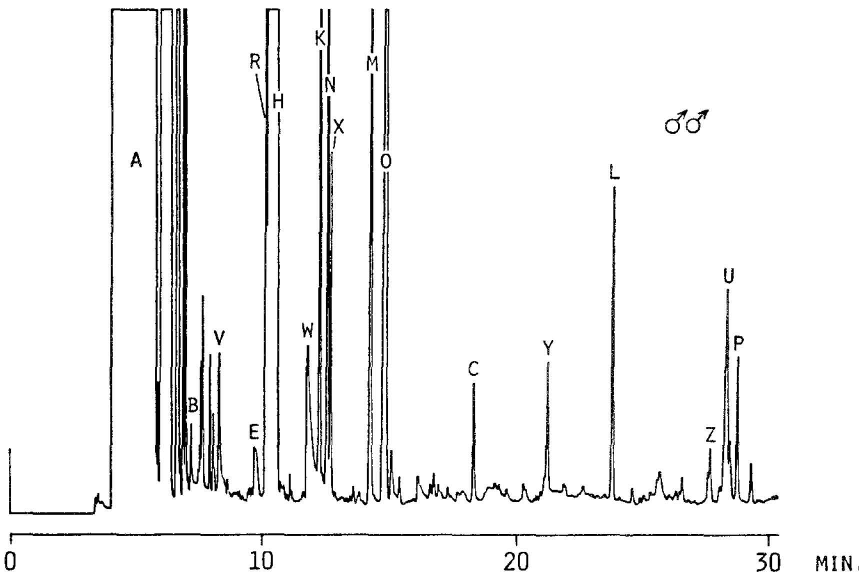

FIG. 5. Profile of the basic extract from urine of male *Tupaia belangeri*, obtained with a different GC capillary (phase OV-101). The capital letters above the peaks label compounds, the mass spectra of which are identical to those of the corresponding peaks in the profile of Figure 4. Peak W does not appear in the profile obtained with phase CW-20 M.

TABLE 4. OCCURRENCE OF TREE-SHREW URINARY SULFUR COMPOUNDS (B AND R) AND PYRAZINES (H–U)[a] IN SCENT PRODUCTS OF OTHER MAMMALS

Animal	Substrate or scent product	Sulfur compounds and pyrazines identified in tree-shrew urine										Reported by
		B	R	H	J	K	L	M	N	O	U	
Hamster	gland secretion	x										Singer et al., 1976
Human	gland secretion		x									Preti et al., 1978
Human	urine			x	x	x			x			Zlatkis et al., 1973
Mouse	urine	x		x								Miyashita and Robinson, 1980
Pig	urine	x										Lunn and van de Vyver, 1977
Rabbit	feces/gland secretion	x				x						Goodrich et al., 1981
Red deer	gland secretion		x									Bakke and Figenschou, 1983

[a]The letters B–U refer to the components so labeled in the profiles of basic urine extract (Figures 4 and 5 and Table 4).

ence of dimethylpyrazines in the basic urine extract from males has been demonstrated by comparison with reference substances in thin-layer chromatography (Figure 2).

The possibility must be considered that male mammals fed on diets like that of the tree shrews might generally excrete pyrazines in the urine as species-unspecific, androgen-dependent metabolites. The comparison of tree-shrew urine with that of fertile male Mongolian gerbils fed for several months on tree-shrew diet contradicts such interpretation. Analysis of the gerbil urine samples revealed no pyrazine compounds; hence pyrazines are probably not a general androgen-dependent urine constituent.

Reaction of Animals to Synthetic Scent Substances. Bioassays with synthetic scent substances were carried out to establish whether odors in general elicit chinning and, if not, what properties (chemical composition, nature of the odor, distribution among mammals) characterize those substances that are effective (Table 5). All scent substances were tested as a 0.1% solution in diethyl ether. They are expected to be perceptible at this concentration, since it is above those measured in normal tree-shrew urine.

The first test series compared the effectiveness of a scent substance that is one of the characteristic components of tree-shrew urine (3-methylthiopropionic acid) with that of amyl acetate, which was not detected in tree-shrew urine. 3-Methylthiopropionic acid is particularly interesting because it is present in higher concentrations in male than in female urine (Figure 3, peak J). Amyl acetate was chosen because it is attractive to tree shrews, presumably because it smells like bananas. In the bioassay, 3-methylthiopropionic acid was overmarked by 19 of 20 animals (average chinning score = 3), but amyl acetate did not elicit chinning at all in half the animals tested. The results suggest that, in general, samples of scent substances typical of the species are overmarked more frequently, and by more animals, than samples of scent substances not produced by these animals ($P < 0.005$); the latter elicit little or no chinning.

In the second series, another odor component of higher concentration in male than in female urine was tested, 2-methylbutyric acid (Figure 3, peak A). In contrast to 3-methylthiopropionic acid, this substance occurs commonly in mammals. 2-Methylbutyric acid was compared with pyrazine-2-carboxylic acid, a pyrazine compound that might be contained in the urine of male tree shrews as a product of the oxidation of alkylpyrazines, although it has not yet been demonstrated in urine samples. Both carboxylic acids fail to elicit chinning in most animals tested. It follows that not all urine components present in different amounts in male and female urine elicit chinning when presented at the high concentration typical of male urine. It appears that for a urine component to be behaviorally effective in isolation, it must have greater species specificity than does 2-methylbutyric acid.

In the third test series, two different dilutions (0.1% and 0.01% in diethyl

TABLE 5. AVERAGE CHINNING SCORES[a] OF TREE SHREWS IN RESPONSE TO SYNTHETIC URINE COMPONENTS AND OTHER SYNTHETIC SCENT SUBSTANCES[b]

Test series	Synthetic scent substances	Median	[25–75%]	R_j[c]	T[d]	N
1	amyl acetate	0.5	0–1	116	7	15
	3-methylthiopropionic acid	3	1–5	164		
2	2-methylbutyric acid	0	0–2	101	15.5*	8
	pyrazine-2-carboxylic acid	0	0–1	109		
3	3-methyl-2-oxovaleric acid	3	1–7	178	9	14
	3-methyl-2-oxovaleric acid 0.01%	0	0–3	121		
4	2,3-dimethylpyrazine (A)	6	2–9	221	A:B = 4.5	17
	2,5-dimethylpyrazine (B)	0	0–2	161	B:C = 32*	13
	2,6-dimethylpyrazine (C)	2	0–3	155	C:A = 2	18
5	pyrazine-2-carboxylic acid m.e. (D)	4.5	1–10	178	D:E = 2.5	15
	2,3,5-trimethylpyrazine (E)	0.5	0–2	113	E:F = 17*	9
	3-ethyl-2,5-dimethylpyrazine (F)	0	0–2	110	F:D = 7.5	14

[a] Each value is the median of the individual chinning scores of all animals tested, 25–75 percentiles in brackets.
[b] Presented as 25-μl aliquots of a 0.1% solution in diethyl ether.
[c] R_j = sum of ranks in Friedman two-way analysis.
[d] Wilcoxon matched-pairs signed rank test; * = not significant, all other differences are significant.

ether) of the typical tree-shrew scent substance 3-methyl-2-oxovaleric acid were compared. The concentration of this component, which smells of meat broth, is about 20 times as high in male as in female urine. In the bioassay the more concentrated samples (0.1%) elicited chinning in 19 of 20 animals (average chinning score = 3), while samples containing only one tenth as much of the substance were nearly ineffective. This result corroborates the above inference that samples containing typical tree-shrew scent substances are overmarked only if the substances are present in the high concentration characteristic of males. Most animals fail to respond to samples with low concentrations, corresponding to those in urine from females.

In the fourth series, the main component of the basic extract from male urine (2,5-dimethylpyrazine) was tested against the isomers 2,3-dimethylpyrazine and 2,6-dimethylpyrazine. 2,6-Dimethylpyrazine is also present in the urine, but in much smaller amounts than 2,5-dimethylpyrazine (ca. 1 : 1000); 2,3-dimethylpyrazine is not detectable. Surprisingly, in the bioassay, 2,3-dimethylpyrazine elicits an intense chinning response in nearly all animals (average chinning score = 6), whereas 2,5-dimethylpyrazine does not stimulate most animals. The average chinning score for 2,6-dimethylpyrazine is 2, larger than that for 2,5-dimethylpyrazine, although the difference is not significant. These

results of bioassay seem inconsistent with those of GC-MS analysis. Perhaps the behavioral responses can be explained by the difference in odor of the dimethylpyrazines; to the human nose, 2,3-dimethylpyrazine has a slight roasted odor like that characteristic of male tree shrews. The other dimethylpyrazines do not smell like this. It may be that 2,3-dimethylpyrazine elicits such a strong chinning response because the tree shrews also detect the resemblance to the sex-specific male scent substance.

The fifth series was designed to test whether what we experience as a roasted odor is a characteristic property of the pyrazines that are overmarked by the tree shrews. Pyrazine-2-carboxylic acid methyl ester has such an odor and is not present in the profile of the basic urine extract; it was compared in the bioassay with 2,3,5-trimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine, both of which are characteristic components in the GC profile of male urine (Figures 4 and 5, peaks K and M). To humans, 2,3,5-trimethylpyrazine has the typical pyrazine odor and 3-ethyl-2,5-dimethylpyrazine smells like chocolate. In the bioassay, pyrazine-2-carboxylic acid methyl ester elicits intense chinning in most animals (average chinning score = 4.5), whereas half of the animals give no response to the other two alkylpyrazines.

This result supports the hypothesis that a roasted odor could be the criterion for effectiveness of a pyrazine compound in eliciting chinning. This roasted odor, as mentioned above, also characterizes peak W of the GC profile and the TLC fraction II of basic extract of male urine, which elicits a stronger behavioral response than any of the other fractions.

The results of these bioassays of synthetic odor substances can be summarized as follows. When tested at the same concentration, the various urine components elicit marking at different rates (Friedman two-way analysis: $x_2^2 = 83$; $P < 0.001$). The marking responses of males and females to a given sample do not differ. Intense chinning is elicited by odor substances that distinguish tree-shrew urine from the urine of other mammals and which, to the human sense of smell, are representative of the typical odor of male tree shrews (certain pyrazine compounds, 3-methyl-2-oxovaleric acid, 3-methyl-thiopropionic acid). On the other hand, odor substances commonly produced by mammals in general (e.g., 2-methylbutyric acid) and substances not produced by tree shrews or other mammals (e.g., amyl acetate) elicit little or no chinning. Substances need not belong to different chemical classes to elicit differential responses; tree shrews also respond quite differently to isomers of a given compound—for example, to 2,3-dimethylpyrazine and 2,5-dimethylpyrazine.

Although various substances at a given concentration are differently effective, the efficacy of a given substance can also depend on its concentration, as the tests of 3-methyl-2-oxovaleric acid demonstrated. Therefore additional experiments are being undertaken to establish threshold curves for the relevant urine components.

These results suggest a number of problems for further study, but for the present it is clear that chinning can be elicited by several urine components, which are also excreted by other mammals. It seems likely that the most pronounced chinning is elicited by a mixture of these components that has a particular quantitative composition.

GENERAL DISCUSSION

Many mammals can distinguish the scent marks produced by conspecifics of different sexes and in different physiological states (Johnston, 1983). In principle, such a discrimination could be based either on single characteristic odor components or on particular mixtures of components. Single-component signals would require mammals to have at their disposal a great variety of scent substances; in the alternative case, a small set of scent substances could be combined to produce many different multicomponent mixtures. As few as 300 substances, combined in groups of 15 components each, would give more than 10^{27} qualitatively different mixtures. The number of different mixtures would be even greater if quantitative differences also counted. This number of odor mixtures would probably suffice to characterize all the mammals now in existence, with regard to species, sex, individual identity, and physiological state.

Of course, no matter how diverse the chemical information mammals can produce, it functions in communication only if an adequate system for detecting and processing the information is available. That the necessary abilities can exist has been demonstrated in training experiments on dogs (Neuhaus, 1956) and mongooses, *Herpestes auropunctatus* (Gorman, 1976), which have learned to distinguish mixtures of several odor components differing in both qualitative and quantitative composition.

The scent substances excreted in mammalian urine are sufficiently numerous to signal all the species and individual characteristics mentioned above (Albone, 1984). It has been shown for the laboratory mouse (Novotny et al., 1980) and the red fox (Jorgenson et al., 1978; Bailey et al., 1980) that various breeds, the two sexes, and different physiological states of an individual can all be characterized by volatile urine components. Similar results have been obtained by the analysis of cutaneous gland secretion (Brinck et al., 1983; Smith et al., 1985).

Male tree shrews also excrete urine that differs from that of females with respect to several volatile components. These components are responsible for the differences in chinning responses of the tree shrews to urine from conspecifics of different sexes. We conclude that these identified pyrazine compounds and monocarboxylic acids are in fact used by the animals to identify the sex of an individual. Male tree shrews can also distinguish their own urine from that

of other male conspecifics, inasmuch as they fail to overmark samples of their own urine (Stralendorff, 1986).

It seems plausible that the quantitative and the qualitative composition of the urine sample contributes to discrimination among individual urine samples, so that the relative proportions of the various components in the urine would be an important feature with regard to identification of the sample. In view of this, it may seem surprising that tree shrews will overmark such crude imitations of the male scent signal as the pure chemicals 3-methyl-2-oxovaleric acid or pyrazine-2-carboxylic acid methyl ester. However, the average chinning score in response to these single components is considerably lower than that to whole urine. Presumably the pyrazine compounds and the monocarboxylic acids are just barely sufficient for an approximate characterization of the male signal—sufficient to distinguish it from the scent signals of female conspecifics. That is to say, odor samples conveying only the general information "fertile male tree shrew" trigger the fixed behavioral response "chinning," whereas urine samples from an identifiable individual may elicit more flexible or graded responses.

But why is the scent signal of an individual overmarked by its conspecifics at all? At first glance, it appears that overmarking would obscure the scent left by the other animal. The sternal-gland secretion deposited during chinning is a complex lipid mixture produced by males at the rate of 10–20 mg/day (Stralendorff, 1977). By spreading it over alien scent marks encountered in his own living space, a male can either eliminate the strange odor or at least change the odor at that spot in such a way that the identity of the animal that produced the original scent mark is no longer discernible.

Unlike the males, female tree shrews produce very small amounts of sternal-gland secretion, less than 1 mg/day. Therefore it is unlikely that chinning by a female would eliminate or disguise the scent left by a male. By adding female-specific scent components, she could modify a male scent mark sufficiently to document her presence. But this goal is probably achievable with urine, so why overmark with sternal-gland secretion? One answer to this question could be provided by a form of behavior observed by chance during the bioassays. That is, if male urine is applied not to the front of a female's cage but rather to the floor, some females rub their entire ventral surface over the spot rather than just the sternal region. They may even roll over on the urine mark, so that the dorsal fur also comes into contact with it. To all appearances, they become sexually excited while doing this. Such behavior gives an observer the impression that the female intends less to eliminate the scent mark than to pick up the male scent in her own fur. Males have never been observed to respond in this way to male urine. We suppose, therefore, that overmarking has a different function for the two sexes.

Studies of animals in large enclosures (Holst, 1985) have shown that both

male and female tree shrews, when in familiar surroundings, vigorously attack strange conspecifics of the same sex. Before, during, and after such fights the males mark the boundaries of their territory or enclosure copiously with urine and sternal-gland secretion. These findings have been confirmed by field observations (Kawamichi and Kawamichi, 1979). In an enclosure, the male that emerges from the conflict as subordinate henceforth ceases marking behavior. Marking at the boundaries of the enclosure accounts for 80% of the marking behavior of male tree shrews. The remaining 20% is done within the remainder of the enclosure and occasionally on the sexual partner. That is, male tree shrews perform chinning predominantly in the territorial context. Assuming that overmarking makes a strange scent mark unrecognizable, this behavior could prevent the strange male from establishing familiarity with the area and thus reduce his chances of winning a new territory. When the occupant of a territory distributes his own scent marks, they could enhance his familiarity with the territory and increase his self-confidence in defending it. These functions of scent-marking have previously been postulated by other authors (Ewer, 1968; Adams, 1976).

Although females overmark the scent marks of strange males with their sternal-gland secretion, they do not attack such males in the territorial context. On the other hand, as mentioned above, a female will violently attack strange females but not overmark their urine marks.

The absolute marking frequency of females in large enclosures is only about $\frac{1}{10}$ that of males (Holst, 1985). Moreover, females do not preferentially mark the boundaries, but rather distribute their marks throughout the area. In doing so, presumably they are overmarking the urine marks of the territorial male. Paired females perform chinning on the head and back of the partner, moving their bodies over the male in the same way as over a male urine sample in the bioassay. This behavior is often observed during mating.

Evidently, then, chinning is done by females not in the context of territorial behavior but in the context of pair formation and pair bonding. If overmarking of male urine marks by a female has the effect of mixing female-specific components with the male scent, the result could be to indicate the presence of a female to strange males. When a female rubs male scent substances into her own fur, the effect could be that when the originator of the male scent later encounters the "perfumed" female she seems olfactorily familiar to him, which might facilitate pair formation or reinforce an existing bond.

If these hypotheses should prove correct, it would follow that identical scent substances (pyrazine compounds and monocarboxylic acids) encode an item of information (fertile male conspecific) that, as a scent signal, elicits a given, fixed behavioral response (chinning). Nevertheless, the "significance" of the scent signal would be different for the two sexes; for males it would mean "rival" and for females, "potential sexual partner."

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FACTORS INFLUENCING DISTRIBUTION OF *Diabrotica* SPP.[1] IN BLOSSOMS OF CULTIVATED *Cucurbita* SPP.

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Abstract—Cultivars representing three species of *Cucurbita* were examined for blossom preference by *Diabrotica* spp. *C. maxima* cultivars were found to be preferred by *D. undecimpunctata howardi* over those of *C. pepo* and *C. moschata*. *D. virgifera virgifera* preferred *C. maxima* and the "Connecticut Field" cultivar of *C. pepo*. *C. moschata* and other cultivars of *C. pepo* were not preferred. Cultivars were examined for differences in floral volatile release, blossom cucurbitacin content, and pollen content of male blossoms. *C. maxima* male blossoms released a larger quantity of volatiles than *C. pepo* or *C. moschata*. Also, only *C. maxima* male blossoms contained cucurbitacins. Cultivars of *C. moschata* contained the largest quantities of pollen, but all three species contained relatively large quantities. The data indicate a correspondence of *D. u. howardi* distribution in the field with high volatile release rates and high cucurbitacin levels that are found in *C. maxima* blossoms. *D. v. virgifera* distribution appears to be somewhat independent of these factors since this species was abundant in blossoms of a *C. pepo* cultivar as well as cultivars of *C. maxima*.

Key Words—*Cucurbita, Diabrotica undecimpunctata howardi, Diabrotica virgifera virgifera,* floral volatiles, cucurbitacins, Cucurbitaceae, Coleoptera, Chrysomelidae, host selection.

INTRODUCTION

Previous studies have shown that *Diabrotica* spp. prefer the blossoms of certain species and cultivars of *Cucurbita* (Fronk and Slater, 1956; Bach, 1977; Fisher et al., 1984). Fronk and Slater (1956) found that the northern corn rootworm

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(*Diabrotica barberi* Smith and Lawrence), the southern corn rootworm (*D. undecimpunctata howardi* Barber), and the related striped cucumber beetle [*Acalymma vittatum* (F.)] prefer the blossoms of *Cucurbita maxima* Duchesne over those of *C. pepo* L. Bach (1977) reported that the "Buttercup" cultivar of *C. moschata* Poir. were preferred by *A. vittatum*. In addition, the "Hubbard" cultivar of *C. maxima* and the "Pumpkin" cultivar of *C. moschata* were preferred by the western corn rootworm (*D. virgifera virgifera* LeConte). Recently, Fisher et al. (1984) found that *D. v. virgifera, D. barberi,* and *D. u. howardi* all preferred the cultivars of *C. maxima* over those of other cultivated species. *D. v. virgifera* differed from the other beetle species in that substantial numbers were also found in certain *C. pepo* cultivars.

The blossom characteristics responsible for preference are not known. It has been demonstrated, however, that the blossoms of *C. maxima* cultivars release indole, an attractant for *D. v. virgifera* adults (Andersen and Metcalf, 1986). Other examples of *Diabrotica* attraction to compounds commonly found in floral odors have recently been reported (Ladd, 1984; Lampman, personal communication). This suggests that differences in odor may be important in determining preference.

Gustatory stimulants may also play a role in blossom selection. Cucurbitacins, a group of oxygenated triterpenoids, occur in the leaves and fruits of wild *Cucurbita* species. These act as nonvolatile feeding stimulants, and have proven to be of importance in host selection by *Diabrotica* spp. (Metcalf et al., 1980). Cucurbitacins do not occur in the fruits and true leaves of cultivated *Cucurbita* (Metcalf et al., 1980). However, their possible occurrence in blossoms has not been investigated. In this report, nutritional and secondary chemical characteristics of blossoms from cultivated *Cucurbita* are examined in relation to beetle preference.

METHODS AND MATERIALS

Field Preference Measurements. Eight cultivars representing *C. moschata, C. pepo,* and *C. maxima* were planted in three rows. The cultivars were arranged in small plots containing six plants each, and the position of each cultivar within a row was randomly assigned. Each row contained one plot of each cultivar, and the distance between the plots was 3 m, preventing any overlap of plants from different plots. Due to severe drought conditions in the summer of 1983, an entire plot (cultivar) was lost in the center row. Consequently, samples were taken from only the two outside rows.

The sampling procedure consisted of selecting two blossoms from each cultivar in each of the outside rows. A small plastic bag was placed over each blossom. The bag was closed, and placed on ice to limit the activity of the insects. In the laboratory, the adults of *Diabrotica* spp. in each bag were

anesthetized with carbon dioxide, identified to species, and counted. Since male blossoms are far more abundant than females, only male blossoms were selected for sampling. Five collections were made during the months of August and September 1983, and these were carried out between the hours of 9:00 and 10:30 AM.

For statistical analysis, the two blossom counts for each row on a given date were averaged, and each mean was treated as a replicate. Data were analyzed by a two-factor ANOVA to test the influence of sampling date and cultivar on beetle distribution. For paired comparisons of means, data were analyzed by one-way ANOVA coupled with the Student-Newman-Keuls test for multiple comparisons. All tests were performed with the Statistical Package for the Social Sciences (SPSS; Nie et al., 1975).

Analysis of Cucurbita *Floral Volatiles.* Floral volatiles from field collected blossoms were obtained by trapping on Porapak-Q using a tubular glass collection chamber described previously (Andersen and Metcalf, 1986). An airstream (2 liters/min) was passed through a column of activated charcoal, through the chamber containing the blossoms, and finally through a short column containing 5 g of Porapak-Q. Volatiles were collected from 20 cut male blossoms in a single batch. The flowers were obtained from field-grown plants on the morning of anthesis and transported to the laboratory on ice. All blossom collections were made in late July and early August, 1983. During this period, infestations of *Diabrotica* spp. were relatively light, so damage to blossoms was minimal.

Volatiles were collected for a period of 4 hr, at the end of which the Porapak-Q was removed from the column and extracted for several hours with diethyl ether in a Soxhlet apparatus. The dilute extract was dried over anhydrous sodium sulfate and concentrated to a volume of 15 ml by slowly removing the ether in a rotary evaporator. Final concentration to 0.5 ml was accomplished under a gentle stream of nitrogen.

For more detailed studies of the qualitative and quantitative composition of floral odors, plants were grown in a greenhouse in large pots. Natural lighting was supplemented by artificial fluorescent and incandescent lighting (16 hr–8 hr light–dark). Applications of resmethrin were made periodically to suppress whiteflies, and no fertilization was provided. Volatiles were obtained from groups of 3–14 blossoms collected from six different plants of each cultivar. The procedure was repeated four times on four separate days. The collection chamber was a 3000-ml three-neck flask having two 24/40 joints and one 45/50 joint. Nitrogen (1.0 liter/min) was filtered through activated charcoal and entered the flask through one of the 24/40 jointed necks. Attached to the second 24/40 neck was a short column containing 2.5 g Tenax TA (60/80). At the end of a 5-hr collection period, the column containing the Tenax TA was backflushed with 150 ml anhydrous diethyl ether. The extract was then concentrated to a volume of ca. 12 ml by slow removal of the solvent through a Vigreux

column. Final concentration to 200–300 μl was performed under a gentle stream of nitrogen.

Gas–liquid chromatography (GLC) of volatile samples from field collected blossoms was performed on a Varian 2700 instrument equipped with a hydrogen flame ionization detector (FID) and a 3.7-m × 2-mm ID glass column packed with 3% OV-17 on Gas Chrom Q (100/120). The temperature was programmed from 110 to 180° C at 4°/min with an initial hold of min at 110° C and a final hold of 15 min at 180°C. Quantitative work was performed using a Varian 3700 instrument equipped with an FID and a 25-m × 0.32-mm fused silica WCOT column having a bonded phase of DB-1701. The temperature was programmed from 40 to 200°C at 6°/min with a 5-min hold at 40°C and a 15-min hold at 200°C.

Electron impact gas chromatography–mass spectrometry (GC-MS) (70 eV) was performed on Hewlett Packard 5985, Finnigan 1020, and Finnigan TSQ quadrupole instruments equipped with fused silica capillary columns of DB-1 or DB-1701 (J&W Scientific). When standards were available, compound identities were verified by retention comparisons with authentic standards on the DB-1701 column. Retention indices were calculated using the homologous *n*-alkane series (Van den Dool and Kratz, 1963).

To quantify individual components, areas of sample capillary GLC peaks were measured by electronic integration and compared to values obtained with known quantities of authentic standards.

Indole was detected in volatile samples from field- and greenhouse-collected blossoms by TLC on silica gel (0.1- and 0.25-mm plates) using a mobile phase of chloroform–benzene–ethanol (83 : 8.5 : 1; v/v/v). The developed plates were sprayed with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) which reacts to form a pink spot (Durkee and Sirois, 1964). Quantification of indole from field-collected samples was made by HPLC. A silica gel radial compression-type column (Waters Assoc.) was employed with a mobile phase of 10% tetrahydrofuran in hexane (2.0 ml/min) (Andersen and Metcalf, 1986). The eluent was monitored at 254 nm using a UV absorbance detector, and the retention time of indole was determined by comparison with an authentic standard. Quantification of indole was made by electronic integration of sample and standard peak areas.

Cucurbitacin Analysis. Blossoms from all cultivars utilized in field measurements of beetle preference were subjected to analysis for cucurbitacins. Ten to fifteen male blossoms were collected from each cultivar on single dates in August and September, 1983, and returned to the laboratory on ice. The blossoms (without stems) were homogenized in a blender with 50 ml distilled water. The homogenate was added to a flask with 250 ml chloroform and stirred vigorously for 2 hr. The mixture was then filtered through cheesecloth into a separatory funnel. The chloroform layer was collected, dried over anhydrous so-

dium sulfate, and rotary evaporated to dryness. The residue was dissolved in chloroform at a concentration of 10.0 g plant material (wet weight)/ml.

A bioassay developed by Metcalf et al. (1980) was used for the detection of cucurbitacins. Twenty microliters of each extract was applied to 0.1-mm silica gel TLC plates which were developed in a mobile phase of chloroform–methanol (95:5; v/v). Cucurbitacins were detected by placing the developed plate in a cage with 100 adult *D. u. howardi* and a source of 5% sucrose solution. Over a period of several days, the insects ingested silica gel from the area of the plate containing cucurbitacins.

Separate extraction of the corolla, nectary, and anther–filament region from five male blossoms of "Pink Banana Jumbo" showed cucurbitacins to be localized in the anther–filament structure of the male blossom. This greatly facilitated isolation for quantitative studies, since the heavily pigmented corolla could be discarded prior to extraction.

The isolation of blossom cucurbitacins was accomplished by preparative TLC of anther–filament extracts on 0.25-mm silica gel plates containing fluorescent indicator. These were developed in diethyl ether–hexane–methanol (70:30:5; Metcalf et al., 1980). Bands were located by observing the quenching of fluorecence under short wave UV light and subsequently were removed from the plate. The bands were extracted with chloroform, and a small portion was chromatographed along with authentic cucurbitacin standards using the same solvent system as above. Cucurbitacins were identified by comparison of R_f values with authentic standards using the beetle feeding assay for detection.

Small quantities of interfering pigments were present after the first preparative chromatography. Consequently, the material was rechromatographed in the same solvent system, and again the UV absorbing bands were recovered. At this point, cucurbitacin levels could be quantified by measuring percent transmittance at 210 nm in absolute methanol with a Hitachi-Perkin Elmer 124 double-beam spectrophotometer. The values obtained were compared with those from a curve generated with authentic standards.

Individual variation in cucurbitacin levels was measured in greenhouse-grown plants (grown under the conditions described above) by the analysis of anther–filament extracts from single male blossoms. Immediately after picking, the anther–filament structures were dissected from both mature (postanthesis) blossoms and immature buds ($>$ 1 day preanthesis). They were then placed in a 150-ml flask with 1 ml methanol and chopped with a spatula. Fifty milliliters of chloroform were added and the mixture was shaken for 4 hr. After filtration through anhydrous sodium sulfate, the extract was evaporated to dryness and taken up in 0.25 ml chloroform.

Analyses were made by TLC on 0.25-mm silica gel plates (ether–hexane–methanol; 70:30:5) and by HPLC with a C_{18} column (Whatman; methanol–water; 60:40; 1 ml/min). The HPLC eluent was monitored at 230 nm with a

UV detector. Compound identity was verified by comparison of HPLC retention time and TLC R_f value with authentic standards. Cucurbitacins were visualized on TLC plates by observing the quenching of fluorescence under short wave UV radiation. Quantification was accomplished by comparison of HPLC peak areas with a curve generated from authentic standards.

Quantification of Pollen Weights. On the evening prior to anthesis, four to five male blossoms on field-grown plants were enclosed in plastic pollination bags to exclude insects. The following morning, the blossoms were harvested and returned to the laboratory. The corollas were removed, and the pollen was rinsed with distilled water from the anther and nectary areas onto a single pre-weighed piece of filter paper. The samples were air-dried for 24 hr, at which time the weight was determined.

Blossoms from greenhouse-grown plants (grown under the conditions described above) were treated in the same manner with two exceptions: The blossoms were not shielded by pollination bags, since no pollen feeding insects were present, and the pollen contents of individual blossoms were kept separate and weighed individually.

RESULTS

Field Preference Measurements. Two *Diabrotica* species. *D. virgifera virgifera* and *D. undecimpunctata howardi*, were common in the study area. *D. v. virgifera* was more abundant on the early sampling dates, while *D. u. howardi* was the most abundant species later (Figure 1). Results of a two-factor analysis of variance revealed a significant preference by *D. v virgifera* for certain cultivars ($P < 0.001$). No significant interaction between cultivar and sampling date effects was seen ($P > 0.05$), indicating that preference was consistent throughout the growing season (Figure 1). Comparisons of *D. v. virgifera* counts combined over the entire sampling period showed that three cultivars, "Blue Hubbard," "Pink Banana Jumbo," and "Connecticut Field," were significantly preferred over all others (Table 1). The former two are *C. maxima* cultivars, and the latter is a *C. pepo* cultivar.

D. u. howardi also showed significant preference for certain cultivars ($P < 0.001$), but unlike *D. v. virgifera*, a significant interaction between cultivar and sampling date was seen ($P < 0.001$). In Figure 1, it is apparent that the numbers of *D. u. howardi* found in *C. maxima* blossoms increased dramatically over the course of the sampling period, while the numbers found in *C. pepo* and *C. moschata* blossoms were similarly low on all sampling dates. Data given in Table 1 demonstrate that both cultivars of *C. maxima* were strongly preferred by this species.

Analysis of Cucurbita *Floral Volatiles.* GLC was performed to compare the volatile compositions or "profiles" from field-collected blossoms of each

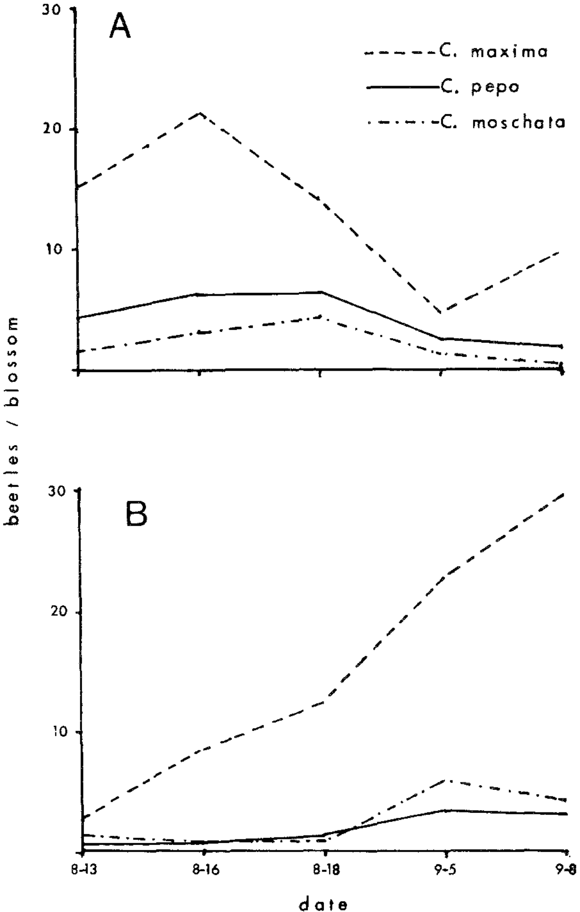

FIG. 1. Seasonal distribution of *D. v. virgifera* (A) and *D. u. howardi* (B) in blossoms of three species of cultivated *Cucurbita* in 1983. *C. maxima* = 2 cultivars; *C. pepo* = 4 cultivars (including *C. pepo* × *C. texana* hybrid); *C. moschata* = 2 cultivars.

Cucurbita species sampled in field preference measurements. Injection quantities representing equal periods of volatile collection were analyzed to detect differences in the qualitative or quantitative composition of the floral volatile mixtures.

It was obvious from these analyses that *C. maxima* cultivars produced a much larger quantity of an apparently more complex mixture. The differences are illustrated in Figure 2 by a comparison of gas chromatograms of "Blue Hubbard" (*C. maxima*), "Connecticut Field" (*C. pepo*), and "Waltham Butternut" (*C. moschata*), representing equal durations of volatile collection.

TABLE 1. MEAN DISTRIBUTIONS (± SE) OF *D. v. virgifera* AND *D. u. howardi* IN BLOSSOMS OF CULTIVATED *Cucurbita* SPP. ON FIVE SAMPLING DATES IN AUGUST AND SEPTEMBER, 1983

Species and cultivar	*D. v. virgifera*[a]	*D. u. howardi*
C. maxima		
"Blue Hubbard"	14.2 ± 2.7a	17.3 ± 2.6
"Pink Banana Jumbo"	11.5 ± 1.8a	13.4 ± 3.1
C. pepo		
"Connecticut Field"	9.0 ± 1.5a	1.9 ± 0.6
"Early White Bush Scallop"	2.7 ± 0.6b	1.6 ± 0.3
"Yellow Summer Crookneck"	3.0 ± 0.5b	2.2 ± 0.4
C. pepo × *C. texana*	3.9 ± 0.9b	1.8 ± 0.5
C. moschata		
"Dickinson Field"	3.4 ± 0.8b	2.7 ± 0.9
"Waltham Butternut"	1.7 ± 0.3b	2.9 ± 0.5

[a]Means followed by different letters are significantly different at the 5% level when analyzed by ANOVA and the Student-Newman-Keuls test for multiple comparisons.

The qualitative composition of *C. maxima* floral volatiles was examined by GC-MS. Samples of the volatile material from field-collected blossoms of "Blue Hubbard" and greenhouse-grown "True Hubbard" were compared. "True Hubbard" was not included in the field-sampling experiment described above, but was found to be highly preferred over "Waltham Butternut" by *D. v. virgifera* and *D. u. howardi* in small field plantings during 1985 (Andersen, unpublished observation).

Volatiles from both cultivars were qualitatively and quantitatively similar, despite differences in growth conditions and sample preparation. The predominant components of both were simple aromatics and sesquiterpene hydrocarbons (Table 2). In separate studies with floral steam distillates (Andersen, unpublished), the diversity of sesquiterpenoids seen in headspace samples was not encountered. These compounds are apparently somewhat labile, and degradation occurs even under the relatively mild conditions of reduced pressure distillation.

The volatile mixture released by greenhouse-grown "Connecticut Field" male blossoms was compared chromatographically to the *C. maxima* volatile mixtures and was found to be similar in composition (Table 2). As with the field-collected samples, however, much smaller quantities of volatile material were released, and consequently fewer components were detected.

In greenhouse studies with "True Hubbard" and "Connecticut Field,"

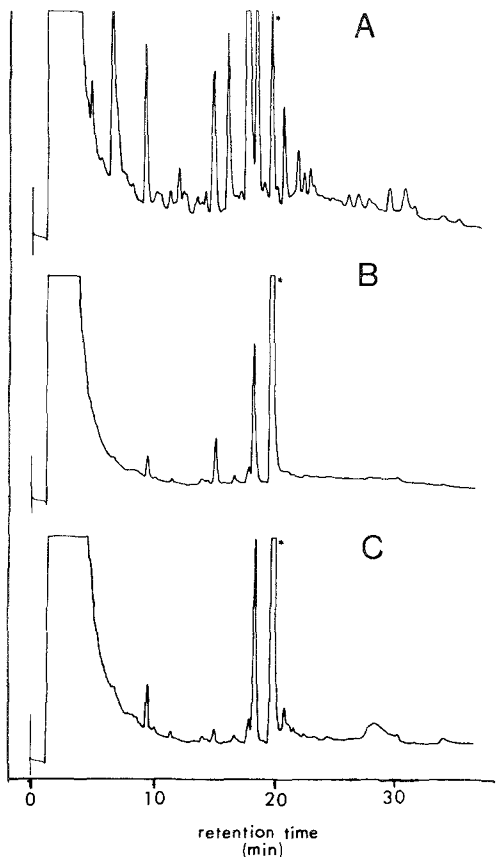

FIG. 2. Gas chromatograms of "Blue Hubbard" (A), "Connecticut Field" (B), and "Waltham Butternut" (C) on 3% OV-17. Each chromatogram represents the equivalent release of one blossom in 0.2 hr. Asterisk indicates butylated hydroxytoluene added as a preservative.

the relative quantities of odor components were not perfectly consistent from sample to sample. With both cultivars, there appeared to be a general increase in the quantities of sesquiterpene hydrocarbons released with time. This variation is shown by the ranges of relative composition given in Table 2. "Connecticut Field" was sampled four times over a period of seven days and "True Hubbard" was sampled four times on four consecutive days. Although these changes were significant, the levels of volatiles released by "Connecticut Field" never approached those exhibited by "True Hubbard."

As an absolute measure of the differences between cultivars, the release rates of the major components from greenhouse-grown plants of "True Hub-

TABLE 2. COMPOSITION OF HEADSPACE SAMPLES FROM CULTIVATED *Cucurbita* CULTIVARS

Compound	Retention index[a]	Prominent ions (EI)[b]	Percent		
			Blue Hubbard	True Hubbard[c]	Connecticut Field[c]
1. Benzyl alcohol	1221	79, *108*, 107, 77	tr[d]	7.9–17.4	tr
2. 2-Phenylethanol	1275	91, 92, 74, *122*	tr	—[e]	—
3. *p*-Dimethoxybenzene	1289	124, *138*, 96	28.9	42.2–69.3	72.5–100.0
4. $C_{15}H_{24}$ hydrocarbon	1437	105, *204*, 161, 119, 189	1.1	tr–0.7	tr
5. $C_{15}H_{24}$ hydrocarbon	1472	119, 93, 105, 69, 161, *204*	3.1	tr–0.9	—
6. $C_{15}H_{24}$ hydrocarbon	1472	91, 93, 133, 105, 161, *204*	1.8	tr–2.9	tr–14.1
7. *p*-methoxybenzyl alcohol	1495	*138*, 109, 77, 121	tr	tr	—
8. (*E*)-cinnamyl alcohol	1514	92, *134*, 78, 105, 115	tr	tr	—
9. 1,2,4-trimethoxybenzene	1538	*168*, 153, 125, 110	0.7	1.1–2.4	—
10. $C_{15}H_{24}$ hydrocarbon	1550	161, 105, 119, 91, *204*	34.5	8.8–25.1	tr–8.6
11. Indole	1565	*117*, 90, 89	3.0	tr	tr
12. $C_{15}H_{24}$ hydrocarbon	1566	121, 93, 107, 79, 161, *204*	2.8	tr–2.5	tr
13. Nerolidol isomer	1675	69, 93, 107, 136, 161	tr	tr–1.5	tr

[a] Calculated on DB-1701 capillary.
[b] In order of decreasing abundance, molecular ion italicized when present.
[c] Range of relative percentages from four collections.
[d] Less than 0.5%.
[e] Not detected.

bard'' and ''Connecticut Field'' were determined by GLC. Peak areas from samples were compared to those of authentic standards for each identified compound. Since none of the sesquiterpene hydrocarbons were fully characterized, release rates were estimated by comparison of peak area with an authentic standard of β-caryophyllene. In these tests, ''True Hubbard'' was found to release far more volatile material than ''Connecticut Field.'' With ''True Hubbard,'' four components occurred in measurable quantities in all samples. These made up 90–95% of the total mixture and showed a combined mean release rate of 11.8 μg/blossom/hr (Table 3). ''Connecticut Field'' released only one consistently measurable component, *p*-dimethoxybenzene, which comprised 72–100% of the volatile mixture. The average release rate of this compound was 2.4 μg/blossom/hr (Table 3).

Indole has previously been isolated from the floral volatiles of *C. maxima* and found to be attractive to *D. v. virgifera* when tested in field sticky traps (Andersen and Metcalf, 1986). Qualitative measurements by TLC with detection by Ehrlich's reagent showed that this compound was present in field-collected blossoms of all three species tested for beetle preference. Quantification of indole in headspace samples of blossoms taken from the field showed that more indole was released by cultivars of *C. maxima* than by *C. pepo* or *C. moschata* cultivars (Table 4). The ''Connecticut Field'' and ''Early White Bush Scallop'' cultivars of *C. pepo* did not release levels of indole detectable by TLC or HPLC.

Indole was also detected in greenhouse-grown blossoms of ''True Hubbard'' and ''Connecticut Field'' by TLC and capillary GLC (Table 2). However, the levels released were very small (<50 ng/blossom/hr) in both cases

TABLE 3. RELEASE RATES OF MAJOR VOLATILE COMPONENTS FROM BLOSSOMS OF ''TRUE HUBBARD'' AND ''CONNECTICUT FIELD''[a]

	Release rate (μg/blossom/hr ± SE)	
	''True Hubbard''	''Connecticut Field''
Benzyl alcohol	1.93 ± 0.36	—
p-Dimethoxybenzene	7.99 ± 0.35	2.41 ± 0.43
RI 1472 (peak 6)	0.07 ± 0.06[b]	0.10 ± 0.09[b]
1,2,4-Trimethoxybenzene	0.23 ± 0.08	—
RI 1550	1.68 ± 0.42	0.05 ± 0.04[b]
RI 1566	0.09 ± 0.05[b]	—

[a] Components not fully characterized are referred to by retention index values (Table 2).
[b] Component did not occur in measurable quantities in all four replicate samples. In these cases it was given a value of zero.

TABLE 4. RELEASE RATES OF INDOLE FROM SINGLE GROUPS OF 20 MALE BLOSSOMS FROM VARIOUS CULTIVARS OF *Cucurbita* SPP

Cultivar	Release rate (ng/blossom/hr)
C. maxima	
"Blue Hubbard"	117.5
"Pink Banana Jumbo"	367.5
C. pepo	
"Early White Bush Scallop"	—[a]
"Yellow Summer Crookneck"	55.0
"Connecticut Field"	—
C. pepo × *C. texana*	12.5
C. moschata	
"Dickinson Field"	47.5
"Waltham Butternut"	5.0

[a]Not detected.

and did not differ greatly between cultivars. Note the difference between the relative quantities of indole contained in head space samples of field-collected "Blue Hubbard" and greenhouse-grown "True Hubbard" (Table 2). Other studies with floral steam distillates have shown these two cultivars to contain similar levels of indole (Andersen, unpublished data).

Cucurbitacin Analysis. Chloroform extraction of single batches of whole field-collected male blossoms followed by the TLC feeding bioassay indicated that cucurbitacins were present only in the two *C. maxima* cultivars. Both "Pink Banana Jumbo" and "Blue Hubbard" portions of the plate showed two obvious feeding spots, while no traces of feeding were apparent on areas of the plate containing other extracts.

Dissection experiments with 13 blossoms showed that cucurbitacins were contained only in the anther–filament structure of male *C. maxima* blossoms. These were identified as cucurbitacins *B* and *D* by comparison of TLC R_f values with those of authentic standards. In both cultivars, cucurbitacin B occurred in considerably larger quantities than D (Table 5).

In greenhouse studies, cucurbitacin levels of individual blossoms of "Connecticut Field," "Waltham Butternut," and "True Hubbard" were compared. Again, only the *C. maxima* cultivar "True Hubbard" was found to contain cucurbitacins, and these were identified as cucurbitacins B and D by TLC. Quantification was accomplished by HPLC with UV detection. The molar absorptivities of cucurbitacins B and D at 230 nm are reported to be 10,500 and

TABLE 5. CURCURBITACIN LEVELS IN ANTHER–FILAMENT STRUCTURES FROM FIELD-GROWN AND GREENHOUSE-GROWN *C. maxima* MALE BLOSSOMS (μg/blossom)[a]

	Cucurbitacin	
	B	D
"True Hubbard" (postanthesis)[a]	146.8 ± 39.9	tr[c]
"True Hubbard" (bud)[a]	73.9 ± 21.7	tr
"Blue Hubbard" (postanthesis)[b]	137.3	3.3
"Pink Banana Jumbo" (postanthesis)[b]	76.5	6.3

[a]Mean values from 12 greenhouse-grown blossoms (± SE).
[b]Values obtained from single groups of field-collected blossoms.
[c]Trace quantity present.

10,000 respectively (Lavie and Glotter, 1971). This allowed relatively accurate quantification of cucurbitacin B using a cucurbitacin D standard which was available in large quantities.

As expected from the results with field-collected material, five mature blossoms from four plants of "Waltham Butternut" and eight blossoms from four plants of "Connecticut Field" contained no detectable cucurbitacins. Also in agreement with field results was the fact that 12 blossoms from 12 plants of "True Hubbard" showed detectable levels of cucurbitacins B and D (Table 5).

In single blossom extracts, levels of cucurbaticin D were too low to quantify, and levels of cucurbitacin B were quite variable (Table 5). The mean cucurbitacin B content, however, was similar to that seen for the "Blue Hubbard" cultivar in the field (Table 5).

Buds from the same 12 "True Hubbard" plants were extracted individually and cucurbitacins B and D were detected in 11 of 12 individuals. Cucurbitacin B was again dominant, and the levels were somewhat lower than in mature blossoms (Table 5). Bud levels of cucurbitacin B were also variable, but showed a strong correlation with the levels found in mature blossoms from the same plants (Figure 3).

Quantification of Pollen Weights. In the field, cultivars of *C. moschata* contained the largest quantities of pollen (mean of 25.8 mg/blossom, Table 6), while *C. pepo* including (*C. pepo* × *C. texana* hybrid) contained the least (mean of 15.5 mg/blossom; Table 6). *C. maxima* was intermediate in pollen content, with a mean value of 19.5 mg/blossom (Table 6).

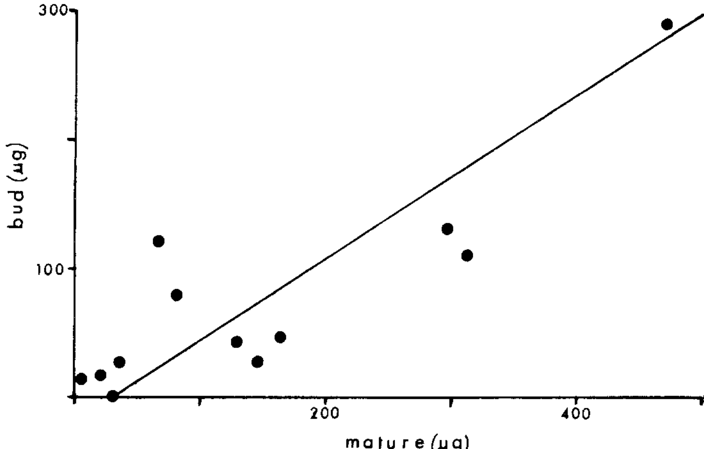

FIG. 3. Relationship of cucurbitacin B levels in postanthesis blossoms of "True Hubbard" (mature) with levels found in immature buds from the same plants ($Y = 0.64x - 20.92$, $r = 0.85$).

TABLE 6. POLLEN CONTENTS OF FIELD-COLLECTED (SINGLE LUMPED MEASUREMENT) AND GREENHOUSE-GROWN MALE BLOSSOMS OF CULTIVATED *Cucurbita* SPP (mg/blossom).

Species and cultivar	Pollen: Field	Pollen: Greenhouse[a]
C. maxima		
"Blue Hubbard"	18.1	—
"Pink Banana Jumbo"	20.9	—
"True Hubbard"	—	21.6 ± 1.9b
C. pepo		
"Connecticut Field"	15.7	21.1 ± 1.8b
"Early White Bush Scallop"	8.9	23.5 ± 1.5b
"Yellow Summer Crookneck"	16.4	18.4 ± 0.8b
C. pepo × *C. texana*	20.9	—
C. moschata		
"Waltham Butternut"	27.1	30.6 ± 1.6a
"Dickinson Field"	24.5	—

[a] Means (± SE) followed by a different letter are significantly different at the 5% level using Student-Newman-Keuls test for multiple comparisons.

In greenhouse measurements, the *C. moschata* cultivar "Waltham Butternut" produced significantly more pollen than *C. pepo* or *C. maxima* cultivars (Table 6). The only large discrepancy between field and greenhouse data involved the *C. pepo* cultivar "Early White Bush Scallop," which produced a very small quantity of pollen in the field. Low pollen weights were also observed on occasion in the greenhouse. For example, the range of values obtained for "Connecticut Field" was 8.9–29.1 mg/blossom. The reason for this occasional drop in pollen production is not known.

DISCUSSION

The species of *Diabrotica* sampled in the field show clear preference for certain cultivars of *Cucurbita* spp. (Table 1). *D. u. howardi* was found almost exclusively in the blossoms of *C. maxima* cultivars, while *D. v. virgifera* preferred the cultivars of *C. maxima* as well as a single cultivar of *C. pepo* ("Connecticut Field"). These data are similar to those of Fisher et al. (1984) who found that *D. u. howardi* and *D. barberi* showed strong preference for *C. maxima* cultivars. *D. v. virgifera* also significantly preferred *C. maxima* over all *C. pepo, C. moschata*, and *C. mixta* cultivars, but blossoms of certain *C. pepo* cultivars did contain significantly more *D. v. virgifera* than *C. moschata* and *C. mixta* (Fisher et al., 1984). In the study of Fisher et al. (1984), *D. v. virgifera* and *D. barberi* were the dominant species numerically. *D. v. virgifera* occurred in much larger numbers than *D. barberi* in blossoms of certain *C. pepo* cultivars, but the two beetle species occurred in similar numbers in *C. maxima* blossoms (Fisher et al., 1984). It appears, then, that *D. barberi* and *D. u. howardi* are similar in their host preferences, each strongly favoring *C. maxima*. *D. v. virgifera*, on the other hand, appears to find a broader range of cultivars acceptable.

Reasons for the strong displays of preference observed here must be related either to higher availability of a food resource in preferred blossoms or to the quality and quantity of sensory stimuli associated with preferred blossoms. An important food resource for *Diabrotica* adults is pollen, which can be obtained from a variety of host plants (Ludwig and Hill, 1975). The quantity of pollen found in field and greenhouse studies did not vary in any discernible way with preference (Table 6). In fact, the least preferred *Cucurbita* species (*C. moschata*) contained the largest quantities of pollen. This suggests that pollen content is not a major factor in the discrimination of preferred and nonpreferred blossoms by *D. v. virgifera* or *D. u. howardi*.

Sensory stimuli involved with host selection can act on the olfactory, gustatory, and visual senses. The importance of olfactory stimuli in host selection by *Diabrotica* spp. has been established indirectly through field trapping ex-

periments. Andersen and Metcalf (1986) isolated indole from headspace samples from *C. maxima* blossoms and found it to be a potent attractant for *D. v. virgifera* and the related *Acalymma vittatum* (F.). Morgan and Crumb (1928) found that cinnamaldehyde and cinnamyl alcohol attracted *D. u. howardi* to field traps. (*E*)-Cinnamyl alcohol was found as a component of *C. maxima* headspace samples, and both of these compounds occurred in steam distillates of *C. maxima* blossoms (Andersen, unpublished data). In field trapping tests, Lampman (personal communication) has found several simple aromatic compounds, including the floral components benzyl alcohol and 2-phenylethanol, to be attractive to *D. u. howardi*. Ladd (1984) has found eugenol, isoeugenol, and 2-methoxy-4-propylphenol to be potent attractants of *D. barberi*. Interestingly, none of the latter were found in *Cucurbita* headspace samples.

In both the field and greenhouse, cultivars of *C. maxima* were found to release larger quantities of volatile material than other cultivated species, even when accounting for variation in the release rates of single components (e.g., indole). This observation corresponds well with preference data for *D. u. howardi* given here, and with published data for *D. barberi* (Fisher et al., 1984). It is impossible at this time, however, to determine the effect on beetle behavior of any single volatile component or group of components. The variety of compounds found to be active in the behavioral studies listed above suggests that multiple floral components may be acting in attraction to blossoms. The efficacy of multicomponent plant-derived attractants has been demonstrated for other phytophagous species (Fein et al., 1982) and, recently, synergistic effects have been observed with three-component attractants of *D. u. howardi* (Metcalf and Lampman, unpublished data). More study of the effects on beetle behavior of single components, multiple components, and variation in multiple-component mixtures needs to be performed in order to determine the nature of the volatile stimulus from *Cucurbita* blossoms.

It is clear from field preference data and quantitative volatile release measurements that floral odor is not of sole importance in blossom selection by *D. v. virgifera*. The "Connecticut Field" cultivar of *C. pepo* is highly preferred by this species, but releases little volatile material (Table 3, Figure 2), while "Waltham Butternut" has a similar volatile profile to "Connecticut Field" (Figure 2), but is almost completely ignored by *D. v. virgifera*.

More clearly understood than the effects of floral odors are the effects of cucurbitacins as specific gustatory cues. Adults of *D. v. virgifera*, *D. u. howardi*, and *D. barberi* respond to these substances by the cessation of locomotion followed by compulsive feeding (Metcalf et al., 1980, 1982). This occurs even when the substrate is an inert material such as silica gel (Metcalf et al., 1980, 1982). In both greenhouse and field tests, only *C. maxima* cultivars contained detectable cucurbitacins. Mature foliage and fruits of this species are reported not to contain these compounds (Metcalf et al., 1980), but the coty-

ledons are a rich source of cucurbitacins B and D (Ferguson et al., 1983). When herbivorous insects were excluded (greenhouse studies), cucurbitacins were found at levels comparable to those in field-collected *C. maxima* (Table 5) and were absent from *C. moschata* and *C. pepo*. This indicates that the presence of cucurbitacins in *C. maxima* blossoms is not due to induced synthesis or translocation caused by feeding damage.

As with floral odor, the distribution of *D. u. howardi* and the reported distribution of *D. barberi* (Fisher et al., 1984) correspond very well to the cucurbitacin levels of male blossoms, suggesting that cucurbitacins may be a determining factor in blossom selection by these species. Also, the preference of *D. v. virgifera* does not correspond to cucurbitacin levels detected in field and greenhouse experiments, since ''Connecticut Field'' contained no detectable cucurbitacins and was preferred along with the *C. maxima* cultivars. A notable field observation with regard to these results was that *C. maxima* male blossoms incurred much higher levels of tissue damage than ''Connecticut Field.'' This was true even when *D. u. howardi* were nearly absent from the sampling area on early collection dates. Feeding by *D. v. virgifera* was concentrated in the anther area and was apparently due to the presence of cucurbitacins. *D. v. virgifera* found on ''Connecticut Field'' blossoms did not feed as actively as those on *C. maxima* and were dispersed about the surface of the corolla.

While gustatory and olfactory cues may act individually, they may also act in concert to produce the pattern of preference exhibited by *D. u. howardi*. High levels of volatile release may increase arrival rates at blossoms, while after arrival, the nonvolatile cucurbitacins would decrease the departure rate by stimulating feeding and arresting locomotion (Howe et al., 1976; Metcalf et al., 1982). Over time, this would lead to the large aggregations of *D. u. howardi* normally found in *C. maxima* male blossoms.

Apparently, neither olfactory or gustatory stimuli serve as the sole blossom selection cues for *D. v. virgifera*. In this instance, visual qualities of blossoms may be most important. *Diabrotica* species are known to orient to yellow-colored surfaces (Hein and Tollefson, 1984), and yellow is the color of *Cucurbita* blossoms. Also, the spectral sensitivity of *D. v. virgifera* has been determined to lie in the yellow-green and near-ultraviolet regions (Agee et al., 1983).

Chemical factors may still play a role in blossom selection by *D. v. virgifera* since Fisher et al. (1984) found a significant preference for *C. maxima* cultivars. The degree of preference, however, was much smaller than that seen with *D. barberi* and *D. u. howardi*. In this study, both *C. maxima* cultivars contained more *D. v. virgifera* than the ''Connecticut Field'' cultivar of *C. pepo*, but the differences were not significant (Table 1). This trend of a small preference for *C. maxima* cultivars over the most preferred *C. pepo* suggests that visual cues may be of primary importance and that these may be augmented

by chemical factors. Specifically, the blossoms of *C. maxima* and "Connecticut Field" may have similar visual characteristics, but the high levels of volatile release or the presence of cucurbitacins would increase insect arrival rates, decrease departure rates, or both.

In addition to spectral qualities, visual characteristics important in blossom selection of *D. v. virgifera* may include differences in blossom size or differences in blossom visibility due to plant growth form. These differences do exist and should be examined more closely, if a complete explanation of blossom selection by *D. v. virgifera* is to be gained.

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FIELD RESPONSE OF SPRUCE BARK BEETLE, *Ips typographus*,[1] TO AGGREGATION PHEROMONE CANDIDATES[2]

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Abstract—Six compounds previously identified from hindguts of unmated male *Ips typographus* (L.) during host colonization: 2-methyl-3-buten-2-ol (MB), *cis*-verbenol (cV), *trans*-verbenol (tV), myrtenol (Mt), *trans*-myrtanol (tM), and 2-phenylethanol (PE), were tested for their attractivity in the field with a subtractive method. The amounts of MB and cV released from a pipe trap were similar to those given off from the commercial bait Ipslure as well as that from a Norway spruce tree, *Picea abies* (L.) Karst., under mass attack. The blend of the compounds became nonattractive when either MB or cV was subtracted, while subtraction of any of the other four compounds had no effect. Addition of ipsdienol (Id) to the blend did not significantly increase the attraction. In a second comparative test, the addition of three compounds as a group (tV + Mt + PE) to MB + cV again had no effect on the attraction, but the addition of Id increased the catch somewhat. Addition of host logs to a bait releasing MB + cV at a rate lower than in previous experiments did not influence the attraction to pipe traps. Sticky traps containing natural pheromone sources (50 males in a log), which released 1–5 mg/day of MB as determined by aerations with deuterated MB as internal standard, were less attractive than a synthetic source releasing similar amounts of MB.

Key Words—2-Methyl-3-buten-2-ol, *cis*-verbenol, *trans*-verbenol, myrtenol, *trans*-myrtanol, 2-phenylethanol, ipsdienol, subtractive assay, *Ips typographus*, Coleoptera, Scolytidae, *Picea abies*, host volatiles.

[1]Coleoptera: Scolytidae.
[2]This study was made within the Swedish project "Odour Signals for Control of Pest Insects."

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INTRODUCTION

The aggregation pheromone system by which the spruce bark beetle of Eurasia, *Ips typographus* (L.), colonizes a patch, such as a tree or a part of a tree, consists of two major signals. One is an attractive signal initiating the attack, and a second is a density-regulating signal that also functions in terminating the attraction (Schlyter et al., 1986). The chemical nature of the first attractive signal is the subject of this study.

The existence of a male-produced aggregation pheromone in the spruce bark beetle has been shown in the field by Bakke (1970) and Rudinsky et al. (1971). In the laboratory, it was later shown that the pheromone was released soon after host contact, with maximum attraction reached after 4–6 hr, followed by a rapid decline after the admission of females (Schlyter and Löfqvist, 1986).

Chemical analysis by gas chromatography of *I. typographus* males indicated the presence of *cis*-verbenol (cV), *trans*-verbenol (tV), ipsenol (Ie), and ipsdienol (Id), which were also found in other *Ips* species (Vité et al., 1972). However, different combinations of these substances had rather weak attractivity in the field (Bakke, 1976). The identification of 2-methyl-3-buten-2-ol (MB) with gas chromatography–mass spectrometry (GC-MS) by Bakke et al. (1977), together with the determination of (−)-(4*S*) as the active isomer of cV (Krawielitzki et al., 1977) formed the basis for the design of lures competitive with natural pheromone sources. Ipslure® (Bakke and Riege, 1982) and Typolure II (Sauerwein and Vité, 1978) both contain MB, cV, and Id, but in different ratios, while Typolure I (Vaupel et al., 1981) and Pheroprax® (Adlung, 1979) contain only MB + cV. However, rigorous tests to determine the attractivity of the individual components MB, cV, and Id are still lacking. These lures have also been tested with α-pinene and host volatiles (resin) to increase attraction, but with less clear results (Vaupel et al., 1981; Bombosch et al., 1982).

A detailed chemical analysis of beetles in different attack phases has showed that unpaired males with a completed nuptial chamber (phase 3) had a blend of at least six major components in their hindguts (Birgersson et al., 1984): MB, cV, tV, myrtenol (Mt), *trans*-myrtanol (tM), and 2-phenylethanol (PE). A seventh compound, verbenone, was only found in trace amounts. After mating and the beginning of egg-laying, some males also had Id and Ie, while the seven other components had decreased. It is possible that all of these natural compounds of related chemical structure have behavioral activity.

In this field study we have analyzed the attractivity of the components of the natural blend by a subtractive assay (Byers et al., 1985), compared the natural blend with three lures, tested synergism between host and synthetic pheromone, and compared natural and synthetic pheromone sources.

METHODS AND MATERIALS

Substances and Release Rates

The substances tested were those of the natural blend identified from male hindguts by Birgersson et al. (1984); see Table 1 for sources, purity, and release rates. Attempts were made to adjust the release rates to represent the ratios found in male hindguts. For comparison, the absolute and relative release rates of cV and MB were measured from commercial Ipslure® dispensers.

An airflow of 100 ml/min was passed over 0.1-m sections of the laminated Borregaard/Hercon® Ipslure bands in a cuvette (diameter 5 cm), and the resulting volatiles were trapped on a plug of 325 mg Porapak®-Q. The adsorbed volatiles were extracted with 2 ml pentane, and an internal standard (100 g C_7Ac) was added. The extracts were then subjected to GC for quantification and GC–MS for identification, conditions as in Birgersson et al. (1984). The release rates were calculated to be 50, 1, and 0.3 mg/day of MB, cV, and Id, respectively, for a 1-m strip (standard length) of Ipslure dispenser after one week of aging.

Field Tests

Subtractive Assay. Dispensers with test chemicals were placed in the lower part of black drainpipe traps with white exterior funnels ("N79 with funnel" type, Regnander and Solbreck, 1981). Traps were placed about 50 m apart on clear-cuts and randomized after each replicate period which lasted a few hours to more than two days, depending on flight activity. The test was carried out at two different sites in 1982: site A (Aborrtjärnsberget), a fresh clear-cut with a very high local beetle density (> 100 trees killed the previous year), and site L (Lilltjärnsberget), an old clear-cut with a low population density (no trees killed within 1 km the previous year), close to Torsby, province of Värmland, middle Sweden, 1982.

Comparative Study. Four different clear-cut areas in 1982 near Torsby were used for the comparison between the "phase-3" (natural) and the three "commercial" compositions. Two were fresh clear-cuts with high beetle densities: site A (as above) and site T (Torkbäcken, > 10 trees killed the previous year), and two were old with lower densities: site B (Boseberget) and site G (Gäddtjärnsberget). Both sites B and G had no trees killed the previous year. The later site had an elevation difference of about 50 m, while the other had less than 20 m. Traps and positioning were as in the subtractive assay.

Host Synergism. Pipe traps without funnel ("N79" type, Bakke et al., 1983) were baited with screened logs (6 cm diameter, 30 cm long, with ten 2- to 3-cm axe cuts) freshly cut from a Norway spruce (*Picea abies* (L.) Karst.) with or without a "medium" dose of MB + cV (Table 1). Together with traps

TABLE 1. CHEMICALS, RELEASE RATES AND DISPENSERS USED IN FIELD TESTS OF AGGREGATION PHEROMONE COMPONENT CANDIDATES IN *Ips typographus*

Compound	Chemical purity (%)[a]	Source	Measured[b] release rate (mg/day) (±95% C.I.)[b]	Dispensers[c]
Subtractive and comparative tests, Värmland, Sweden, 1982				
2-Methyl-3-buten-2-ol (MB)	97	Aldrich	57.0 ± 0.8	hard vial, 1-mm hole
(4*S*)-*cis*-Verbenol (cV)	96	Borregaard	1.0 ± 0.05	hard vial, 9-mm hole
(4*S*)-*trans*-Verbenol (tV)	98	KTH	0.27 ± 0.004	"730" with 4.2-mm hole
(1*S*)-Myrtenol (Mt)	92	Aldrich	0.084 ± 0.01	"730" with 200-μl capillary
(1*S*)-*trans*-Myrtanol (tM)	>99	Fluka	0.037 ± 0.003	"730" with 150-μl capillary

2-Phenyl-ethanol (PE)	99	Kebo	0.17 ± 0.005	"730" with 150-μl capillary
Ipsdienol (Id)	95	Borregaard	0.34 ± 0.02	"730" with 2.9-mm hole
			0.21 ± 0.02	"730" with 150-μl capillary
Host synergism test, Lardal, Norway, 1983				
2-Methyl-3-buten-2-ol (MB)	97	Aldrich	5.8 ± 0.3	"730" with 50-μl capillary
(4*S*)-*cis*-Verbenol (cV)	99	Borregaard/KTH	0.05 ± 0.001	"730" with 150-μl capillary
Comparison of natural and synthetic pheromone, Gribskov, Denmark, 1984				
2-Methyl-3-buten-2-ol (MB)	97	Aldrich	5.8 ± 0.3	"730" with 50-μl capillary
			0.5 ± 0.05	"730" closed
			0.2 ± 0.02	0.25-mm-diam capillary
(4*S*)-*cis*-Verbenol (cV)	99	Borregaard/KTH	0.05 ± 0.001	"730" with 150-μl capillary
			0.01 ± 0.001	"7570," 1.1 mg of cV
			0.002 ± 0.001	"7560," 0.5 mg of cV

[a] Chemical purity estimated by capillary GC. Optical purity for cV > 94% (−)-(4*S*), Id was racemic.

[b] The release rates from polyethylene vials were estimated in the laboratory by measuring the weight loss of the vials, placed in a wind tunnel at 0.7 m/sec and 20°C, during a month's period. The rate of release was calculated as the slope, with its 95% confidence interval, from the regression of weight on time.

[c] Dispensers were polyethylene vials (Kartell, Italy) of two types: "730," a 1-ml vial of soft polyethylene; and "hard," a 2-ml vial of hard polyethylene, with capillaries or drilled holes in their lids. For the two lowest rates of cV we used polyethylene tubes, 3 cm long, heat sealed in both ends, with 0.40 mm (7570) or 0.55 mm (7560) wall thickness.

with only MB + cV, these formed an equilateral triangle, a trap group, with 10 m to a side, randomized within the group after each replicate. A total of four groups were used in an old clear-cut, north of Skien, Lardal, southern Norway in May–June 1983.

Comparison of Natural and Synthetic Pheromone. Logs (diameter 12 cm, 30 cm long) infested with 50 males or 30-cm sections of pipe traps with dispensers for synthetic pheromone (Table 1) were enclosed by a sticky trap (hardware cloth No. 4 coated with Stickem Special, Byers and Wood, 1980). Logs and pipe-trap sections were both screened with brass net (No. 60) to exclude arriving beetles and to give a similar appearance. The logs were cut from three Norway spruce [*Picea abies* (L.) Karst.] trees that represented different stages of vigor and resin content (Table 4). The trees were all cut in the evening on May 17, 1984. Male beetles were introduced, after sex separation (Schlyter and Cederholm, 1981), the day after. The males (50/log) were introduced into predrilled holes on the evening before May 18, but had a free choice to start excavating the nuptial chamber or to leave, in which case they were replaced after a few hours. Both beetles from our laboratory stock (from Lardal, southern Norway) and wild, Danish beetles (caught in pipe traps baited with synthetic pheromone in the area the days before) were used (Table 4). In all cases, each set had one log from each of the three trees infested with 50 males. The traps in a set were placed in two lines with a minimum of 10 m between traps. The two trap sets were at least at 25 m away from each other and other pheromone sources such as trees under attack. Trap positions were randomized after each replicate. Trapping sites were in clear-cuts, cleared of logging residues, near groups of trees killed the previous year in Esrum forest district, Grib Skov, north of Hillerød, Denmark, 1984.

Aerations of Natural and Synthetic Pheromone Sources. Male-infested logs and sections of pipe traps (containing dispensers with synthetic compounds) were aerated after each day of trapping. Logs and pipe-trap sections were placed individually in glass jars (ID 15 cm, 30 cm high) through which 150 ml air/min (charcoal filtered, ID 2 cm × 20 cm) was passed with a pneumatic suction ejector (AGA). The volatiles in the effluent were trapped on Porapak-Q (mesh 80–100, 170 mg). As internal standards, trideuterated-MB and α-isophorone were evaporated from fused silica capillaries (ID 0.20 mm), one end sealed with beewax, attached to the side of logs or pipe-trap sections. After 3 hr of aeration, the Porapak-Q plugs were extracted with 2 ml of diethyl ether, 500 ng C_7Ac was added as a standard for the concentration, and the extracts stored at −20°C. The extracts were concentrated and subsequently analyzed by GC −MS; conditions as in Birgersson et al. (1984).

Sample Handling and Statistics

Sample Handling. Beetles were preserved in ethanol and identified to sex by pronotal bristle density (Schlyter and Cederholm, 1981). In the subtractive

assay, at least 260 beetles (or the total catch if less than 260) from each site and bait were identified to sex. Beetles from the comparative study were not identified to sex, and, for samples of >100 individuals, their numbers were estimated by measuring the volume of *I. typographus* (42 beetles/ml).

Statistics. Raw catch data (y) were subjected to a series of transformations y^{-1}, $y^{0.5}$, $\log(y + \text{constant})$, p^{-1} to make them suitable for ANOVA by achieving homogeneous variances (Cochran's C and Barlett box tests, $P > 5\%$) and approximately normal distributions. The transformation $\log(y + \text{constant})$ was chosen for the first two tests as it made the data homoscedastic and gave high F ratios in ANOVA. The high number of zero catches in the two later tests caused significant heteroscedacity to remain after transformations, which made it more appropriate to perform a nonparametric test [Wilcoxon matched-pairs with significance levels adjusted for number of comparisons (Kirk, 1969)] of the untransformed data in lieu of ANOVA.

RESULTS

Subtractive Assay

Subtraction of either MB or (−)-(4*S*)-cV from the natural phase-3 blend dramatically reduced the catch of *I. typographus* (Figure 1), indicating that both MB and cV are equally essential for attraction. The relative response in the two sites was very similar, in spite of a 10-fold difference in total catch (Figure 1). Thus, (+)-(4*S*)-tV or Mt could not substitute for cV (or MB). The inclusion of tM, a component of the natural phase-3 blend, in the later replicates also did not increase the attractivity. The addition of (*R/S*)-Id to the phase-3 blend seemed to increase the catch of *I. typographus* slightly, although the difference was not statistically significant. The sex ratio was little affected by subtraction or addition of compounds. Exceptions were the removal of PE or MB, which resulted in a somewhat higher percentage of males in the catch (Table 2).

I. duplicatus Sahlb. was attracted only to the bait with Id added to the phase-3 blend. Several other scolytid species were caught in low numbers in the pipe traps with funnel, but none showed a clear pattern of attraction like the two *Ips* species. *Pityogenes chalcographus* (L.) was caught on all baits with an increase on phase 3 plus Id but the numbers were low (Table 2).

Comparison between Lures

The two baits containing Id (Ipslure, Typolure II) generally caught more beetles, although the differences were small and not statistically significant at the site with the highest number of replicates (Figure 2). The bait with the highest catches, Typolure II, had both a higher dose of Id and of cV, while Ipslure was almost identical to phase 3 at the best site. This means that it is difficult to assign a clear synergistic function of Id based on these data. When

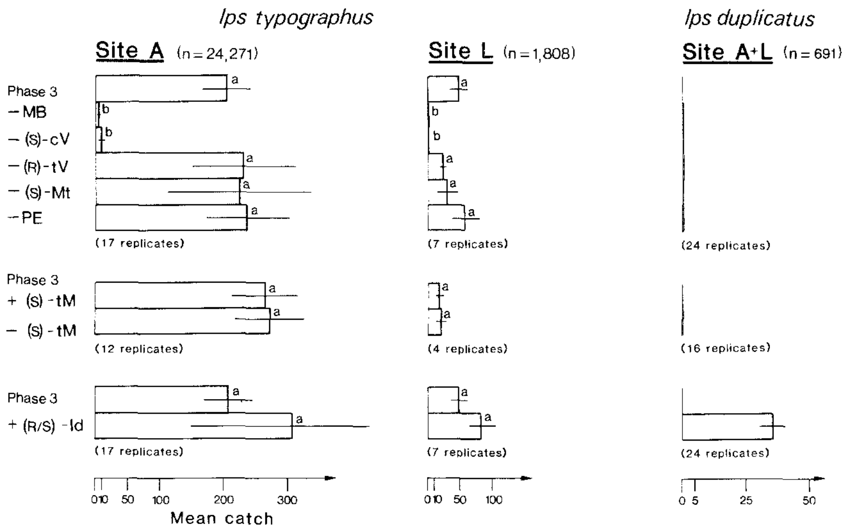

FIG. 1. Mean catches (±SE) per replicate in pipe traps with the subtractive assay of *Ips typographus* pheromone components. Subtraction or addition of a component of the phase-3 blend is indicated by + and −, respectively. Phase 3 is a bait representing the pheromone content in hindguts of unmated *I. typographus* males, which included the components MB, cV, tV, Mt, and PE during the first five replicates. For the remaining replicates, an additional trap was added which had a sixth compound, tM, which is also found in males from phase 3. One bait had the phase-3 blend plus Id, which is found in mated males. Total catch is given by (n). Release rates and names of chemicals and sex ratios of catch are shown in Table 1. Chirality of compounds with optical activity is given by *R* and *S*. Bars with the same letter are not significantly different within a site ($P > 5\%$) by ANOVA of log (catch + $\frac{1}{4}$) followed by Duncan's multiple-range test.

comparing phase 3 with Typolure I, it is evident that the addition of tV, Mt, and PE to our standard MB + cV bait (=Typolure I) did not increase the attraction, as the catches of the two baits were virtually identical in all sites (Figure 2).

Host Synergism

Volatiles from a spruce log did not enhance the attractivity of a lower, "medium" dose of MB and cV, as shown by the nearly identical mean catches (Table 3). The bait consisting of a log alone in the pipe traps without funnel did not catch a single beetle during the entire experiment, while 705 beetles were caught by the two pheromone baits. The sex ratio was not altered significantly by the addition of the log to the pheromone source. Few other scolytids were collected, probably due to the low pheromone release and the use of pipe traps without an exterior funnel.

TABLE 2. SUBTRACTIVE ASSAY OF AGGREGATION PHEROMONE CANDIDATES IN *Ips typographus*: BAITS, SEX RATIOS, AND CATCHES OF OTHER SPECIES IN PIPE TRAPS, VÄRMLAND, SWEDEN, MAY 17, TO JUNE 8

	Release rates (mg/day)[a]							Sex ratio of *Ips typographus*		Catches of other species[b] (means, site A)[b]				
										Scolytidae				*Cleridae*
Baits (Designation)	MB	cV	Id	tV	Mt	tM	PE	Male (%)	95% C.I.	Pc	Hc	Da	Tl	Thf
Phase 3 (natural composition)	57	1	—	0.3	0.1	—	0.2	20.4	17–24	0.8c	1.4	0.2	0.2	1.4
−MB	—	1	—	0.3	0.1	—	0.2	33.7	25–43	1.3c	3.3	0.5	0.1	1.0
−cV	57	—	—	0.3	0.1	—	0.2	19.4	14–26	0.8c	1.9	0.1	0.1	1.2
−tV	57	1	—	—	0.1	—	0.2	19.3	16–23	2.2c	1.4	0.3	0.3	2.8
−Mt	57	1	—	0.3	—	—	0.2	17.6	14–21	5.6de	1.8	0.4	0.2	1.6
−PE	57	1	—	0.3	0.1	—	—	31.2	27–35	1.5c	1.4	0.5	0.3	1.6
+Id	57	1	0.2	0.3	0.1	—	0.2	15.6	13–19	8.2e	1.5	0.2	0.1	2.5
+tM	57	1	—	0.3	0.1	0.04	0.2	(9.7)[c]	7–13	(3.2)[c]	2.9	0.2	<0.1	1.0

[a] For confidence intervals of release rates and abbreviations of compounds see Table 1.

[b] Pc = *Pityogenes chalcographus* (L.), Hc = *Hylastes* cf. *cunicularius* Er., Da = *Dryocoetes autographus* (Ratz.), Tl = *Trypodendron* (*Xyloterus*) *lineatum* Oliv., Thf = *Thanasimus formicarius* L. Values followed by the same letter are not significantly different by ANOVA on log (catch + 0.25) followed by Duncan's multiple-range test.

[c] tM was included in the test only during the last 12 replicates, which means that catches and sex ratios are not comparable with other baits with 17 replicates (see Figure 1).

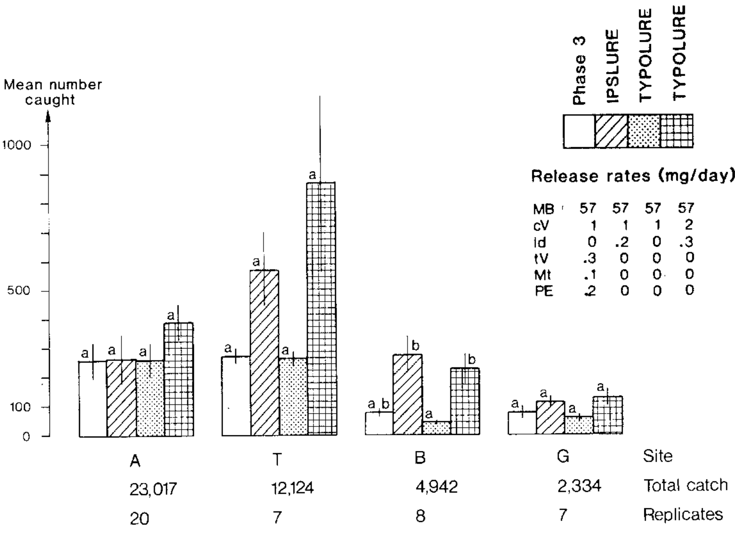

FIG. 2. Mean catches (±SE) per replicate of *I. typographus* in pipe traps with the comparative test of baits representing commercial lures and phase-3 blend (representing the natural hindgut composition). For more extensive data on release rates and names of chemicals see Table 1. Bars with the same letter are not significantly different within a site ($P > 5\%$) by ANOVA of log (catch + $\frac{1}{4}$) followed by Duncan's multiple-range test.

TABLE 3. HOST VOLATILES AND PHEROMONE SYNERGISM TEST OF *Ips typographus* WITH PIPE TRAPS, LARDAL, SOUTHERN NORWAY, JUNE 7–11, 1983, 28 REPLICATES

Baits, synthetics (mg/day)			Catch (number of beetles)[b]		Sex ratio	
MB	cV	Log[a]	Mean	Max–min	Males (%)	95% C.I.[c]
5.8	.05	No	14.2c	49–0	47.4	42–52
5.8	.05	Yes	13.5c	35–4	44.6	40–49
0	0	Yes	0.0d	—	—	—
Total catch			705			

[a]Tree cut June 1, logs 6–8 cm diam. × 28 cm (with 2- to 3-cm long axe cuts, 10 per log).
[b]Values followed by the same letter are not significantly different by Wilcoxon matched-pairs signed ranks test ($P > 5\%$) for totals, males, or females.
[c]95% Binomial confidence intervals (Byers and Wood, 1980).

Natural and Synthetic Pheromone: Attraction and Release Rates

The strongest bait in this test, the "medium" MB + cV synthetic pheromone source consistently caught more beetles than did the male-infested logs (Table 4). There was no consistent difference in the catch between logs infested with laboratory-reared males originating from Norway and logs with wild males of local origin, and the data are pooled in Table 4. The sex ratio was about 40% males on most traps but, of the synthetic baits, only the catches of the two stronger baits were significantly different from an equal sex ratio. If the catches from all three traps with male-infested logs are pooled, the proportion of males (35%) is also significantly different from an equal sex ratio (95% binomial confidence interval, Byers & Wood 1980).

The release of MB could be accurately estimated by the deuterated MB internal standard and showed that the release of MB from the logs was high and close to the "medium" synthetic bait, which was the strongest in this test. The release rates estimated by weight loss and by entrainment were in good agreement for the MB in both the "medium" and "low" baits (Table 4). The standard for the monoterpene alcohols, α-isopherone, was not released in sufficient quantities to obtain an estimate of their absolute release rates. However, the ratios between the monoterpene alcohols could be measured with precision and accuracy and were used to show that verbenone, together with readily detectable amounts of cV and tV, was released from the logs at some times in approximately equal ratios to the verbenols (Table 4).

DISCUSSION

Tests of Synthetic Compounds

MB and cV could be identified as essential for the attraction of *I. typographus*, while subtraction from the phase-3 blend of tV, Mt, or PE, or addition of tM had no apparent effect on the catch. Thus, the released amounts of (+)-tV, (−)-Mt, PE, and (−)-tM could not substitute for or increase the attraction to MB and cV. We could not substantiate the report by Dickens (1981) that (+)-(4*S*)-tV could substitute for cV and was as attractive as (−)-(4*S*)-cV when tested in sleeve olfactometers. The addition of Id increased the catch somewhat, but not significantly, as was earlier indicated by Dickens (1981) and Bakke et al. (1983). Thus Id plays, at most, a minor role in the aggregation pheromone, as tested here. However, a small, but significant, increase in catch when small amounts of Id were added to MB + cV was demonstrated by Schlyter et al. (1987).

Only combinations including ipsdienol (Id) were attractive to *I. duplicatus*, in agreement with the results of Bakke (1975). The comparative test of baits

TABLE 4. RELEASE RATES OF AND ATTRACTION TO NATURAL AND SYNTHETIC PHEROMONE SOURCES OF *Ips typographus* GRIB SKOV, DENMARK, MAY 19–JUNE 5 1984, STICKY TRAPS, 12 REPLICATES

	Release rate by weight loss (mg/day)		Release rates by entrainment per day				Catch (number of beetles)		
Baits (designation)	MB	cV	MB (mg)[a]	cV (μg)[b]	tV (μg)	Vn (μg)	Mean	Max–min	Sex ratio (% males)
Medium	5.8	0.05	2.8	0.8	0	0	22.7	55–14	40[e]
Low_1	0.5	0.01	0.2	0.6	0	0	5.5	22–0	38[e]
Low_2	0.1	0.002	0.1	0.5	0	0	2.1	5–0	46
Log + 50♂♂									
Tree MI[c]	—	—	1.0	0.3	0.3	0.3	3.6[d]	12–0	44
Tree MII[c]	—	—	4.6	0.1	0.4	0.5	1.7[d]	6–0	(29)
Tree N[c,f]	—	—	1.5	0.4	0.8	0.7	1.6[d]	6–0	(20)
Control (blank)	—	—	0	0	0	0	1.7	5–0	(24)

[a] MB release estimated by GC-MS via the m/z 71:74 relative to the release of d_3-MB in the aeration vial. Values given for release by entrainment are means of three or more aerations of the same bait. For release by weight loss and abbreviations of compounds see Table 1.

[b] cV, tV, Vn calculated relative to the 500 μg C_7Ac added after extraction of the adsorbent.

[c] Two sets of traps were used, with five and seven replicates each, and results pooled as no difference between the sets could be detected. In one of the two sets the laboratory-beetle-infested logs were replaced with local, wild beetles caught in pheromone traps after the second replicate. No differences in relative or absolute catches could be noted between wild and laboratory beetles.

[d] Significantly different from "Medium" at $P < 5\%$ by Wilcoxon matched-pairs signed ranks test, significance level (α) corrected for number of planned comparisons (Kirk, 1969).

[e] Significantly different from 50% males (95% binomial C.I.), values within ( ) indicate less than 20 beetles caught.

[f] Tree N of low apparent vigor (crown narrow with low needle density, "transparent") with a mean annual increment for the last five years of 3.3 mm/year, while MI and MII were of high apparent vigor with dense, dark green crowns with 6.8 and 6.7 mm increments, respectively.

representing commercial lures showed that even the addition of tV, Mt, and PE as a group did not enhance the attractivity of the MB + cV combination. The addition of a small amount of Id, in combination with a doubled dose of cV (Typolure II), produced the bait with the highest catch, but significantly so in only one of four test sites. As the known attractant cV also was increased, the effects of Id might be confounded, so we cannot, in this case, confirm a benefit of Id in the attractant blend. However, the bait representing Ipslure also caused high catches, which, in one of four sites, was significantly different from one of two possible controls. One reason that none of the hindgut components other than MB and cV (and possibly Id) were found active in this study could be that the tV, Mt, tM, and PE tested had impurities of an inhibitory nature or they were not of the appropriate enantiomeric composition. However, the tV tested was the same enatiomer, (+)-(4*S*)-tV, as tested by Dickens (1981), and the (4*S*)-enantiomer is probably the one naturally produced (Klimetzek and Francke, 1980; Birgersson et al., unpublished). The natural enantiomeric composition of Mt and tM produced by the beetles is not known, and we used only one enantiomer. Thus, the possibility remains that a full, enantiomerically correct blend of phase 3 might be more active than MB + cV alone.

Host Synergism and Natural/Synthetic Comparison

Examples exist in several bark beetle genera of an aggregation pheromone synergized by volatiles from living host material: *Scolytus multistriatus* and elm logs (Peacock et al., 1984), and *Tomicus minor* and pine logs (Lanne et al., 1987), or by identified host compounds: *Dendroctonus brevicomis* and myrcene (Bedard et al., 1969), *D. ponderosae* or *D. frontalis* and α-pinene (Pitman, 1971; Renwick and Vité, 1969), and *Gnathotrichus* spp. and α-pinene (Borden et al., 1980). Tests with *I. typographus* in Germany have indicated that α-pinene or spruce resin added to traps with synthetic pheromone did not increase trap catches (Vaupel et al., 1981). However, spruce logs in one experiment increased catch considerably (Bombosch et al., 1982), although proper controls and replication appear to be lacking. Our test showed no increase in catch in pipe traps with spruce logs added. Comparisons between experiments using logs are made difficult by the fact that, as in our experiment, the release rates of the host compounds are usually not known, but only the size of logs used. In our test the logs were rather small and volatile release may have declined too soon to show possible weak pheromone synergism effects. However, the lack of any strong host attraction or synergism found in this study is not surprising, as other *Ips* species have not conclusively been shown to have a long-range host attraction behavior or synergism between pheromone and host odors (Wood, 1982).

The poor attractivity of the natural pheromone sources (50 males in log) is more surprising, especially as the chemical analysis of aerations showed that the release of MB from the logs was similar to "medium," the strongest syn-

thetic bait in this test. MB is believed to be produced de novo by the beetle in the appropriate biological phase. The high production of MB, similar to that of beetles mass-attacking trees (Birgersson, unpublished), indicates that the beetles in the test logs were in the appropriate physiological and behavioral condition for maximal pheromone production. The period of the test (18 days for one set of logs) is not unreasonably long, as walking beetles in a Y-tube bioassay were attracted to male-infested logs for 16 days (Schlyter and Löfqvist, 1986). However, a low and declining release of cV (produced from the host monoterpene α-pinene) may well explain the low attractivity of the logs. Byers (1981) showed for *I. paraconfusus* Lanier males that the pheromone components, ipsenol and ipsdienol (produced from host myrcene), began to decline after about six days and were undetectable (GLC) after two weeks. Furthermore, verbenone (Vn) decreases trap catches (Bakke, 1981), and although its release rate was low, it could also have contributed to the low catches. The release of Vn from infested logs is probably due to the activity of microorganisms in the host tissue as they convert pinene and verbenols to Vn (Brand et al., 1975; Leufvén et al., 1984), while male hindguts contain very little Vn (Birgersson et al., 1984).

If the pheromone system in *I. typographus* in fact consists of only two components (MB + cV), one might ask if such a simple system is specific enough to ensure species specificty of the pheromone signal. The monoterpene alcohol cV is found in several related and/or sympatric species (Vité et al., 1972; Wood, 1982). However, the enantiomeric ratios and behavioral roles of cV are not well characterized in these species. As yet, the very large amount of the isoprene alcohol MB in the two-component system appears unique to *I. typographus* among *Ips* species and probably plays the major role to ensure the species specificity of the pheromone.

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HUMAN AXILLARY EXTRACTS: Analysis of Compounds from Samples which Influence Menstrual Timing

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Abstract—Previous studies have shown that menstrual cycle length and fertility are affected by a regular pattern of either: (1) intimate contact with men; or (2) contact with extracts from male axillary secretions; and (3) the axillary extracts of women. Experiments utilizing axillary extracts employed a controlled, double-blind research design using either extract or placebo. The study reported here employed quantitative gas chromatography–mass spectrometry (GC-MS) techniques to determine the concentration of several steroids in the male and female axillary secretion extracts. The steroids examined were: androsterone sulfate, dehydroepiandrosterone sulfate, and the volatile steroid androstenol. The results suggest the following: (1) The concentration of androstenol produced by secretion donors varied during the collection period. Males appear to produce more androstenol at certain times; women's secretions show a menstrual variation in androstenol; the highest concentrations of this compound appear to be produced in the midfollicular phase, prior to ovulation. (2) The concentration of dehydroepiandrosterone sulfate is significantly greater in men than in women. (3) In addition to the steroids, a series of aliphatic acids which range from C_2 to C_{18} in chain length were found. The more volatile members of this series may contribute to the odor of the secretions.

Key Words—Primer pheromones, human steroids, androstenol, axillary secretions, menstrual synchrony, menstrual cycle regularization.

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INTRODUCTION

The possibility that human odors can affect reproductive biology has been discussed at some length in both the scientific and the popular press. Much of this discussion has centered upon the possible existence of releaser pheromones (i.e., substances which release a behavioral response in a conspecific). Although specific data to support the existence of a releaser pheromone in humans has not been forthcoming, data from several studies suggest the possibility of another more subtle pheromonal effect, i.e., a primer pheromone. Such a substance would cause a slow changing or priming of the endocrine system (see Albone, 1984; Freeman, 1978, for a discussion of primer and releaser pheromones). Several studies reveal that interpersonal relations between women may result in menstrual synchrony (McClintock, 1971; Graham and McGrew, 1980; Quadagno et al., 1981), while sexual interactions between men and women can influence cycle length (Cutler et al., 1979, 1980, 1985; Russell et al., 1980). These data therefore imply that humans may exert a primer pheromone influence on human reproductive endocrinology.

Studies recently completed in our laboratories employed axillary extracts obtained from men and women to influence the menstrual cycle length of nulliparous women (Cutler et al., 1986; Preti et al., 1986). Female subjects who reported having a history of aberrant length cycles (<26 days and >33 days) received extract from male donors while those who reported having normal length cycles received extract from female donors. Women who received the male extracts for 13.5 ± 1 weeks experienced more regular (i.e., less aberrant) cycles than the controls who received placebo applications. The women who received female extracts showed a significant shift towards a synchronization of their menstrual cycle onset with menstrual onset of the donor females. These two studies demonstrated that axillary constituents can shift the length and onset of the menstrual cycle when subjects are chronically exposed to them and that this effect can occur in the absence of direct social contact. In addition, the studies confirmed olfactory–reproductive system relationships in humans that had been previously demonstrated among several nonhuman mammals.

The stimuli used in our studies were derived from the axillary region, a body area with unique odor-producing characteristics. In the axillae, apocrine, sebaceous, and eccrine glands provide an excellent substrate for a large permanent population of microorganisms (Labows et al., 1982). The apocrine secretion as collected at the skin surface is odorless. However, incubation with the resident bacteria results in the production of a characteristic odor which appears to be unique to the organism used (Leyden et al., 1981; Labows et al., 1982). The micrococci bacteria present in the axillae give an acidic odor to the secretion that has been characterized by headspace analysis as isovaleric acid (Labows, 1979; Labows et al., 1982). The diphtheroid bacteria give similar

chromatographic headspace profiles with isovaleric acid being present; however, the odor is more distinct and pungent and suggests the presence of other unidentified volatiles (Labows et al., 1982).

Previous analyses of both the apocrine secretion and the total axillary sweat have shown the presence of a variety of both volatile and nonvolatile steroids (Brooksbank, 1970; Brooksbank et al., 1974; Claus and Alsing, 1976; Labows et al., 1979; Bird and Gower, 1980). In addition, the axillary bacteria are also thought to produce volatile aliphatic acids (Labows, 1979; Labows et al., 1982).

Analysis of freshly collected apocrine secretions in our laboratory has shown the presence of two androgen steroid sulfates: 17-oxo-5α-androstan-3α-yl (androsterone) sulfate and 17-oxo-5α-androsten-3β-yl (dehydroepiandrosterone) sulfate (Labows et al., 1979). Both dehydroepiandrosterone sulfate and androsterone sulfate are present in high concentrations and may serve as precursors for the volatile, odiferous androst-16-enes. The androst-16-enes, 5α-androst-16-en-one (androstenone) and 5α-androst-16-en-3α-ol, (androstenol) act as releaser pheromones in the mating behavior of the pig (Gower et al., 1981). Several studies have examined, with limited success, the potential behavioral effect of androstenol and androstenone in human subjects (Cowley et al., 1977; Kirk-Smith and Booth, 1978; Benton, 1982; Black and Biron, 1982).

GC-MS techniques were employed to analyze the female and male axillary extracts which, as described above, had affected the length and onset timing of recipient's menstrual cycles. Several steroids were examined in a quantitative fashion. These were dehydroepiandrosterone sulfate (DHEAS), androsterone sulfate (AS), and 5α-androst-16-en-3α-ol (androstenol). In addition, a series of aliphatic acids were identified in several of the male extracts.

METHODS AND MATERIALS

Collection of Axillary Secretions. The axillary secretions used to prepare the axillary extracts employed in this study were collected from both male and female volunteer donors. Four females (ages 25, 26, 29, and 35) and three males (ages 41, 35, and 32) provided the samples and were recruited from among coworkers and members of the university community during the autumn of 1982. Each was engaged in a stable, ongoing heterosexual relationship and had large numbers of lipophilic diptheroids in the axillary regions. As noted above, lipophilic diphtheroids are associated with a more pungent axillary odor (Leyden et al., 1981). This pungent odor is similar to that of C_{19}-androst-Δ^{16}-enes such as androstenol and androstenone. Our secretion donors were selected for their ability to produce a complete spectrum of axillary odorants (Labows et al., 1982).

Each female donor had a history of normal length (29.5 $\pm$ 3 day) men-

strual cycles. None of the donors shaved their axillary regions. During the 14–16 weeks in which donors collected secretions, all were required to (1) not use deodorant, deodorant soap, or perfumes in the axillary region; (2) wash once each morning with Ivory soap; and (3) not shave their underarms. Secretions were collected on 4 × 4-in. cotton pads which had been previously extracted, autoclaved, dried, and wrapped in solvent-extracted foil (Preti and Huggins, 1975). Each donor wore one pad in each axilla three times a week for a 6- to 9-hr span which was most convenient for him or her. Each day after removal, the pads from any one donor were placed in an individual acid-cleaned-glass-jar and frozen at −60°C until extraction.

Preparation of Axillary Extracts—Female Samples. Each female donor collected secretions for three complete menstrual cycles. However, the axillary pads included in the preparation of the stimulus came from only five of the donated cycles which met the following more rigorous criteria: (1) each was 29 ± 2 days in length; (2) the basal body temperature charts were clearly biphasic and presumably ovulatory with basal body thermal rises which lasted 12 or more days, a characteristic of a fertile cycle (Cutler et al., 1979, 1985; Treloar et al., 1967; Vollman, 1977); and (3) menstruation occurred within 7 days of a full moon (Cutler, 1980; Friedman, 1981). We have previously shown that women who cycle as often as the moon cycles tend to be the most fertile: These women have an increased propensity for menstruation at the time of the full moon. This timing of donor cycle selection did yield significant effects not directly related to this report (Preti et al., 1986; Cutler, et al., unpublished data).

Because of the similarity in cycle lengths employed, it was assumed that each of the three-day sequences from each donor (i.e., cycle days 1, 2, and 3) would be endocrinologically similar, and consequently the pads from these days were combined. To prepare the sample, pads were grouped in three-day segments: e.g., all pads from cycle days 1–3 were combined and called "combined donor day 2" (the first day of menstruation = day 1 of a menstrual cycle); all pads in days 4–6 were combined and called "combined donor day 5"; all pads in days 7–9 were combined to form "combined donor day 8"; and so on. In this fashion, 10 separate extracts were prepared, each containing odors from different portions of the menstrual cycle to form the "donor cycle" of combined donor days 2, 5, 8, 11, 14, 17, 20, 23, 26, and 29.

All pads from each three-day group were placed in a glass column and allowed to soak in doubly distilled ethanol for 1 hr at room temperature. Fifteen milliliters of ethanol were used for each pad in the column. After 1 hr, the ethanol-extracted materials were allowed to run out of the bottom of the column through a Teflon stopcock as the pads were squeezed with a Teflon disk. Approximately $\frac{2}{3}$ of all ethanol put on the pads was recovered. The ethanol extracted axillary secretions were subsequently stored at −60°C until used for behavior testing or chemical analysis.

Preparation of Axillary Extracts—Male Samples. To prepare the male extracts, batches of glass jars containing the pads were grouped consecutively according to the date received. Each batch of pooled extract was prepared from six consecutively collected jars and contained pads from all three individuals. A total of 14 separate extracts were prepared in this manner. All pads from each sample were extracted in the same manner as described above for females.

Analysis of Extracts and Standard Samples by Gas Chromatography and Mass Spectrometry. A Finnigan 4510 GC-MS data system equipped with a split-splitless injector, a fused silica capillary column, and with capabilities for operation in both electron impact and chemical ionization modes was used for analysis. The column employed for chromatography was a methylsilicone permanently bonded phase, CP SIL-8, 25 m × 0.32 mm with a 1.2-μm coating (Chrompack, Inc., Bridgewater, New Jersey). Analyses of both male and female donor extracts as well as extracts containing the standard mixture extracted from pads were performed by concentrating to a low volume, generally 20–80 μl and diluting with an equal volume of the injection standard, androstane, to give 100 ng of androstane for each injection. The chromatographic program used for the analysis was as follows: 150°C for 8 min and then 3°/min to 300°C with a hold for 20 min. The mass spectrometer is interfaced with a Nova 3 computer which utilizes the Incos software for data acquisition, analysis, and quantitation. The mass range employed during these analyses was m/z 50–450. This mass range was scanned once each second and a typical run included 3600 scans.

The data system also includes the NBS library of 31,000 compounds. Identifications were based on comparison of unknown spectra with both the NBS library and interpretation of the resulting comparison with mass spectra generated from commercially available standard compounds. In addition, the relative chromatographic retention times of unknowns and known standards were compared. A mixture of both fatty acid ethylesters as well as hydrocarbons was used to determine relative retention times. In the case of the compounds being quantitated, commercially available, authentic compounds were used for comparison of retention times, mass spectra, and generation of standard curves.

Determination of Extraction Efficiency of Target Compounds from Pads. Because the extracts of the axillary pads were to be placed on humans (with associated requirements for human subject experimentation), no internal standards were added to the pads. Thus, the efficiency of extraction could not be judged directly. Subsequently, post hoc tests were performed. A standard solution was made which contained the following steroidal compounds: DHEAS, 103 ng/μl; AS, 18 ng/μl; and androstenol, 0.3 ng/μl. Pads were prepared in the same way as those pads that had been worn by the donors.

Pads were put together in several different sized batches reflecting the original experimental conditions in order to evaluate whether the number of pads affected the extraction efficiency. For example, the number of pads in each

female extract, as noted above, varied depending on the yield received: e.g., combined donor day 2 contained 10 pads; combined donor day 5, 16 pads; combined donor day 8, 16 pads. Six repetitive measures were performed on the smallest batch (6 pads) and the largest batch (16 pads). For the men, a greater consistency was possible because each batch contained 12 pads. In order to test for extraction efficiency from the male samples, ten separate batches of 12 pads each simulated the actual extraction protocol.

Each pad had 100 μl of the standard solution added to it. Each batch of pads was then placed in the column used previously to extract the donor derived pads. Next, the pads were put through the same soaking, extracting, and solvent concentration protocol as had been performed on the actual donor pads. The resulting mixture was analyzed on our Finnigan 4510 system as described above.

Quantitation of Compounds Extracted from Pads of Human Donors and Standard Pads. The Incos software (supplied by the Finnigan Corporation) was used to quantitate the desired compounds by using the Targeted Compound Analysis (TCA) package. This software package locates and quantitates individual compounds by employing retention times and the intensities of key ions designated by the user. For each of the steroids, we used quantitation based principally on the molecular ion and the most intense ion fragment in the mass spectrum. The standard curves for androstenol, DHEAS, and AS consisted of the absolute computer-generated intensities of key ions for each steroid at different concentrations vs. the ratio of that intensity divided by the intensity of the molecular ion of androstane generated by 100 ng of that compound. The concentrations of androstenol, DHEAS, and AS used for each standard curve were 1 ng, 5 ng, and 10 ng. A representative of standard curve for DHEAS may be seen in Figure 1.

RESULTS

Extraction efficiency from standard mixtures was higher for androstenol (67% $\pm$ 22.1%; mean $\pm$ standard deviation) than for DHEAS (22% $\pm$ 11%) or AS (21.4% $\pm$ 10.5%). Thus for the two sulfates, approximately five times the concentration extracted was initially present on the donor pads. For androstenol, about 1.5 times the concentration extracted was initially present in the donor pads. No variation in efficiency emerged as a function of the number of pads in a batch.

The data shown in Figures 2 and 3 display the actual concentrations of the three steroids isolated from the sequentially collected donor pads of men and women in Autumn, 1982. In Tables 1, 2 and 3, the respective concentrations for androstenol, DHEAS, and AS are listed. In these tables we have also provided the 95% confidence level (1.96 $\times$ standard deviation) around each data point. This confidence level was calculated from the data set used to determine

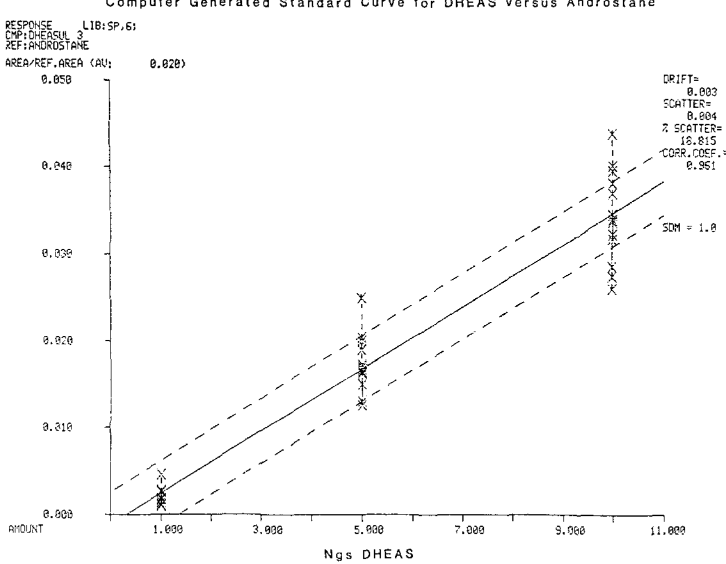
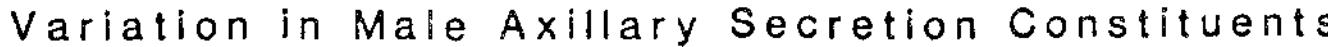


FIG. 1. The standard curve generated for dehydroepiandrosterone sulfate (DHEAS). The standard calibration curve is generated by the Target Compound Analysis package of the Incos software supplied by the Finnigan/MAT Corporation, San Jose, California. The curve was generated by injecting three different concentrations of DHEAS sulfate in conjunction with a fixed amount of injection standard, androstane. The three separate concentrations employed for DHEAS were 1, 5, and 10 ng of DHEAS with 100 ng of androstane. Each of the Xs at the three separate concentrations in the graph represent a separate injection. Fifteen injections were done at each of the three concentrations. The percent variation shown in the graph is the standard deviation assigned to the injection technique.

efficiency by the following procedure: (1) multiplying the standard deviation obtained from the standard extraction by 1.96; and (2) adding (as well as subtracting) this derived confidence interval to the value obtained in the actual samples. Because internal standardization and multiple measurements at each time interval were not obtained, this was the best 95% confidence limit available and served to estimate statistical variation around each data point. Data points whose confidence intervals do not overlap may be interpreted as being generated by biological or physiological variation rather than by variation due to extraction and analysis methods.

When one examines the data points and compares them to their 95% confidence intervals, one may see that several of the combined female donor days and male batches of pads appear to be significantly more concentrated in androstenol than the rest. Figure 4 shows the menstrual variation in androstenol concentration for the women. The data suggest that women produce signifi-



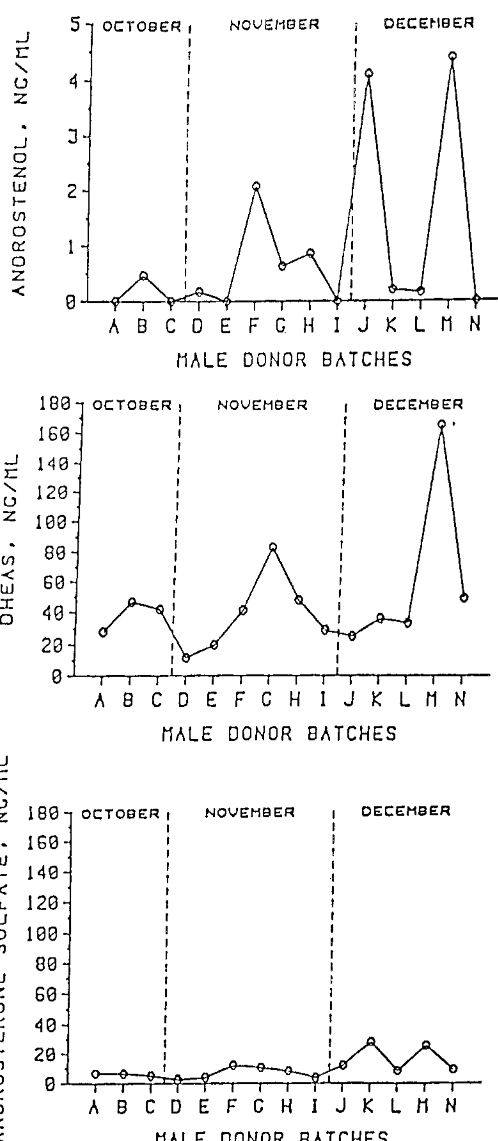
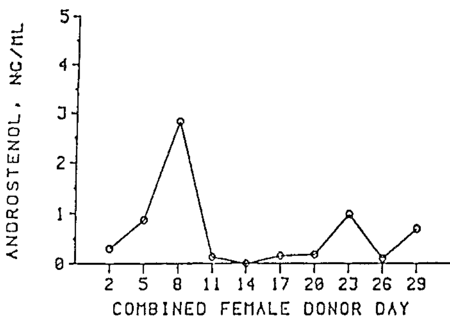
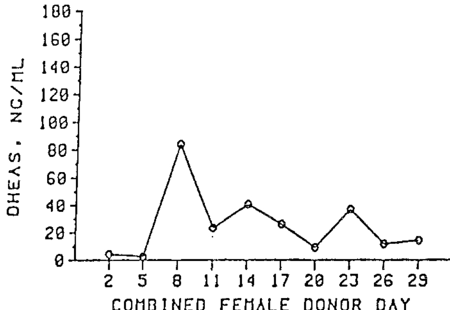


FIG. 2. The concentration of the three steroids measured from the male donor axillary secretions. The concentrations listed are in nanograms per milliliter of axillary secretion extract. These show the actual concentrations of material which were applied to the subjects as part of our previous studies. The compounds listed from top to bottom are androstenol, DHEAS, and AS. The graph also shows the month in which the pads for each extract were collected.

Variation in Female Axillary Secretion Constituents





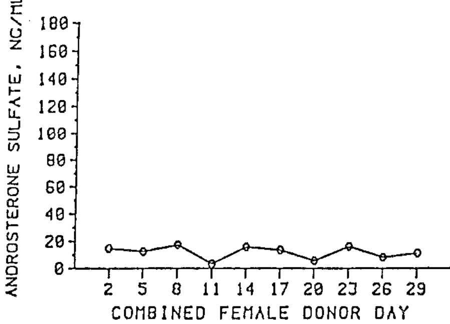


FIG. 3. The concentrations of the three steroids measured from the female donor axillary secretion extracts. The concentrations are nanograms per milliliter of secretion extract. The compounds listed from top to bottom are androstenol, DHEAS, and AS. The concentrations are the actual concentrations isolated from the pads.

TABLE 1. ANDROSTENOL CONCENTRATION (ng/ml) IN DONOR PAD EXTRACTS (±95% CONFIDENCE LIMIT)

Male donor batches			Combined female donor day	
October	A	NP[a]	2	0.302 ± 0.210
	B	0.4607 ± .320	5	0.877 ± 0.609
	C	NP	8	2.827 ± 1.963
November	D	0.1639 ± 0.058	11	0.1347 ± 0.0935
	E	NP	14	NP
	F	2.065 ± 1.43	17	0.1617 ± 0.112
	G	0.629 ± 0.437	20	0.1878 ± 0.130
	H	0.854 ± 0.593	23	0.108 ± 0.075
	I	NP	26	0.108 ± 0.075
	J	4.0805 ± 2.83	29	0.701 ± 0.486
December	K	0.1960 ± 0.136		
	L	0.1550 ± 0.108		
	M	4.369 ± 3.03		
	N	NP		

[a]NP < 0.1 ng/ml.

TABLE 2. DHEA SULFATE CONCENTRATION (ng/ml) IN DONOR PADS (± 95% CONFIDENCE LIMIT)

Male donor batches			Combined female donor day	
October	A	27.94 ± 29.311	2	4.28 ± 4.490
	B	46.50 ± 48.783	5	2.77 ± 2.906
	C	41.87 ± 43.930	8	83.89 ± 88.001
November	D	11.64 ± 12.211	11	23.43 ± 24.578
	E	19.83 ± 20.803	14	40.64 ± 42.411
	F	41.29 ± 43.319	17	26.14 ± 27.421
	G	83.20 ± 87.291	20	9.03 ± 9.472
	H	47.87 ± 50.216	23	36.96 ± 38.774
	I	29.09 ± 30.519	26	11.55 ± 12.116
	J	25.45 ± 26.701	29	14.24 ± 14.938
December	K	36.35 ± 38.135		
	L	33.57 ± 35.219		
	M	165.66 ± 173.832		
	N	49.29 ± 51.713		

TABLE 3. ANDROSTERONE SULFATE CONCENTRATION (ng/ml) IN DONOR PADS (± 95% CONFIDENCE LIMIT)

Male donor batches			Combined female donor days	
October	A	6.521 ± 6.840	2	14.496 ± 15.206
	B	6.386 ± 6.699	5	12.358 ± 12.963
	C	4.973 ± 5.217	8	17.200 ± 18.043
November	D	2.528 ± 2.652	11	3.283 ± 3.444
	E	3.974 ± 4.169	14	15.636 ± 16.402
	F	12.013 ± 12.602	17	13.372 ± 14.027
	G	10.483 ± 10.997	20	5.624 ± 5.899
	H	8.098 ± 8.495	23	16.111 ± 16.900
	I	3.729 ± 3.912	26	8.208 ± 8.610
December	J	12.044 ± 12.634	29	11.267 ± 11.819
	K	27.781 ± 29.142		
	L	8.203 ± 8.605		
	M	25.285 ± 26.524		
	M	9.191 ± 9.641		

cantly more androstenol during the preovulatory period, i.e., donor day 8 ± 1 of the 29-day, normal menstrual cycle. The five menstrual cycles used to generate the pads for this study all showed a definitive normal ovulatory type basal body temperature chart (Vollman, 1978) in which ovulation appeared to occur during days 13–16. The preovulatory blood estrogen rise in a typical 29-day cycle with midcycle ovulation generally commences on or about day 8. Concentrations of male axillary androstenol show several maxima in concentrations (e.g., batches F, J, and M).

The data from Tables 2 and 3 show that the overlap for each of the data points for the steroid sulfates was too large to suggest a significant variation due to physiological causes. This occurs despite the suggestion of such a variation in women (Figure 3). Although the concentration appears to maximize during combined donor day 8, corresponding to the androstenol peak, the variation due to experimental procedure for isolation of this compound was too great. We must await further experimental data before concluding the presence of a menstrual variation for DHEAS.

Inspection of the data in Table 2 and Figures 2 and 3 did suggest that the differences in levels of DHEAS between males and females might be significant. To test this, the Mann-Whitney U test was used. The results revealed that men do secrete more DHEAS ($U = 33, p = 0.025$). Figure 5 shows the median value and interquartile range of DHEAS concentrations for men and women.

Several of the male samples were also surveyed for the presence of ali-

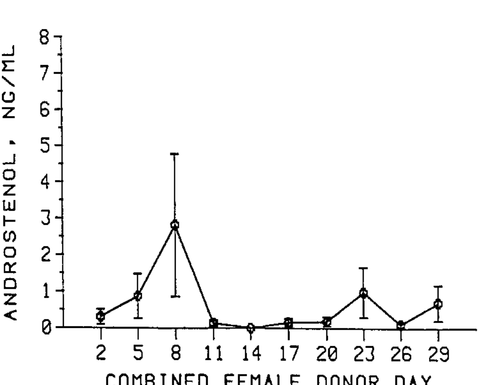


FIG. 4. This figure represents the variation in androstenol concentration across the combined female donor secretions. For each data point we have plotted 1.96 × standard deviation of the isolation technique. Androstenol appears to be produced in greater amounts in the pads comprising combined donor day 8. This is the midfollicular phase for the subjects.

phatic acids. Acids which range from C_2 to C_{18} chain length were found. Both the chromatographic retention times and mass spectral data of lower-molecular-weight acids from two of the male batches suggest the presence of acetic, propionic, isobutyric, butyric, isovaleric, valeric, and isohexanoic acids. Although C_8 and C_{10} acids are present, we cannot say which members of this series are represented.

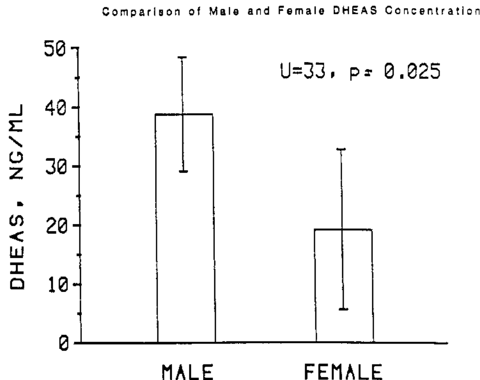


FIG. 5. A comparison of the concentration of DHEAS in male and female subjects. The data show the median value for this compound in each of the subject groups and the interquartile range. The Mann-Whitney U test performed on these data reveals significant differences.

DISCUSSION

The results of these experiments suggest that men secrete more DHEAS in their axillary region than do women. They also suggest that there are different rates of production of androstenol in the axillary region of women at different times of the menstrual cycle and perhaps at different times for men. In women, there has already been revealed a cycle variation in menses onset with respect to the lunar cycle. We have previously reported that women who menstruate every 29.5 $\pm$ 1 days (the same cycle length as the moon's repeating cycle), tend to menstruate in the full moon part of the lunar cycle (Cutler, 1980; Friedman, 1981). The presence of a seasonal variation in plasma androgens of men and their related implications for a pheromonal influence has been recently reviewed (Cutler and Garcia, 1984). It is therefore reasonable, although not yet studied, that men also will show a seasonal variation in reproductive endocrine rhythm that may be reflected in the constituents of the axillary secretions.

While these results do not definitively show which axillary constituents influence women's cycles (e.g., menstrual synchrony and/or menstrual cycle regularization), they do suggest that there are different patterns of production for at least two of the steroids. Moreover, the extracts do contain a number of other odoriferous compounds which should be quantitatively measured to see if there is a concerted increase in the entire odor profile at the key times in the female menstrual cycle (i.e., combined donor day 8) and during the fall season in males.

The use of ethanol as the solvent for the extraction of the original pads was not ideal, given our expectation of large amounts of lipophilic substances in the axillary secretions. However, the choice of the solvent was dictated by safety requirements for human testing and, thus, all the analyses of axillary products had to employ ethanol. Despite its low lipophilicity, ethanol still managed to remove a great number of lipid-like substances, as indicated by the GC-MS analysis. In addition, the most important consideration was the fact that the extracts did alter the menstrual cycle (Cutler et al., 1986; Preti et al., 1986), and therefore, ethanol does remove sufficient active components to elicit a biological effect.

Several other investigators have examined the steroid substances of axillary secretions in a quantitative fashion. Bird and Gower (1980) made quantitative measurements of androstenone in male and female axillary secretions using radioimmunoassay techniques. An earlier study done by Brooksbank et al. (1974) estimated the amount of androstenol taken from the combined extract of seven male donors who wore pads under their arms for a number of days in succession. The amounts obtained from this study suggested that each male produced approximately 50–100 ng per day in his axillae. This number, when corrected for the extraction efficiencies which we found, agrees very well with our results. Our male donors show amounts in their extracts which range be-

tween 0.1 ng and 5 ng. In addition, although other studies (Labows, 1986) have suggested the presence of isovaleric acid in the axillary secretions, our data suggests that an entire series of aliphatic acids is present in male samples.

As axillary extracts alter female menstrual cycles, further studies of the components of this extract continue in our laboratory with the goal of determining which individual constituents and/or groups of constituents alter this important physiological function.

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MALE-PRODUCED AGGREGATION PHEROMONE IN PEA AND BEAN WEEVIL, *Sitona lineatus* (L.)[1]

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Abstract—The attraction of *S. lineatus* to live baits comprising *S. lineatus* feeding on *Vicia faba* (L.) was studied in a field experiment in the early spring. There was clear evidence that male *S. lineatus* produced an aggregation pheromone which attracted approximately equal numbers of both sexes from overwintering sites. No evidence was obtained for the production, in the spring, of any semiochemical by female weevils.

Key Words—*Sitona lineatus*, pea and bean weevil, pea leaf weevil, Coleoptera, Curculionidae, aggregation pheromone.

INTRODUCTION

The pea and bean weevil, *Sitona lineatus* (L.), a pest of leguminous crops (Bardner et al., 1983; El-Lafi, 1977), is widely distributed throughout Western Europe, the Middle East and the northwest region of North America. It has recently been found also in eastern North America (Hoebeke and Wheeler, 1985). The biology of this insect was first documented in detail in Britain by Jackson (1920), and it has since been studied intensively by several workers, including Andersen (1931) and Hans (1959) in Germany.

Adult weevils overwinter in sheltered situations in long grass, clover, lucerne, or the stubble of pea and bean fields. They migrate to the preferred food plants, peas, beans, and vetches (Jackson, 1920; Hans, 1959), in the following spring when mating occurs and egg laying commences. Adult feeding damage, consisting of characteristic semicircular notches in the margins of leaves is most obvious, but more serious damage is caused by the larvae which live in the soil and feed on the root nodules.

[1] Coleoptera: Curculionidae.

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The production of aggregation pheromones by several weevils, including *Anthonomus grandis,* three *Sitophilus* spp., and three *Pissodes* spp., is well-documented (Hardee et al., 1969; Tumlinson et al., 1969; Walgenbach et al., 1983; Fontaine and Foltz, 1982; Booth et al., 1983). We are therefore investigating the production by *S. lineatus* of pheromones which might be used to detect, monitor, and mass trap these insects or to time insecticide application. In this paper we present evidence for the presence of a male-produced aggregation pheromone in *S. lineatus*. The identification of and initial field studies on some components of this pheromone have been published elsewhere (Blight et al., 1984).

METHODS AND MATERIALS

Insects. S. lineatus used in the experiments were adults which had overwintered in the field. Weevils were collected in early spring from cultured broad bean (*Vicia faba*) plants which had been placed out on Rothamsted farm in areas where field beans had grown in the preceding year. They were sexed by examination of the ventral posterior abdominal segments (Jackson, 1920). Males and females were maintained separately, until use, on broad bean plants, under a controlled 16:8 (light–dark) photoperiod with a temperature of 15–16°C in the light and 9–10°C in the dark period.

Traps. The nonsticky cone traps (Figure 1) were similar in design to the Leggett trap (Leggett et al., 1975) used for capturing the cotton boll weevil, *Anthonomus grandis*. The base, A, consisted of an inverted yellow polythene bucket (26 cm diameter at lower edge, narrowing to 16 cm diameter × 25 cm high) from which the bottom had been removed. Surmounting the base was a cone, B, (24 cm diameter at lower edge, narrowing to ca. 5 cm diameter at upper edge × 27 cm high) made of woven stainless-steel square mesh wire cloth, aperture size 0.40 mm. The cone was held away from the base by three plastic spacers, G. A transparent polystyrene circular collecting pot, C, (7.5 cm diameter, narrowing to 6 cm diameter × 11 cm high) was held on the top of the cone with nylon shock cord. It contained a small (5 cm diameter × 8 cm high) wire cloth cone, D, which hindered the escape of live weevils. The assembled trap was held down on the soil by nylon shock cord guys, E, attached to tent pegs, F. Insects were able to gain access to the trap either by walking over the outside of the base and entering under the bottom edge of the large cone or by walking straight up the inside of the base from the ground. As the latter appeared to be the preferred route, six semicircular holes (3 cm radius) were cut out at the lower edge of the base to allow easier access.

Live Baits. Each bait consisted of a 13-cm pot of five broad bean (Sutton variety) shoots enclosed in a fine mesh terylene bag. The bag was taped tightly to the pot, the weevils were added, and the bag was closed securely with a

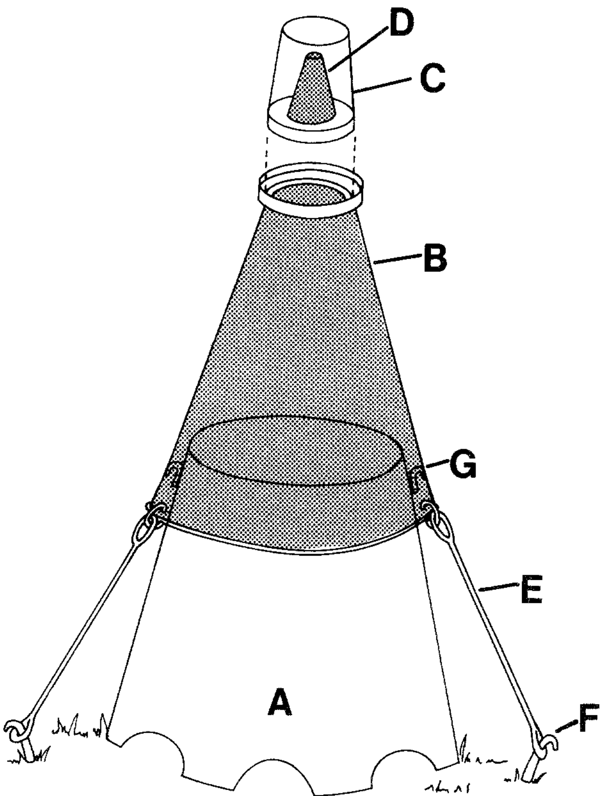

FIG. 1. Diagram of cone trap. A, base; B, cone; C, collecting pot; D, small cone; E, shock cord guy; F, tent peg; G, spacer.

rubber band. The five treatments (baits) were a blank control (pot containing soil only, enclosed in a bag), broad bean plant, and plants with, respectively, 40 females, 40 males, and 40 males plus 40 females. When the treatments were set out in the experiment, each pot was sunk into the ground so that the top of the pot was level with the soil surface, and a cone trap was then placed over the top. The plants were watered every day. When the experiment was terminated the numbers of live weevils of each sex present on the baits were recorded.

Experimental Design. The experiment was carried out on Rothamsted Farm, Harpenden, Hertfordshire, April 12–26, 1984. Three blocks were set up, two of which were towards the edges of fields where field beans had grown in 1983, and the third was on a field approx. 4 m from a wood. On each site, the treatments, 8 m apart, were placed in a row, parallel with the hedgeline. On two of the sites, the treatments were approx. 4 m out from the hedgerow and

wood, respectively, but on the third, because of farming operations, they were 1 m in from the hedge.

A randomized complete block design, with periodic rerandomization within the blocks, was used. Each treatment occurred once in each block, and each block was rerandomized when a minimum of 50 weevils had been caught. Weevils were removed from the traps each day and were stored alive at 6°C until sexed and counted.

Total catch data were subjected to the transformation $x = \log_e (t + 1.0)$ where x and t were the transformed and untransformed counts, respectively. Factorial analyses of variance were performed on these data and on the ratio of males to males-plus-females caught by each treatment.

RESULTS AND DISCUSSION

The catch data, derived from 12 replicates, are shown in Table 1. Because of uneven weevil distribution among the three sites, eight of the replications were performed on one site, with the other two sites yielding two replicates each.

Only low numbers of weevils were caught by either the blank control or the bean plant alone (5.4% and 5.6%, respectively, of the total catch). Although a few more weevils (7.3% of the total caught) were trapped by treatment 2 containing females, the "female effect" was not significant (Table 2). However, treatments 1 and 3, containing males, were highly attractive to both sexes, which were trapped in approximately equal numbers ("male effect" significant at $P < 0.001$, Table 2). The presence of females with males had no effect on

TABLE 1. FIELD RESPONSE OF *S. lineatus* TO TRAPS BAITED WITH MALE AND/OR FEMALE *S. lineatus* FEEDING ON BEAN PLANTS[a]

Treatment	Mean no. of weevils caught per replicate (range)	Sex Ratio (♂ : ♀)[b]
1. Bean plant + 40 ♂♂	31.3 (13–78)	1.0:1
2. Bean plant + 40 ♀♀	5.4 (0–14)	0.9:1
3. Bean plant + 40 ♂♂ + 40 ♀♀	29.3 (8–72)	0.9:1
4. Bean plant	4.2 (0–13)	0.9:1
5. Blank control	4.0 (0–10)	0.6:1

[a]The total number of weevils caught in 12 replicates was 891. At the end of the experiment, at least 85% of each sex of the *S. lineatus* comprising the baits remained alive.

[b]Factorial analysis of variance of the ratio of males to males plus females showed no significant differences between the five treatments.

TABLE 2. ANALYSIS OF VARIANCE: FIELD RESPONSE OF *S. Lineatus* TO TRAPS BAITED WITH MALE AND/OR FEMALE *S. Lineatus* FEEDING ON BEAN PLANTS
[Variate = $\log_e$ (catch + 1.0)]

Source of variation	Degrees of freedom	Mean square	Variance ratio
Replication	11	0.6414	1.681
Treatments, broken down into			
Blank control vs. other treatments	1	9.9472	26.065[b]
Treatments with males vs without	1	41.1379	107.794[b]
Treatments with females vs. without	1	0.0026	0.007
Interaction between males and females	1	0.2610	0.684
Residual	42(2)[a]	0.3816	
Total	46	1.4647	
Grand total	57		

[a] Missing values.
[b] Significant at $P < 0.001$.

this attraction (Table 2), and there were no significant differences between the sex ratios of weevils trapped by the five treatments.

These results indicate clearly that *S. lineatus* produces an aggregation pheromone in the spring. In common with most curculionids, it is male-produced (see references cited in the Introduction). However, in the experiment reported here, the possibility that some of the bean volatiles synergized the activity of the male-produced pheromone cannot be excluded. Indeed, we have independent evidence that the three synthetic bean volatiles, (*Z*)-3-hexen-l-ol, (*Z*)-3-hexen-l-yl acetate, and linalool, synergize the male-produced attractant, 4-methyl-3,5-heptanedione (Blight et al., 1984).

There was no evidence that female *S. lineatus* produced any semiochemical, although they may do so at other times of the year. Females of some curculionids, viz., *A. grandis* and *Cylas formicarius elegantulus* (Coffelt et al., 1978) produce a sex pheromone. In the case of *A. grandis,* it has weak attractancy compared to the male-produced aggregation pheromone (McKibben et al., 1977). However, the aggregation pheromone apparently functions mainly as a sex attractant after the insects are established on the host plants (Lloyd et al., 1983). A similar situation could exist with *S. lineatus* (Blight and Wadhams, unpublished).

The adaptive significance of an aggregation pheromone in these weevils is not clear. It has been suggested (Shorey, 1973) that coleopteran aggregation pheromones arose initially as mechanisms by which insects could cause others of the same species to aggregate at a suitable food source. The male-produced

Sitona pheromone presumably does assist the aggregation of the weevils in legume fields for the purposes of both feeding and mating. However, in this artificial monoculture it must be of far less importance to survival than in an environment comprising widely scattered food resources.

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INDUCIBLE VERSUS CONSTITUTIVE PI 227687 SOYBEAN RESISTANCE TO MEXICAN BEAN BEETLE, *Epilachna varivestis*

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Abstract—Contrary to constitutive resistance, inducible resistance to Mexican bean beetle (MBB) (*Epilachna varivestis*) herbivory in PI 227687 soybean leaves was positively correlated with total phenolic content and temporally unique, increased L-phenylalanine ammonia-lyase (PAL) and L-tyrosine ammonia-lyase (TAL) activities. Initial expression of the induced resistance was localized at or near the site of herbivory. Systemic parameters of the induced resistance also were observed. Inducible MBB resistance in PI 227687 soybeans apparently involves increased phenylpropanoid metabolism.

Key Words—Plant resistance, constitutive, inducible, soybean, Mexican bean beetle, *Epilachna varivestis*, Coleoptera, Coccinellidae, PI 227687, phenylpropanoid metabolism, PAL, TAL, total phenols.

INTRODUCTION

Plant resistance to insect pests involves at least two (i.e., constitutive and inducible) categories of parameters (Kogan and Paxton, 1983; Chiang and Norris, 1985). Constitutive parameters are expressed by the plant independent of environmental stresses. Such chemical parameters which are effective against Mexican bean beetle (MBB), *Epilachna varivestis* Mulsant, feeding have been studied partially in *Glycine max* (L.) Merr. PI 227687 soybeans by Chiang et

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al. (1986). In this work, L-phenylalanine ammonia-lyase (PAL) and L-tyrosine ammonia-lyase (TAL) activities in given-age leaves of nonstressed PI 227687 plants showed characteristic temporal patterns which were distinct from those of such leaves on nonstressed, insect-susceptible "Davis" soybeans. Activities of these enzymes have previously been positively correlated with the biosynthesis of phytoalexins and other flavonoids which include antifeedants for insects (Ebel et al., 1984).

Inducible versus constitutive (i.e., stress-dependent versus stress-independent) chemical parameters of PI 227687 resistance to MBB feeding have now been compared. This study contributes new understanding toward an ultimate holistic comprehension of PI 227687 soybean resistance to *E. varivestis* and other herbivores.

METHODS AND MATERIALS

Rearing of E. varivestis. Egg masses of the Mexican bean beetle (MBB) were removed from snapbean (*Phaseolus vulgaris* L.) plants on disks cut from leaves with a No. 8 cork borer. Masses on disks were sterilized for 10 min in Clorox® bleach–distilled H_2O (1:50), and then rinsed for 10 min in distilled H_2O. Egg masses on leaf disks were then placed on a dry filter paper in the bottom of a Petri dish which contained a water-wetted filter paper attached to the inside of the lid. Such eggs were held for three to four days at 25°C for hatching. Larvae were returned to vigorous snapbean plants growing within cages in 16-hr photoperiod, 27°C, and 65% relative humidity in a greenhouse. Feeding larvae and adults were provided daily with vigorous snapbean plants.

Growing of Soybean Plants. Relatively insect-resistant *G. max* PI 227687 plants were grown and studied. Seeds were germinated in moistened vermiculite in flats under the same environmental conditions as were used for the experimentation. Such conditions were: (1) 15-hr photophase, using Metalarc® high-intensity (1000-W), full-spectrum metal halide lighting, 27°C, and 65% relative humidity in a greenhouse or (2) 14-hr photophase with 12 hr of full light intensity, i.e., 300–500 $\mu E/m^2/sec$; day temperature, 27 ± 1°C, and night temperature, 20 ± 1°C; and relative humidity, 65 ± 5%, in the University of Wisconsin Biotron.

Germinated plants were transplanted at the first-leaf stage into individual plastic pots. In greenhouse experiments, plants were watered two times per day in a sterilized potting mixture (i.e., compost–soil–sand–vermiculite, 5:5:2:1). Plants in biotron studies were potted in a support medium and were provided with 30–40 ml of one-half-strength Hogland's nutrient solution (Hammer et al., 1978) four times per day (i.e., every 6 hr). All experiments involved plants between the V2 and V3 stages of development (Fehr and Caviness, 1977).

Mexican Bean Beetle Feeding Assays. The substrate utilized in feeding bioassays was an 18-mm-diameter leaf disk. Each disk was positioned with its abaxial side up. Assay insects were given a choice between two PI 227687 disks, each from a plant which had received a specific "stress" treatment, presented in an opposed arrangement in a Petri disk arena adapted from Norris and Baker (1967).

Only adult female *E. varivestis* beetles that were 2 weeks old and had been starved 24 hr, but were water-satiated, were used in bioassays. Two MBB females were released in each Petri dish arena. Assays were run for 22 hr in complete darkness at 24 ± 1°C and 60 ± 5% relative humidity.

Evaluation of beetle feeding in the bioassays involved measuring area (cm^2) of leaf disk eaten using a model LI-3100 Area Meter (LICOR, Inc., Lincoln, NE).

Estimation of Phenols. The used analytical method was based on techniques of Price and Butler (1977). Single fresh leaves, just removed from the plant, were weighed and then homogenized in 5 ml 60% methanol in a 10-ml ground-glass hand homogenizer. Homogenate plus 1 ml 60% methanol rinse was filtered through Whatman No. 1 paper in a glass funnel using vacuum. The approximate 6 ml of filtrate were added to 50 ml double-distilled (dd) H_2O in a 250-ml flask. Three milliliters of each of two reagents (i.e., A and B) were next added to the flask. Reagent A was 0.1 M $FeCl_3$ in 0.1 N HCl and reagent B was 0.008 M $K_3Fe(CN)_6$. The mixture was allowed to react for 10 min for color development. After 10 min, the optical density of samples was determined with a B&L Spectronic 20 at 720 nm. A blank of identical composition except for omission of the homogenate was analyzed and its reading at 720 nm was subtracted from all other sample readings.

Results were expressed as catechin equivalents (CEs), using a standard curve prepared daily from fresh solutions of commercial D-catechin. A catechin equivalent is the milligrams of catechin/100 mg of soybean leaf tissue that would be required to give the observed absorbance.

Assays for L-Phenylalanine Ammonia-Lyase (PAL) and L-Tyrosine Ammonia-Lyase (TAL). Extraction and assay of PAL from soybean tissue employed the method of Zucker (1965) as modified by Ciepiela (1984). A 1.5-g aliquot of dried acetone extractables from PI 227687 soybean leaves was homogenized in 10 ml of 0.1 M borate buffer, pH 8.8, using a glass homogenizer. The homogenate was centrifuged at 14,000 *g* for 15 min at 4°C. An aliquot (0.7 ml) of the resultant supernatant was mixed directly with 1 ml 0.03 M L-phenylalanine, 1 ml 0.1 M borate buffer, pH 8.8, and enough dd H_2O to bring the total volume to 3 ml. Incubation was 60 min in a 37°C water bath, and enzyme activity in such aliquot mixtures was then determined spectrophotometrically as the increase in absorbance at 290 nm. The enzymatic reaction in samples was killed by addition of 1 ml 1 N HCl. Specific enzyme activity was

expressed as units per milligram protein in the enzyme extract. One unit of enzyme activity equals the amount of PAL required to produce 1 μmol of *t*-cinnamic acid in 1 hr under specific conditions. Soluble protein content of samples was determined using the reagent and methods of Bradford (1976).

Extraction and assay of TAL were conducted similarly to the methods used for PAL, except that the substrate, L-tyrosine, is not dissolved completely in 0.1 M borate buffer, pH 8.8, so the reaction mixture must be continuously shaken at 37°C. At the completion of incubation, 1 ml 1 N HCl was added to kill the reaction and to clear the otherwise cloudy solution so required spectrophotometry could be readily performed. Activity of TAL was measured as the change in absorption at 333 nm.

RESULTS

Comparative Antiherbivory to Mexican Bean Beetle. Experimentally inflicted MBB herbivory on the middle leaflet of the second leaf (i.e., first trifoliolate leaf) of PI 227687 soybeans resulted in a significant ($P < 0.01$ or 0.05) reduction in subsequent MBB feeding on other leaflets of this second leaf at both 12 and 24 hr after the stress (i.e., MBB herbivory) treatment (Table 1). Systemic MBB antiherbivory effects of the experimentally inflicted MBB herbivory on PI 227687 second leaves were observed on the first (i.e., unifoliate) leaves ($P < 0.05$) at 48 hr after the stress treatment (Table 1). Other antiherbivory comparisons of first or second leaves between MBB herbivory-stressed

TABLE 1. COMPARATIVE MEXICAN BEAN BEETLE (MBB) FEEDING ON LEAF DISKS FROM LEAVES (OR LEAFLETS) ON PI 227687 PLANTS EXPERIENCING PRIOR MBB HERBIVORY OR NO HERBIVORY IN STANDARDIZED BIOASSAY

Hours after treatment	Leaf No.	Mean ± SD cm^2 eaten per leaf disk[a]	
		Herbivory stressed	No herbivory
12	2[b]	0.30 ± 0.1[c]	0.53 ± 0.2
24	2[b]	0.22 ± 0.04[d]	0.32 ± 0.02
48	1	0.21 ± 0.03[e]	0.33 ± 0.01

[a] Results from all other bioassays of leaves 1–3 at various times after treatment were not significantly different, $P < 0.10$, *t* test, $N = 4$.
[b] Significantly different feeding on "other" leaflets, *t* test, $N = 4$.
[c] Significantly different, $P < 0.01$.
[d] Significantly different, $P < 0.05$.
[e] Significantly different feeding, $P < 0.05$, *t* test, $N = 4$.

versus -unstressed PI 227687 at 12, 24, 48, or 72 hr gave no significant difference ($P < 0.10$) attributable to treatment.

Total Phenolic Content of Leaves. At 4 or 8 hr after MBB herbivory, no leaves of the stressed PI 227687 soybeans showed a significantly ($P < 0.05$) greater total phenolic content than corresponding leaves on nonstressed plants. However, by 12 hr after this stress treatment, the herbivory-wounded (i.e., fed on) middle leaflet of the first trifoliolate leaf contained a significantly ($P < 0.05$) larger amount of total phenolics (Table 2). At 24 hr after the stress treatment, this wounded (middle) leaflet of the first trifoliolate leaf still contained a significantly larger ($P < 0.05$) amount than was in such leaflets from unwounded PI 227687 plants (Table 2). By 48 hr after the stress treatment, the greater average CEs ($P < 0.05$) were found in the nonwounded (other) leaflets of the wounded second trifoliolate leaf and in the unwounded first (unifoliate) leaf. By 72 hr after the MBB herbivory, none of the leaves (i.e., 1–3) on the wounded (stressed) plants had significantly ($P < 0.05$) more total phenols than did corresponding leaflets or leaves from nonstressed PI 227687 soybean plants.

L-Phenylalanine Ammonia-Lyase (PAL) and L-Tyrosine Ammonia-Lyase (TAL) Activities. MBB herbivory on the middle leaflet [Leaf 2(M), Figures 2 and 4] of the first trifoliolate leaf of PI 227687 soybeans significantly ($P < 0.05$ or 0.01) increased levels (i.e., units per milligram protein) of both PAL and TAL in plants (Figures 1–4). The temporal pattern of activity for each enzyme also differed distinctly from that in the nonstressed plants. Major in-

TABLE 2. COMPARATIVE TOTAL PHENOL CONTENT IN PI 227687 SOYBEAN LEAVES AT INDICATED INTERVAL AFTER MBB HERBIVORY (TREATMENT) ON MIDDLE LEAFLET OF FIRST TRIFOLIOLATE LEAF OR IN LEAVES OF NONSTRESSED (CONTROL) PLANTS

Hours after treatment	Leaf No.	CE[a] (Mean ± SD per leaf)	
		Herbivory stressed	No herbivory
4	2[b]	0.58 ± 0.03[NS]	0.63 ± 0.05
8	2[b]	0.52 ± 0.04[NS]	0.54 ± 0.02
12	2[b]	0.83 ± 0.07**	0.58 ± 0.02
24	2[b]	1.82 ± 0.09**	0.63 ± 0.03
48	2[c]	1.76 ± 0.05**	0.88 ± 0.05
48	1	1.33 ± 0.09**	0.49 ± 0.03

[a] Results from all other assays of leaves 1–3 at various times after herbivory treatment were not significantly (NS) different from nonwounded controls, $P < 0.10$, t test, $N = 8$, ** significantly different at $P < 0.01$. A catechin equivalent (CE) is milligrams of catechin/100 mg of soybean leaf tissue that would be required to give the observed absorbance.
[b] Middle (wounded) leaflet on the first trifoliolate (second true) leaf.
[c] Other lateral (nonwounded) leaflet on the first trifoliolate leaf.

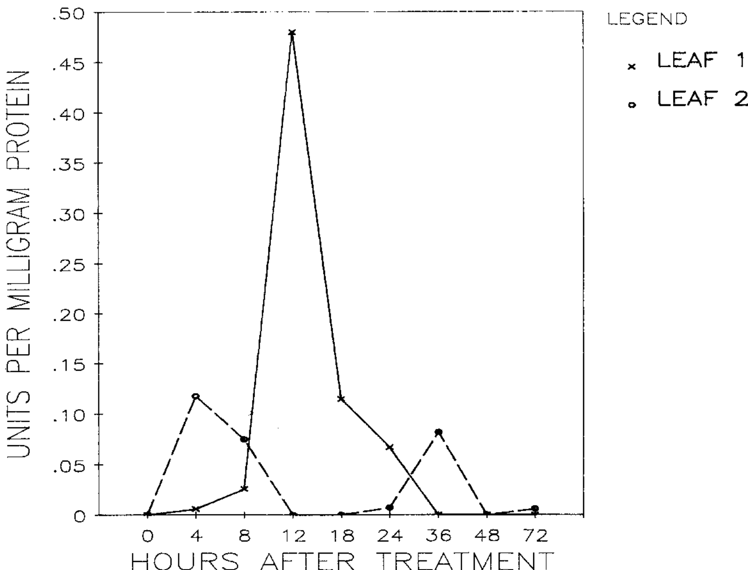

FIG. 1. L-Phenylalanine ammonia-lyase (PAL) activity in the indicated leaf of healthy (nonstressed) PI 227687 soybean plants, at the given time after treatment, growing in a biotron room.

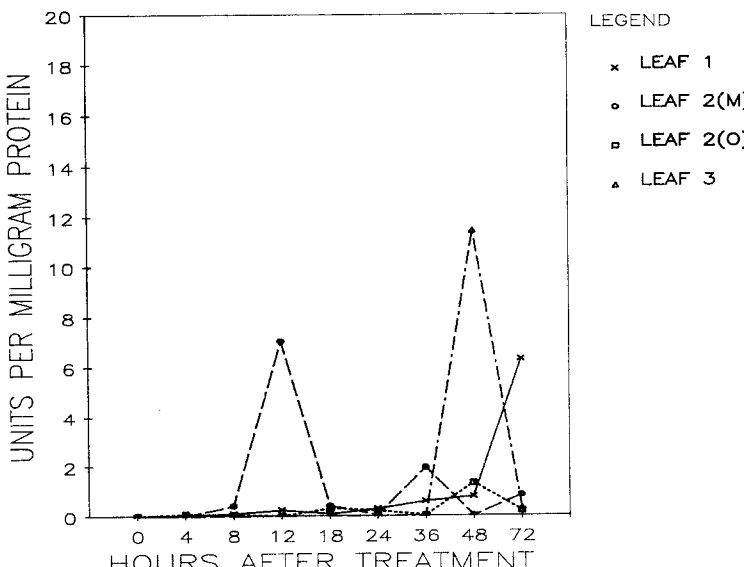

FIG. 2. L-Phenylalanine ammonia-lyase (PAL) activity in the indicated leaf, or leaflet, of experimentally stressed PI 227687 soybean plants, at the given time after the herbivory (stress) treatment, growing in a biotron room. LEAF 2(M) refers to the middle leaflet of the first trifoliolate leaf; and LEAF 2(O), to the outside (lateral) leaflet(s) of such a leaf.

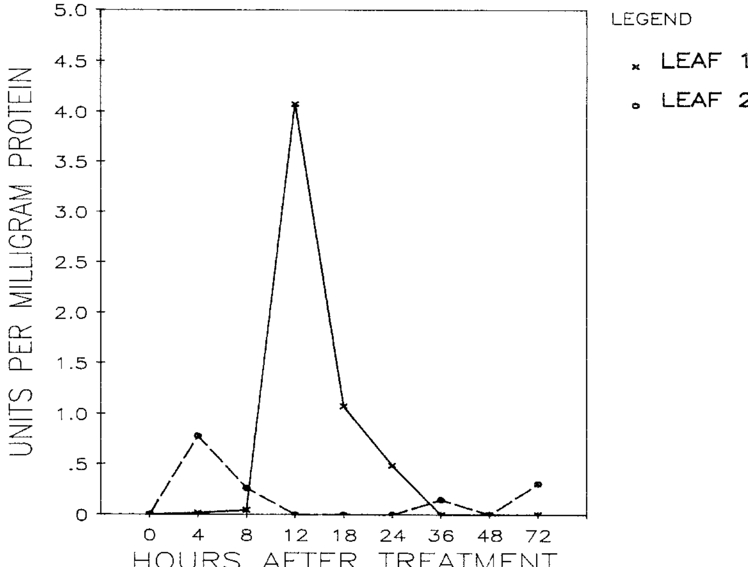

FIG. 3. L-Tyrosine ammonia-lyase (TAL) activity in the indicated leaf of healthy (nonstressed) PI 227687 soybean plants, at the given time after treatment, growing in a biotron room.

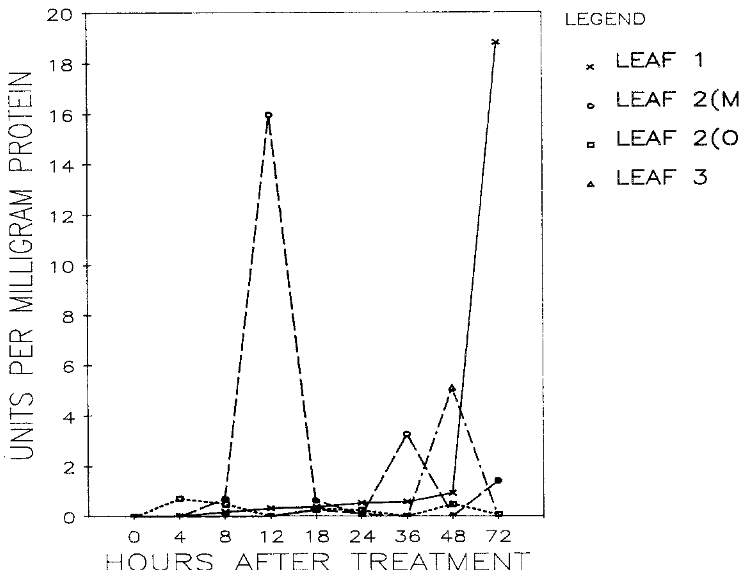

FIG. 4. L-Tyrosine ammonia-lyase (TAL) activity in the indicated leaf, or leaflet, of experimentally stressed PI 227687 soybean plants, at the given time after the herbivory (stress) treatment, growing in a biotron room. LEAF 2(M) and LEAF 2(O) have the same meanings as in Figure 2.

duced activity ($P < 0.01$) for each enzyme was limited to the "attacked" middle leaflet [Leaf 2(M), Figures 2 and 4] until 48 hr after the herbivory treatment. In each case, induced activity ($P < 0.01$) next appeared in leaf 3 (i.e., the youngest leaf present) (Figures 2 and 4). Activity of both enzymes increased ($P < 0.01$) markedly in the unifoliate leaf (i.e., the oldest leaf present) by 72 hr after treatment.

DISCUSSION

Stress (MBB herbivory) -induced higher levels of PI 227687 resistance to subsequent MBB feeding occurred first locally at or near the site of the initial stress (i.e., herbivory), which was the middle leaflet of the first trifoliolate leaf. This increased antiherbivory was positively correlated with an elevated level of total phenolics and temporally and quantitatively altered patterns of active PAL and TAL enzymes in such tissues. Such altered phenolic and enzyme levels occurred within 12 hr after the MBB herbivory. Systemic effects from the herbivory were evidenced as significant increases in PAL and TAL activities in leaf 3 by 48 hr after treatment. Increased antiherbivory activity and altered levels of total phenolics and active PAL and TAL enzymes in leaf 1 by 48 hr after the initial treatment provided further evidence of systemic effects.

Temporal aspects of the observed induced greater MBB resistance, including increases in the total phenolics and PAL and TAL activities, are compatible with the reported timetables of similar events associated with microorganismal induction of phytoalexins in soybeans (Sequeira, 1983; Darvill and Albersheim, 1984; Ebel et al., 1984).

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FACTORS CONTRIBUTING TO RESISTANCE OF EXOTIC MAIZE POPULATIONS TO MAIZE WEEVIL, *Sitophilus zeamais*[1]

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Abstract—Factors contributing to resistance of maize to infestation by the corn weevil *Sitophilus zeamais* were investigated in four populations of indigenous and improved maize from Belize. Resistance was related to the antifeedant properties of grain as well as sugar content and mechanical hardness. Grain extracts of all populations of maize significantly reduced insect feeding on treated artificial diets when compared to control diets. Consumption of treated diets was negatively correlated with phenolic content of the grain extract. An analysis by GC-MS indicated that ferulic acid and *p*-coumaric acid were the principal phenolics present in the extracts, and insect feeding was strongly deterred when pure substances were added to insect diets. Fluorescence associated with ferulic acid and related compounds in grain sections was located in the pericarp and aleurone layer and was especially intense in the most resistant grain variety.

Key Words—*Sitophilus zeamais*, Coleoptera, Curculionidae, maize weevil, maize, resistance, antifeedant, sugar content, hardness, phenolic content, ferulic acid, *p*-coumaric acid.

INTRODUCTION

Postharvest losses of grain to stored product insects remain among the most serious problems faced by small farmers throughout the tropics, accounting for

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substantial losses in less-developed countries (CIMMYT, 1970–1971). In attempts to reduce losses it must be remembered that small farm conditions for storage are often very poor, and, because grain is intended for human or animal consumption, pesticide use should be avoided. For this reason, selection of lines that are resistant to stored product insects is one practical way to deal with these pests (Dobie, 1977).

Unfortunately, breeding programs have been concerned mainly with increased yield and resistance of vegetative growth tissues to insect pests and fungal pathogens. Resistance in storage has received less attention, resulting in an increase in the severity of storage problems as traditionally used varieties are replaced by improved ones (Fortier et al., 1982).

In order to reduce gain losses of the small farmer at the postharvest level, it is important to identify resistant lines in the maize gene pool, pinpoint the mechanisms of resistance, and focus on stable heritable characters for breeding programs.

It has long been known that resistance to insect attack is strongly correlated with physical factors such as kernel hardness, husk protection (Singh and McCain, 1963), and low moisture content (Rivnay, 1972). Feeding stimulants correlated with susceptibility are sugar (Singh and McCain, 1963), and tryptophan (Betanzos, 1980), but do not include protein content or fat in seeds (Singh and McCain, 1963). Attractants are also known to occur in seeds. Yamamoto et al. (1975) have isolated a number of volatiles (e.g., hexanoic acid) that are attractants to *Sitophilus* spp.

No studies have been made of secondary metabolites and their relationships to the resistance of corn seeds to maize weevil.

The present study examines factors correlated with exceptional resistance in lowland tropical races of maize from Belize (Fortier et al., 1982), with special emphasis on the presence of phenolics in the grain.

METHODS AND MATERIALS

Maize Seed. Maize grain *Zea mays* L. collected in Belize, C.A., as described in Fortier et al. (1982), was used. Two populations had exceptional resistance to corn weevil (designated as Belize local yellow and Belize local white) and one population, Belize local black, was moderately susceptible. One improved double-cross hybrid variety, Pioneer 230 which was highly susceptible to weevils, was also included. Ordination and cluster analysis of 16 taxonomic characters, derived from measurements on the local populations indicated that all had affinities to the ancient indigenous group of maize races (Fortier et al., 1982). A very close relationship between Belize local yellow and the land race Nal-Tel amarillo was evident. Belize local black was linked to land race Negro de Tierra Caliente and Belize local white linked with land race Dzit-

Bacal. The hybrid had greatest affinity with upland races of the exotic group of races of maize.

Insect Culture. Corn weevils (*Sitophilus zeamais* Motsch.) from a stock maintained in our laboratory were cultured and kept in screened Mason jars with locally produced maize grain. Jars were placed in a controlled environment chamber at 27°C and 70% relative humidity. Photoperiod was 16:8 hours light–dark.

Choice Tests. For relative consumption of maize populations, 20 unsexed, 14-day-old adults from the stock culture were offered 10 g of each population of grain in a feeding arena. Grain was equilibrated to constant humidity for six weeks before the test and weighed weekly for three weeks after the introduction of the insects.

For emergence tests, 120 insects were offered 20 g of each variety in a feeding arena. After one week of exposure, the number of live adults in each variety were counted and removed, and percentage of seeds with oviposition sites determined by counting under a microscope. Each maize population was then transferred to an individual screened jar and new emergence assessed at 22 days.

Extraction Procedures. Extraction of 10 g milled grain was performed using the hydrolytic procedure of Krygier et al. (1982) for total phenolic acids and dissolved in a final volume and 1 ml ethanol. Acetone extractions for total soluble phenolics and phenolic glycosides were performed as follows: 10 g of corn seeds were placed in liquid nitrogen, ground in a ball mill, and put into 35 ml acetone. After centrifugation (15 min 1000 *g*), the supernatant was reduced to dryness in vacuo, dissolved in 10 ml of 95% ethanol, microfuged, and the supernatant retained. Four replicates of each extraction were assayed in the feeding tests.

Preparation of Diets and Antifeedant Assay. A diet based on wheat flour was prepared as follows: 1 g white wheat flour, 1 ml of extract from corn in ethanol (the equivalent of 1 g corn grain), and 5 mg sorbic acid were placed in a 5-g mortar. The ethanol was evaporated and 0.5 ml water added to make a paste. The paste was rolled to a sheet 2 mm thick and seven 1-cm disks were cut. Control diets were made with addition of only ethanol. Diets treated with pure substances were made in a similar manner from stock solutions of the compounds in ethanol.

For the feeding assay, four weighed disks of each diet type were placed in a 9-cm Petri dish with five unsexed corn weevil adults and kept at 27°C, 70% relative humidity, 16:8 light–dark in an incubator. The disks were later weighed at two-day intervals to day 10. Three disks were kept under similar conditions to monitor weight changes not associated with insect feeding. Calculations of consumption were made from weight changes corrected for nonfeeding changes. Wheat flour was chosen as the artifical diet because of its exceptionally low phenolic content as compared to corn (Krygier et al., 1982).

Chemical Analysis. Total sugar content of grain was assessed by the method of Dubois et al. (1956) and total phenolics of extracts by the method of Swain and Hillis (1959). Extracts were analyzed on a Beckman HPLC using a reverse phase 5 μm ODS column and isocratic elution with 1 : 1 MeOH–1% HOAc in H_2O. GC-MS analysis was performed on TMS derivatized extracts using a VG7070-E spectrometer with a 3% OV-1 column programmed at 150°C and 10°C/min. Two-dimensional TLC of extracts was performed on TLC micro-polyamide foils (F1700, Schleicher and Schüll) using aqueous developing solvent: butanol–acetone–glacial acetic acid, 10 : 5 : 5; and organic developing solvent: chloroform–methanol–butanol–water, 55 : 22 : 20 : 3.

Hardness Tests. Mechanical hardness of seeds was tested with an Instron model 4201 instrument fitted with an electronic force transducer. Hardness was measured as the force to peak (N) in a compression test between two flat steel plates (Timbers et al., unpublished).

Fluorescence Microscopy. Autofluorescene associated with ferulic acid was detected by fluorescence microscopy described previously (Fulcher et al., 1972, 1981). Briefly, individual corn kernels were sectioned 20–30 μm thick on a freezing microtome, the sections fixed to glass microscope slides, and mounted in immersion oil under a cover glass for detection of phenolic autofluorescence. Phenolic fluorescence was also enhanced in the outer tissues by staining sections with boronate (Fulcher and Wong, 1980). With or without boronate staining, all sections were examined with a Zeiss Universal microscope equipped with III RS epi-illuminating condenser and HBO 200-W illuminator. Fluorescence filters included a 365-nm exciter filter and long-pass 420-nm barrier filter. Boronate has been used as a fluorescent indicator for flavonoid compounds (Fulcher and Wong, 1980), and it also markedly enhances the *in situ* fluorescence of known deposits of ferulic acid and related cinnamates in sections of wheat, oats, and barley. Parallel sections were also treated with dimethylaminocinamaldehyde for demonstration of possible aromatic amines (Fulcher et al., 1981).

RESULTS AND DISCUSSION

The exceptional resistance of two populations of maize (local white and local yellow) is evident in the choice test (Table 1) when the consumption of grain by adults and new emergence at 22 days for these populations is compared to the data for a susceptible land race (local black) and a highly susceptible improved variety (Pioneer 230). An examination of two factors (correlated with resistance or susceptibility), mechanical hardness and sugar content, shows (Table 2) that the least susceptible populations (local yellow and local white) had the greatest hardness and lowest sugar content. The more susceptible maizes (Pioneer 230 and local black) had higher sugar content and less hardness.

The presence of extractable antifeedants in the grain is demonstrated in

TABLE 1. VARIATION IN CONSUMPTION, SURVIVAL, AND REPRODUCTIVE CAPACITY OF *S. zeamais* WITH RESPECT TO FOUR MAIZE POPULATIONS

Population of maize	Consumption by adults (mg/week)	No. of live adults found after 1 wk exposure	Seeds with oviposition sites (%)	Total new emergence at 22 days
Pioneer 230	15.1	70	100	77
Local black	3.9	32	100	31
Local white	1.8	9	40	10
Local yellow	1.7	3	26	0

Table 3. Treatment of artificial insect diets with hydrolyzed total phenolic extracts of each grain population led to significantly lower consumption of diets as compared to consumption of untreated controls ($P \leq 0.05$). The antifeedant properties of the hydrolyzed extracts of the three land races were significantly higher than Pioneer 230, and the order of increasing antifeedant activity is the same as the order of resistance in Table 1.

As the total phenolic extracts were hydrolyzed, a second extraction was undertaken of grain in acetone at liquid nitrogen temperatures to extract the soluble glycosides. The consumption of diets treated with these extracts at concentrations found in corn grain was also significantly different from controls ($P \leq 0.05$) but not significantly different among varieties.

Consumption as percent of control was reduced more by the hydrolyzed than unhydrolyzed extracts (except for Pioneer 230). This finding is presumably related to a greater total amount of phenolics and is similar to the observations of Woodhead and Cooper-Driver (1979), who examined the effect of phenolics

TABLE 2. HARDNESS AND SUGAR CONTENT CHARACTERISTICS OF MAIZE POPULATIONS USED

	Total sugars[1] (%)[a]	Instron hardness (mean force at peak compression, *N*)[b]
Pioneer 230	4.0	408
Local black	1.3	602
Local white	1.0	753
Local yellow	0.9	796

[a] Measured by the method of Dubois et al. (1956).
[b] Measured in compression tests to the fracture point of the seed.

TABLE 3. ANTIFEEDANT PROPERTIES OF MAIZE EXTRACTS TOWARDS *S. zeamais* EVIDENCED BY CONSUMPTION OF TREATED ARTIFICIAL DIETS

	Consumption of treated diets[a]	
Maize population from which extract was made	Total phenolic extract (mg/insect/day)	Acetone extract (mg/insect/day)
Control	4.88a	4.24a
Pioneer 230	4.48b	3.09b
Local black	3.89c	3.43b
Local white	3.67c	3.57b
Local yellow	3.66c	3.25b

[a]For ANOVA with total phenolic data: $F = 3.76$; $P = 0.001$; $df = 51$; and with acetone extract data: $F = 4.62$; $P = 0.0001$; $df = 51$. Means followed by the same letter are not significantly different ($P = 0.05$) in Duncan's multiple-range test. Phenolic contents of the extracts are reported in Figure 1.

from sorghum on feeding of *Locusta*. They also emphasized the possibility of synergism, as mixtures of compounds were markedly inhibitory.

The antifeedant properties of the total phenolic grain extracts as measured by consumption (data from Table 3) were negatively correlated ($r = -0.84$) with the phenolic content of the extracts (Figure 1A). For the acetone extracts the correlation was -0.96 (Figure 1B). These results suggest that phenolics are the major substance contributing to reduced feeding.

An analysis of the total phenolic extracts by GC-MS and HPLC indicated that more than 80% of the phenolic material in all varieties was present as ferulic acid (Figure 2), with *p*-coumaric acid being the second most abundant substance (up to 20%). Other phenolic substances such as syringic, caffeic, protocatechuic, and vanillic acids, as have been reported by Sosulski et al. (1982), were evident at much lower concentration in two-dimensional chromatography of the extracts on polyamide.

A preliminary phytochemical investigation of the acetone extracts showed that they contained a variety of phenolic substances including free and esterified phenolic acids, as described by Sosulski et al. (1982). We also investigated the possibility that glycosides of hydroxamates, which are correlated with resistance of maize leaves to *Ostrinia nubilalis* (Klun and Robinson, 1968), were present in these extracts. A GC-MS analysis of the acetone extracts, before or after hydrolysis, indicated that none of the major peaks were hydroxamates, although these substances were readily detected by the same GC-MS procedure after germination, as described by Woodward et al. (1979).

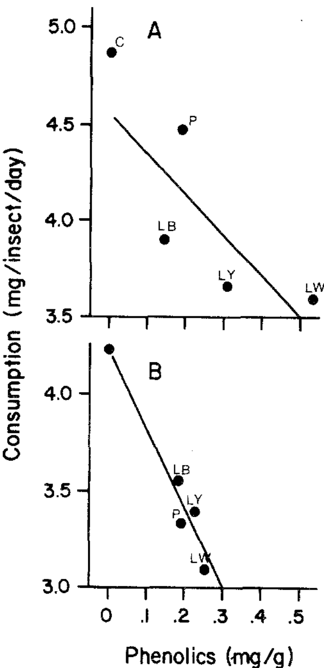

FIG. 1. Consumption by maize weevil of artificial diets treated with extracts from the four maize populations investigated plotted aganist the phenolic content of the extract. (A) Total phenolic extracts (hydrolyzed); (B) acetone extracts (unhydrolyzed).

The feeding response of *Sitophilus* to a number of pure phenolic substances (Table 4) indicates clearly that these substances have significant antifeedant properties ($P \leq 0.05$) at a concentration of 0.3 mg/g, close to the phenolic content of grain. Ferulic acid was a highly effective antifeedant at concentrations as low as 0.05 mg/g (data not shown). Ferulic acid is present in maize grain as a free acid in small quantities, but soluble glycosides and polysaccharide-bound ferulic acid are probably released as the free acid during digestion in the insect. Free ferulic acid release could make a substantial contribution to reduction of feeding by *Sitophilus* on grain. Undoubtedly, the real situation in grain is considerably more complicated due to presence of phenolics such as glycosides, synergism, etc.

Fluorescence microscopy was used to locate phenolics in sections of maize seeds. All varieties showed strong blue autofluorescence in the outer tissues, much of which has been associated with the presence of ferulic acid in other cereals (Fulcher et al., 1972). The exceptional fluorescence was enhanced by boronate staining (Figure 3) and was particularly intense in the pericarp of local

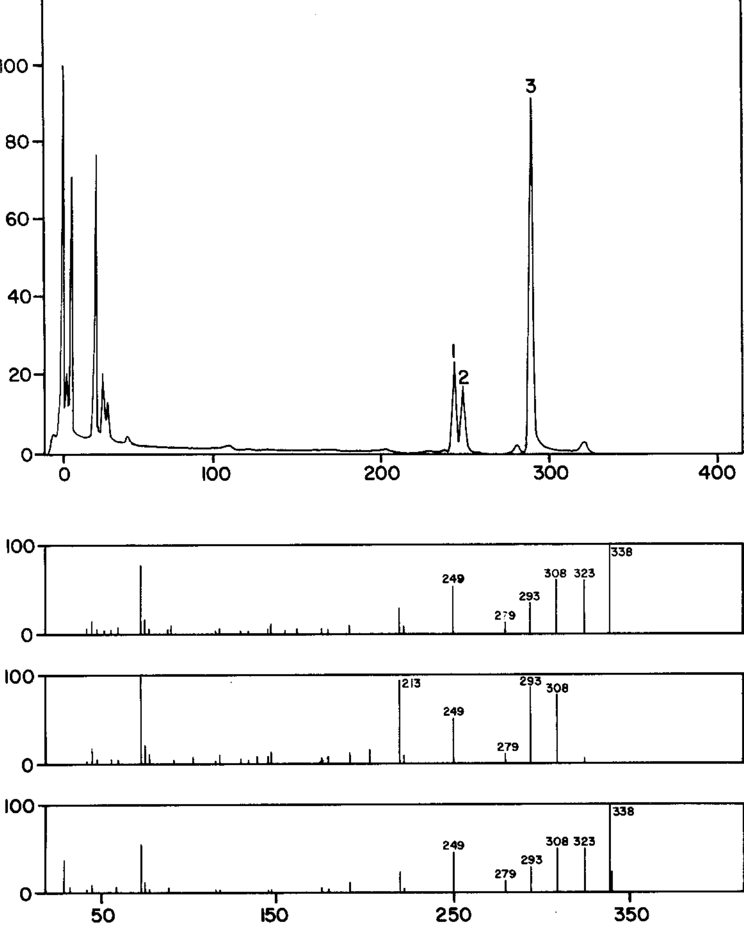

FIG. 2. GC chromatogram and normalized spectra of TMS-derivatized total phenolic extracts from local yellow corn: (1) *cis*-ferulic, (2) *p*-coumaric, and (3) *trans*-ferulic.

white and to a lesser extent in local yellow. Both local white and local yellow were highly resistant to attack by *Sitophilus*, and such resistance appears to be correlated with a higher proportion of phenolics bound to the cell wall, since the susceptible Pioneer and local black had feeding deterrent properties comparable to that of the resistant population in the soluble acetone extracts but not the total phenolic extracts which includes bound material. In the local white pericarp (Figure 3a), the distribution of fluorescent compounds was relatively homogeneous, while in that of the local yellow, the fluorescence was notably more intense in the outer regions of the pericarp (Figure 3b). The local black

TABLE 4. CONSUMPTION OF DIETS TREATED WITH PURE PHENOLIC SUBSTANCES BY *Sitophilus zeamais*[a]

Treatment	Consumption (mg/insect/day)
Control	3.49a
BOA	2.70b
B-Aminophenol	2.58b
6-MBOA	2.24c
Ferulic acid	2.04c

[a]Concentration was 0.3 mg/g diet. Means followed by the same letter are not significantly different ($P \leq 0.05$) in Duncan's multiple-range test. (ANOVA values $F = 15.48$; $P = 0.0001$; $df = 51$).

pericarp showed a somewhat lower level of fluorescence (Figure 3c), while the Pioneer pericarp showed an exceptionally low level of fluorescence (Figure 3d).

In addition, the cytoplasm of the aleurone cells stained pink to red with dimethylaminocinnamaldehyde, suggesting the presence of aromatic amines (Fulcher et al., 1981). Intensity of staining was greater in the three local populations than the hydrid (not shown). The presence of these substances in the outer tissues of maize seeds is consistent with their proposed defensive role. In addition, their discontinuous distribution suggests that their concentration in these tissues is probably higher than that used in the feeding deterrence assays. The secondary chemistry of maize is complex, especially if spatial organization of substances in tissues and the full range of phenolics in free esterified and bound forms in maize is to be considered. Besides their contribution to reduced feeding, phenolics may also contribute to physical factors such as hardness. For this reason, more detailed phytochemical-biological activity investigations are in progress.

There are few other reports of chemical deterrents to *Sitophilus* spp. Maize flour is known to contain inhibitors of the digestive proteases of *Sitophilus* (Baker, 1982). These are proteins themselves and would not likely be present in any of the extracts used in the present study. A group of secondary metabolites, the sesquiterpene lactones, isolated from species of the Apiaceae (Nawrot et al., 1983) and Asteraceae (Nawrot et al., 1982) have antifeedant activity to *Sitophilus granarius*.

Deterrent effects of phenolics to other maize feeding insects are well documented. Recently, a new flavanoid glycoside, maysin, isolated from corn silk of the land race, "Zapalote chico" has been shown to have exceptional growth-reducing properties to *Heliothis zea* (Waiss et al., 1979).

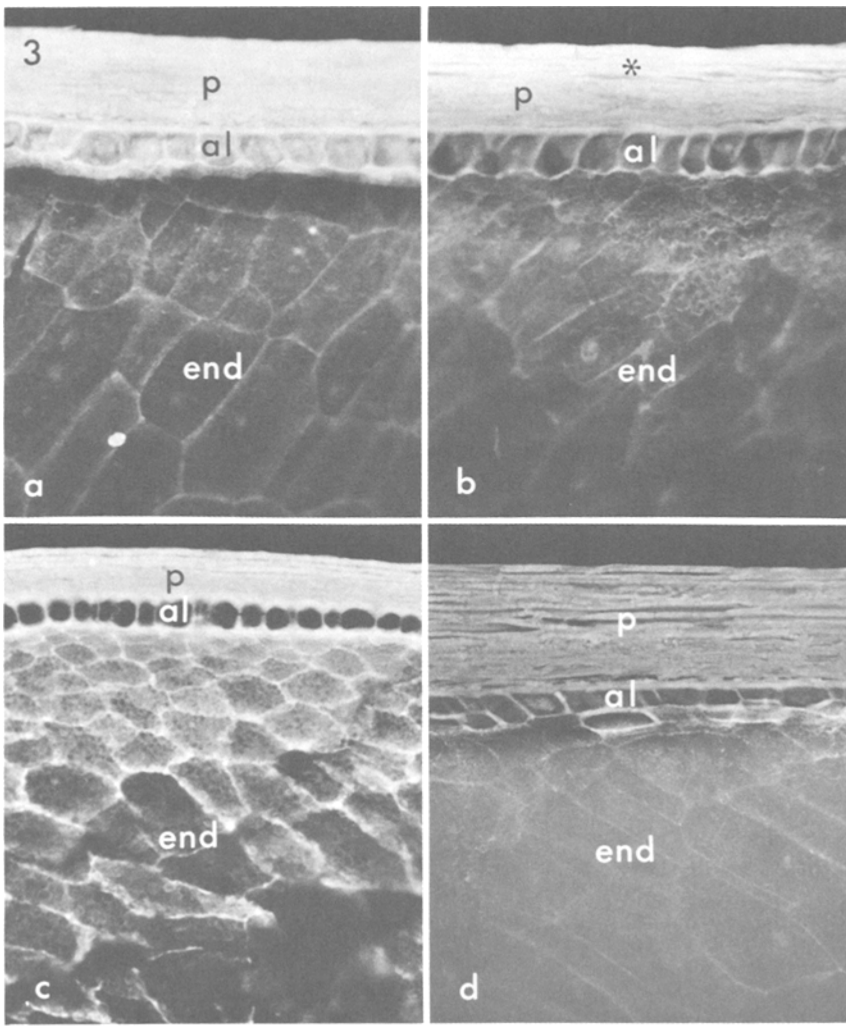

FIG. 3. Fluorescence micrographs of sections of corn kernels showing pericarp (p), aleurone layer (al), and endosperm (endo) tissues after staining with diphenyl boronate. Fluorescence intensity is highest in the pericarp of the local white variety [LW (3a)], while in the local yellow [LY (3b)], a similar intensity is concentrated primarily in the outer layers of the pericarp (*). The local black [LB (3c)] sample showed noticeably lower intensity in the pericarp than either the white or yellow kernels, while the Pioneer line [P (3d)] showed very low intensity.

The exploitation of phenolics as part of a complex of factors for *Sitophilus* control is possible but must be approached with caution. Heritability of phenolics (Styles and Ceska, 1977; Dunn, 1981) in corn is well studied, and the increase in levels of these substances in improved varieties in a conventional breeding program is envisaged. The obvious danger of this approach is that the grain may also become toxic to humans and animals or influence palatability. To avoid this danger, we are examining the existing land races of maize. As these traditional varieties have been used for human consumption for thousands of years without any apparent detrimental effects, their analysis may yield information on acceptable levels of phenolics which will contribute to the resistance to stored grain pests.

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PHENOLIC ACETOGENINS SECRETED BY RHODODENDRON LACE BUG, *Stephanitis rhododendri* HORVATH (HEMIPTERA: TINGIDAE)[1]

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Abstract—Nymphs of the rhododendron lace bug produce from specialized setae a liquid secretion from which a number of related acetogenins have been identified. Two (2,6-dihydroxyphenyl)-1,3-diketones are the principal components and are accompanied by the corresponding chromones as well as by chromones bearing an additional phenolic oxygen.

Key Words—Hemiptera, Tingidae, *Stephanitis rhododendri*, lace bug, setal exudate, chromones, acetogenins.

INTRODUCTION

Nymphs of many genera of lace bugs exude clear fluids that collect as microdroplets on secretory setae on the abdomen, abdominal protruberances, and antennae segments (Livingstone, 1978). We recently reported the identification of several components of secretions produced by nymphs of the azalea lace bug, *Stephanitis pyrioides* (Scott) (Oliver et al., 1985), one of three *Stephanitis* species known to occur in North America. This unusual insect secretion included decanal, undecan-2-one, the novel phenolic acetogenin 2,6-dihydroxyacetophenone, and some derivatives including the diketone **2b** and chromone **3b.** We have since turned our attention to a congeneric species, the rhododendron lace bug, *S. rhododendri* (Horvath). This species is thought to have originated in

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

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eastern North America (Bailey, 1951; Johnson, 1936), but taxonomic relationships to other congeneric species are not firmly established. The other two species in North America, *S. pyrioides* and *S. takeyai* Drake and Maa are known to be introductions from Japan (Drake and Ruhoff, 1965).

The rhododendron lace bug is morphologically distinct from other species by the presence of an irregular biseriate hypocosatal ridge that is uniseriate in other species (Bailey, 1951), and the presence of only four nymphal instars (Crosby and Hadley, 1915; Johnson, 1936) is unusual in the genus and the Family. Thus, in addition to questions concerning the functions of lace bug secretions, a chemical comparison of the secretions of the azalea and rhododendron lace bugs seemed of interest because it might contribute to an understanding of the relationship between these species.

METHODS AND MATERIALS

Gas chromatography (GC) was performed on a Shimadzu model GC-9A instrument equipped with a flame ionization detector, a split–splitless injector operated at a ca. 50:1 split ratio, and a 15-m DB-1 fused silica column with helium as a carrier. A frequently employed temperature program (injection block temperature 300°) consisted of a 2-min interval at 160° followed by a 5°/min increase to 260° which was maintained an additional 10 min. High-performance liquid chromatography (HPLC) was carried out on a Spectra Physics model 8700 system with a variable wavelength UV detector operated at 254 or 220 nm, fitted with a 150 or 250 × 4.1-mm Hamilton PRP-1 "resin" column; various gradient elution systems were performed using water and either methanol or acetonitrile as the primary and secondary solvents, respectively. One such program began with 65% methanol at 1.5 ml/min. for 2 min, then increased the methanol content to 80% between 2 and 6 min. At this point the flow was increased to 2 ml/min, solvent programming from 80 to 100% methanol was continued from 6 to 10 min then the composition was maintained at 100% methanol for an additional 8 min.

Mass spectra were obtained from a Finnigan model 4510 gas chromatograph–mass spectrometer fitted with a 30-m × 0.32-mm-id DB-1 fused silica column. The instrument was scanned from *m/z* 50 to 650 with a total scan cycle time of 1.0 sec. Electron ionization spectra were collected at 70 eV and a source block temperature of 150°C. Data were analyzed via an Incos data system.

Lace bugs were reared on cuttings of mountain laurel, *Kalmia latifloria* L., in a Sherer Environment Rearing Chamber at 26 ± 1°C and 16:8 (light–dark) photoperiod. As was the case with *S. pyrioides*, the cleanest samples of exudate were collected by carefully blotting the material from the surfaces of late instar nymphs with small pieces of filter paper, then extracting the filter paper with dichloromethane. Alternately, nymphs, or their cast moult skins, could be dipped directly in dichloromethane.

Synthesis of Trihydroxydiketone **4c** *and Dihydroxychromone* **5c.** A mixture of 2,4,6-trihydroxyacetophenone (0.34 g, 2 mmol) and *t*-butylchlorodimethylsilane (0.90 g, 6 mmol) in dichloromethane (10 ml) was slowly treated with triethylamine (0.8 ml, 5.7 mmol) at room temperature. After 1 hr, the reaction mixture solution was washed with water and with 1 N $NaHCO_3$, then was dried, concentrated in vacuo, and the residue was further dried in a vacuum desiccator. The resulting oil was dissolved, along with 0.50 g (2.2 mmol) of ethyl dodecanoate, in 3 ml dry pyridine, and this solution was added dropwise to a stirred suspension of sodium hydride (0.4 g) in 5 ml pyridine under nitrogen. After the initial gas evolution, the mixture was refluxed 2 hr, cooled, and added to ice. The solution was rinsed twice with ether, then was neutralized with a mixture of ice and aqueous HCl.

Extraction with ether, followed by water and bicarbonate washes, drying, and evaporation of solvent gave a tan oil that was chromatographed on 20 g of silica gel with benzene containing increasing amounts of ethyl acetate. Fractions 3 (0.17 g, 12% EtOAc) and 4 (0.29 g, 16% EtOAc) contained most of the material of interest. Crystallization of fraction 4 from benzene gave pure diketone **4c,** mp 128.5–129.5°C. Mass spectrum (70 eV) *m/z* (relative intensity): 350 (M^+, 3%); 333 ($M^+ - OH$, 1.9%); 332 ($M^+ - H_2O$, 2.5%); 205 (14.6%); 196 (11.8%); 195 (100%); 153 (38.4%); 69 (3.2%); 57 (3.4%). Chromatographic fraction 3 (a mixture of **4c** and **5c**) was boiled about 3 min in methanol containing a few drops of conc. HCl; this converted all the diketone **4c** to chromone **5c** whereupon cooling and filtration gave pure **5c** as a white solid, mp 96–97°C. Mass spectrum (70 eV) *m/z* (relative intensity): 332 (M^+, 19.8%); 261 (4.0%); 205 (100%); 192 (28.2%); 153 (17.1%); 69 (3.1%); 55 (3.6%).

Diketone **4b** *and Chromone* **5b.** These were similarly obtained when ethyl decanoate was used in place of ethyl dodecanoate: **4b,** mp 131–132°C; **5b,** mp 102–102.5°C. Their mass spectra were analogous to those of **5c** and **6c,** respectively.

Dihydroxydiketones **2c** and **2d,** and hydroxychromones **3c** and **3d,** respectively, were synthesized by the methods previously described (Oliver et al., 1985) for the preparation of **2b** and **3b; 2c,** mp 74.5–75.5°C; **2d,** mp 73–74°C; **3c,** mp 54–54.5°C; **3d,** mp 60–60.5°C. Mass spectra of these compounds were analogous to those of **2b** and **3b** described earlier (Oliver et al., 1985).

RESULTS

GC and HPLC analyses of *S. rhododendri* secretions revealed a more complex mixture than that encountered from *S. pyrioides*. GC-MS (Figure 1) suggested, however, that most of the volatile components were closely related to those previously described and that the two most abundant components were the C_{11} and C_{13} dihydroxydiketones (**2c** and **2d,** respectively) homologous to

RCO_2H

1 e,f

2 a–e

3 a–e

4

5 b–d

a	R = n-C_7H_{15}
b	n-C_9H_{19}
c	n-$C_{11}H_{23}$
d	n-$C_{13}H_{27}$
e	n-$C_{15}H_{31}$
f	n-$C_{17}H_{35}$

6 b,c: unidentified; probably $C_{18}H_{22}O_4$ & $C_{20}H_{26}O_4$

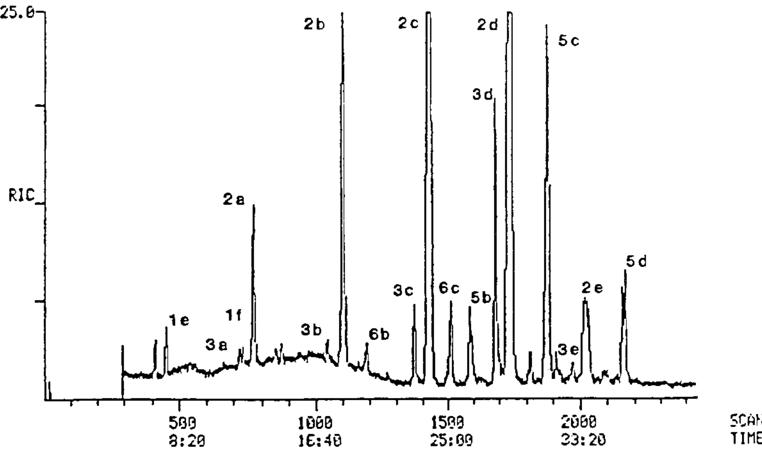

FIG. 1. Reconstructed ion chromatogram of *S. rhododendri* secretion.

the C_9 analog **2b** which had been the major component identified from *S. pyrioides*. Diketone **2b** was also present in the *S. rhododendri* sample along with small amounts of C_7- and C_{15}-alkyl derivatives **2a** and **2e**, respectively.

Cyclodehydration (thermal or acid-catalyzed) of dihydroxydiketones **2a–2e** readily gives 2-alkyl-5 hydroxychromones **3a–3e**, and indeed **3a–3e** were all detectable by GC-MS in the *S. rhododendri* sample. It has been our experience that some cyclization of dihydroxydiketones **2** to hydroxychromones **3** frequently occurs during gas chromatography, and although small peaks consistent with **3d** and **3e** were detectable by HPLC, it is possible that at least portions of the 5-hydroxychromones encountered here may have been formed during analysis. In any event, it is evident that the dihydroxydiketones are the principal components of the exudate. This contrasts to the *S. pyrioides* secretion in which chromone **3b** constituted a substantial portion of the exudate. The C_{11}- and C_{13}-alkyl diketones **2c** and **2d**, and the corresponding chromones **3c** and **3d** were

synthesized as described earlier for **2b** and **3b** (Oliver et al., 1985); the authentic samples matched (GC, HPLC, mass spectra) those identified in the exudate. The C_7- and C_{15}-alkyl analogs were not synthesized; their structures are assigned on the basis of their mass spectra [the C_7-alkyl analogs **2a** and **3a** had also been tentatively identified as very minor components of the *S. pyrioides* exudate (Oliver et al., 1985)].

Among the less abundant components of the *S. rhododendri* mixture were three members of a third series **5b–5d** not encountered in the *S. pyrioides* exudate. Mass spectra of these compounds closely paralleled those of hydroxychromones **3a–3e** except that major ions of **5b–d** were shifted by 16 amu; in fact, these spectra closely resembled a published mass spectrum of 5,7-dihydroxy-2-nonadecylchromone (Tringali and Piattelli, 1982). The n-C_9- and C_{11}-substituted 5,7-dihydroxychromones **5b** and **5c,** respectively, were synthesized and matched the respective components of the secretion. The assignment of the structure of **5d** followed from its analogous mass spectrum. While synthesizing **5b** and **5c,** we observed that the precursor trihydroxydiketones **4b** and **4c** seemed somewhat more prone to cyclodehydration than had been the dihydroxydiketones **2b, c.** Thus the absence of diketones **4** in the gas chromatograms or reconstructed ion chromatogram (Figure 1) might have reflected thermal instability rather than the true composition of the insect exudate. HPLC showed, however, that in contrast to the less highly oxygenated analogs, the chromones were in fact the more abundant components of the **4–5** pairs, at least by the time the samples were collected and analyzed (solutions were stored at $-10°C$ after collections were made, but the viscous microdroplets had in most cases been accumulating on the surfaces of the nymphs for several days prior to collection).

The compositions of these exudates remained quite reproducible over a number of samples. All samples consisted of exudates of a number of nymphs because of the small size of the latter, so individual varieties would not have been recognized. Because of severe limited availability of material, all initial structural assignments were made on the bases of GC-MS; no individual components were available for NMR or IR spectra. Syntheses by standard procedures from defined intermediates completed the structural assignments.

Synthesis of Authentic Samples. Syntheses of dihydroxydiketones **2c** and **2d,** and of 5-hydroxychromones **3c** and **3d,** were achieved as reported for the preparations of **2b** and **3b,** by condensing the monobenzylether of 2,6-dihydroxyacetophenone with appropriate esters in the presence of sodium hydride (Oliver et al., 1985). Our first thought was to carry out analogous condensations between a dibenzylether of 2,4,6-trihydroxyacetophenone **7** and the same esters; however, although **7** has appeared in the literature, it has been observed that O-alkylation of 2,4,6-trihydroxyacetophenone with benzyl chloride tends to be accompanied by considerable C-alkylation (Jain et al., 1972), and we

Y
O O
O OH
Y

6 Y = H

7 Y = $CH_2C_6H_5$

8 Y = $Si(CH_3)_2t\text{-}Bu$

$6 \xrightarrow{a} 8 \xrightarrow{b} 4 + 5$

a. $ClSi(CH_3)_2t\text{-}Bu$

b. RCO_2Et, NaH, Py

FIG. 2. Syntheses of compounds **4b, 4c, 5b,** and **5c.**

experienced difficulties obtaining pure samples of the desired dibenzyl ether **7.** We found, however, that **6** reacted smoothly at room temperature with a slight excess (i.e., 2.2 equiv.) of *t*-butylchlorodimethylsilane to give the diether **8** and that **8** satisfactorily underwent condensation with esters under the same conditions employed earlier (Figure 2). Deprotection (and some cyclization) of the initial condensation product occurred during the neutralization step of the workup, and mixtures of **4b** + **5b** or of **4c** + **5c** were obtained that were separated by column chromatography.

DISCUSSION

Although *S. rhododendri* secretes a larger number, and somewhat greater diversity, of components than does *S. pyrioides*, the compounds secreted by the two species are closely related and, in fact, at least two, and probably four (**2b, 3b;** and probably **2a** and **3a**), components are common to the two insects. The implications of the similarity will not be certain until other species in other genera are examined.

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ISOFLAVONOIDS AS INSECT FEEDING DETERRENTS AND ANTIFUNGAL COMPONENTS FROM ROOT OF *Lupinus angustifolius*

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Abstract—Crude extracts of the root of the resistant legume, *Lupinus angustifolius*, showed feeding deterrent activity to *Costelytra zealandica* and *Heteronychus arator* larvae. Nine 5-hydroxyisoflavones were isolated from an active fraction and their feeding deterrent activity and antifungal activity was measured. High feeding-deterrent activity was associated with high antifungal activity with some exceptions.

Key Words—*Costelytra zealandica, Heteronychus arator, Lupinus angustifolius*, insect resistance, feeding deterrent, antifungal activity, 5-hydroxyisoflavones.

INTRODUCTION

The isoflavan vestitol was isolated from the root of the resistant pasture legume *Lotus pedunculatus* Cav. and shown to be a potent feeding deterrent for third-instar larvae of the two pasture scarabs *Costelytra zealandica* (White) and *Heteronychus arator* Fabr. (Russell et al., 1978, 1979). Vestitol was already known as an antifungal phytoalexin from *Lotus corniculatus* L. (Bonde et al., 1973). An investigation of the effect on insect feeding of a range of isoflavonoids including other recognized phytoalexins (Russell et al., 1979; Sutherland et al., 1980) showed that antifungal activity and insect feeding-deterrent activity were closely associated, suggesting a dual defensive role for isoflavonoids in legumes against both insects and fungi.

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Lupin (*Lupinus*) species have been shown to be resistant to both *C. zealandica* and *H. arator* larvae (Farrell and Sweney, 1974; Farrell and Stufkens, 1977; Wallace, 1945), and we have investigated the possible role of plant-derived feeding deterrents in this phenomenon. Crude root extracts of *Lupinus angustifolius* cv. "Borre" significantly reduced feeding of *H. arator* larvae (Sutherland and Greenfield, 1978). We have found feeding of *C. zealandica* larvae as well as *H. arator* larvae to be significantly reduced by an extract of *L. angustifolius* cv. 'Uniharvest' root. This feeding deterrent activity has been investigated, a series of structurally related isoflavones isolated from active fractions, and their effects on insect feeding and fungal growth determined. The isolation of angustone A (VI) as a feeding deterrent has been briefly reported in a preliminary communication (Russell et al., 1979).

METHODS AND MATERIALS

Insect Feeding Assays. These were performed as described previously (Russell et al., 1978). Field-collected third-instar larvae of *C. zealandica* and *H. arator*, which had been starved for 24 hr, were enclosed individually in 5.2-cm-diameter Petri dishes with a 4% agar–4% cellulose powder disk (1.5 cm) containing a standard feeding stimulant (0.1 M sucrose and 0.01 M ascorbic acid for *C. zealandica*, and 0.1 M maltose for *H. arator*) plus the test fraction or compound. Feeding-deterrent activity was assessed by comparing 24-hr fecal pellet counts of larvae offered disks containing the test compound (TC) with similar counts of larvae offered disks containing feeding stimulants only (control = C) and of a third group offered blank disks (B) prepared with distilled water. Twenty larvae were tested with each medium. The results are presented as percentage feeding response defined as: $100 \times (TC - B)/(C - B)$ (May 1976). Crude extracts and fractions were incorporated in the feeding disks at a concentration equivalent to 10 g dry wt/50 ml medium, with the exception that chromatography fractions (Scheme 1) were tested with *H. arator* at 25 g dry wt equivalent/50 ml medium. Pure compounds, some of which were isolated in only limited quantities, were tested over a range of concentrations up to 200 μg/ml if possible. In all cases, the test material was added to the cellulose powder in solution, and the solvent removed by evaporation. Cellulose powder in control discs was pretreated with solvent.

Antifungal Assays. Antifungal activity in crude fractions was monitored by fungal spore germination assays on TLC plates. Eluted plates were sprayed with a suspension of conidia of the pigmented fungus *Cladosporium cladosporioides* (Fresen.) de Vries in a nutrient solution (Bailey and Burden, 1973) and examined after three days.

The antifungal activity of purified compounds was determined by sporeling growth assays (Skipp and Bailey, 1976) with *C. cladosporioides*, and *Colleto-*

trichum gloeosporioides (Penz.) Sacc. as an additional suitable test organism. These assays have been found to be useful for discriminating the relative antifungal activities of isoflavonoids, showing little variation in inhibitory concentration for a range of test fungi (Skipp and Bailey, 1977). Assays were performed using germinated spores of the test fungi as inoculum, supported on sterile, 4-mm-diameter disks of cellophane film. Disks were placed on the surface of a sucrose–acid casein hydrolysate medium (SCH, containing: sucrose 1.5%, Difco Casamino Acids 0.46%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.5%, agar 20%) in Petri dishes and were inoculated with 2-μl drops of test fungus spore suspension (50–100 spores per drop), and incubated for 20 hr at 20°C.

A range of concentrations of each test compound was prepared by placing appropriate volumes of an ethanolic solution of each (2000 μg/ml) in duplicate in the wells of Boerner Micro Test Slides using a micrometer syringe. Drops were air dried and the residues in the well redissolved in 2-μl drops of dimethyl sulfoxide (DMSO). Drops (0.1 ml) of liquid SCH medium (without agar) were added to the wells. The final concentration of DMSO in the medium was 2.0%, and the concentration ranges of the test compounds were: 0, 10, 20, 30, 40, and 50 μg/ml and 0, 40, 60, 80, and 100 μg/ml. Disks bearing sporeling inoculum were placed in the drops of test solution. Four disks were also mounted in lactophenol, and the lengths of 10 sporeling germ tubes on each disk were measured with an eyepiece micrometer. Sporelings were incubated at 20°C in test solutions for 24 hr; then 100 on each disk were examined to determine the percentage of sporelings which had grown (i.e., had germ tubes of greater length than the largest of the sporelings used as inoculum). The lowest concentration of each compound which prevented the growth of over 95% of sporelings (ED_{95}) was determined.

Extraction of Roots and Feeding-Deterrent Activity of Extract. Roots (3.1 kg dry wt) of 3-month-old field grown *Lupinus angustifolius* L. cv. "Uniharvest" (Barnard, 1972) were air dried (80°C), ground, and extracted with 95% EtOH in a Soxhlet for 48 hr, and the solvent was evaporated under reduced pressure. The crude extract was tested for insect feeding deterrent activity (Table 1). Feeding of *H. arator* larvae was significantly reduced by the extract, as found for *L. angustifolius* cv. "Borre" (Sutherland and Greenfield, 1978). Feeding of *C. zealandica* larvae was also significantly reduced.

Fractionation of Feeding-Deterrent Activity. The extract was fractionated by solvent partitioning and column chromatography, and the feeding-deterrent activity of each fraction monitored in the feeding assay. In preliminary testing, a partitioning scheme for lupin alkaloids (Ruiz, 1978) was used to separate an organic base fraction. Most of the activity did not partition into this fraction, but under neutral conditions partitioned into solvents of medium polarity. The partitioning scheme adopted is shown in Scheme 1 and fractions exhibiting feeding deterrent activity are indicated.

TABLE 1. EFFECT OF CRUDE EXTRACTS OF *Lupinus angustifolius* CV. "UNIHARVEST" ROOT ON FEEDING BY 3RD INSTAR *C. zealandica* AND *H. arator* LARVAE (TOTAL FECAL PELLETS PER 20 TEST INSECTS)

	Blank	Control	Control + extract	Feeding response (%)
C. zealandica	230	582	206	−6.8[a]
H. arator	160	669	284	24.2[a]

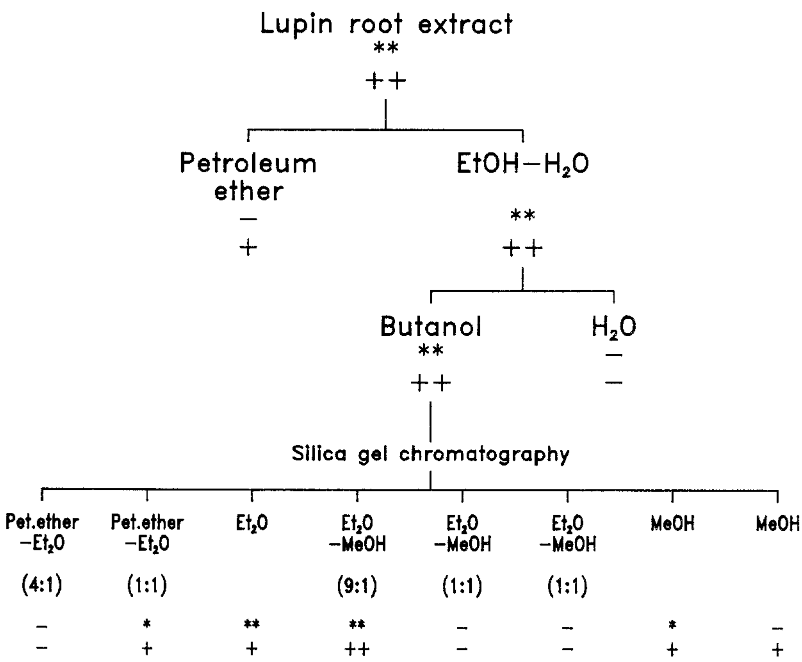
[a] Significant reduction in feeding: $P < 0.05$, Wilcoxon's rank sum test applied to untransformed counts.

The aqueous ethanol and subsequent butanol fractions showed high activity to *C. zealandica*. A similar pattern was observed for *H. arator*, with the petroleum ether fraction also showing some activity. The active butanol fraction was evaporated and eluted through silica gel (2 kg) with a sequence of solvents and fractions collected and tested as shown (Scheme 1). Major insect feeding-deterrent activity appeared to be concentrated in the Et_2O and Et_2O–MeOH (9 : 1) eluates, and these were investigated further.

Isolation of Compounds from Active Fractions. Separations were monitored by TLC on silica gel 60 F_{254} aluminum sheets (Merck) with $CHCl_3$–MeOH (19 : 1), pentane–Et_2O–HOAc (75 : 25 : 3), and petroleum ether–EtOAc (1 : 1); on cellulose sheets (Macherey-Nagel) with H_2O–HOAc (2 : 1), and polyamide (Wang) with acetone. Spots were visualized with UV light (254 nm and 366 nm) and by spraying with fast blue B salt solution, and antifungal components detected with the TLC fungal bioassay. HPLC was performed on Whatman 10-μm Partisil-ODS columns with MeOH–H_2O and UV detection at 254 nm.

A series of compounds (Fig. 1) was isolated for identification and bioassay from the Et_2O–MeOH (9 : 1) eluate and the Et_2O eluate of the initial silica gel column chromatography (Scheme 1). The Et_2O–MeOH (9 : 1) eluate was evaporated to dryness (8.38 g) and a portion (2.8 g) was chromatographed on silica gel with a $CHCl_3$–EtOH gradient. The EtOH fraction was rechromatographed on Sephadex LH-20 with $CHCl_3$–EtOH (1 : 1) to give III (151 mg).

The Et_2O eluate from the initial silica gel chromatography (Scheme 1) was evaporated to dryness (10 g) and rechromatographed on silica gel (5% H_2O) with a series of solvents (petroleum ether–Et_2O, 4 : 1, 3 : 1, and 1 : 1; Et_2O; Et_2O–MeOH, 9 : 1) to give several fractions from which pure compounds were isolated by rechromatography on silica gel and Sephadex LH-20. A petroleum ether–Et_2O (3 : 1) fraction (182 mg) was rechromatographed on silica gel with



SCHEME 1.

$CHCl_3$ and on Sephadex LH-20 with petroleum ether–CH_2Cl_2 (1 : 1) to give IX (28 mg). A further petroleum ether–Et_2O (3 : 1) fraction (431 mg) was rechromatographed on silica gel with $CHCl_3$ and with petroleum ether–Et_2O (3 : 1) to give VIII (71 mg).

Chromatography of the petroleum ether–Et_2O (1 : 1) fraction (3.85 g) on silica gel with $CHCl_3$–MeOH (19 : 1) and with petroleum ether–Et_2O (4 : 1) and on Sephadex LH-20 with petroleum ether–$CHCl_3$–EtOH (10 : 10 : 1) gave VII (114 mg) and VI as a gum (728 mg), a portion (50 mg) of which was further purified by HPLC and crystallized (12 mg) from petroleum ether–Et_2O.

The Et_2O fraction (3.36 g) was rechromatographed on silica gel with $CHCl_3$–Et_2OH (19 : 1) and on Sephadex LH-20 with petroleum ether–$CHCl_3$–EtOAc–EtOH (4 : 4 : 1 : 1) to give V (331 mg) and II (23 mg). The Et_2O–MeOH (9 : 1) fraction (1.12 g) was rechromatographed on silica gel with petroleum ether–Et_2O (1 : 1) and on Sephadex LH-20 with petroleum ether–$CHCl_3$–EtOH

Fig. 1. Lupin isoflavones.

(9:4:2) to give a solid (51 mg). Crystallization from EtOH-H_2O gave I (33 mg) and HPLC of the mother liquor gave IV (6 mg).

RESULTS

Identification of Isoflavones. Isoflavones were identified by comparison of the mp and spectroscopic properties with published data, and by TLC comparison with authentic samples where these were available. The determination of the structures of angustone A (VI), angustone B (VIII), and angustone (IX) is the subject of a separate report (Lane and Newman, 1986). Yields, shown as percent dry weight, are estimated total yields from TLC and HPLC of mixed fractions.

Genistein (I). Yield 0.03%; mp 304–306°C (decomp.) (lit. 300–301°C) (Bradbury and White, 1951); UV and EI-MS data in agreement with reported values (Ollis, 1962; Biggs, 1975). Coelutes with authentic sample (ICN Pharmaceuticals).

Wighteone (II). Yield 0.002%; mp 216–217°C (lit. 220–221°C) (Deshpande et al., 1977); UV, EI-MS, and [^{1}H]NMR data in agreement with reported values (Ingham et al., 1977; Deshpande et al., 1977).

2'-Hydroxygenistein (III). Yield 0.04%; mp 273–275°C (decomp.) (lit.

272 °C) (Whalley, 1957); UV and EI-MS in agreement with data reported by Biggs (1975). Coelutes with authentic sample (Dr. D. R. Biggs).

Luteone (IV). Yield 0.0005%; mp 222–223 °C (lit. 225–227 °C) (Fukui et al., 1973); UV and EI-MS data in agreement with these reported by Fukui et al. (1973). Coelutes with authentic samples (Professor H. Fukui, Dr. J. Ingham).

Licoisoflavone A (V). Yield 0.02%; mp 111–114 °C (lit. 111–113 °C) (Kinoshita et al., 1978); UV and EI-MS data in accord with reported values (Kinoshita et al., 1978; Woodward, 1979). Coelutes with authentic sample (Professor S. Shibata).

Angustone A (VI). Yield 0.06%; mp 159–160 °C; (Lane and Newman, 1986) (lit. 155–157 °C) (Tahara et al., 1984a).

Licoisoflavone B (VII). Yield 0.008%; UV, EI-MS and [^{1}H]NMR data in agreement with reported values (Ingham et al., 1983; Kinoshita et al., 1978). The mp (185–186 °C) differed from that reported by Kinoshita et al. (1978) (245–248 °C) for material prepared from V. However, acetylation (Ac_2O-pyr) gave a triacetate with mp 188–190 °C (lit. 186–188 °C) (Kinoshita et al., 1978), and UV, EI-MS MS, and [^{1}H]NMR data in agreement with these authors.

Angustone B (VIII). Yield 0.003%; mp 160–161 °C (Lane and Newman, 1986).

Angustone C (IX). Yield 0.001%; mp 170–180 °C (Lane and Newman, 1986).

Isolation of Wighteone (II) and Luteone (IV) from Lupinus luteus *Pods.* To provide additional material for biological testing, wighteone (II) and luteone (IV) were isolated from immature seed pods of *Lupinus luteus* cv. "Weiko" (Fukui et al., 1973; Ingham et al., 1977). Freeze dried pods and seeds (1.5 kg) were ground and extracted with MeOH in a Soxhlet for 60 hr. The extract was concentrated and partitioned (3×) between petroleum ether and 80% MeOH. The combined MeOH fraction was evaporated and partitioned (3×) between Et_2O and H_2O, and the combined Et_2O fraction evaporated and chromatographed on silica gel. Elution with petroleum ether–Et_2O (4 : 1) and rechromatography on silica gel (CH_2Cl_2–EtOH, 19 : 1) and Sephadex LH-20 (CH_2Cl_2–petroleum ether–EtOAc–EtOH, 3 : 4 : 2 : 1) gave wighteone (II) (19 mg). Elution with petroleum ether–Et_2O (1 : 1) and rechromatography on Sephadex LH-20 ($CHCl_3$–petroleum ether–EtOH, 2 : 2 : 1) gave luteone (IV) (38 mg).

Feeding-Deterrent and Antifungal Activity of Isolated Compounds. All nine isoflavones isolated were assayed for feeding-deterrent activity with *C. zealandica*, and six reduced feeding significantly at one or more concentrations (Table 2). Four of these, licoisoflavone B (VII), luteone (IV), 2-hydroxygenistein (III), and licoisoflavone A (V) reduced feeding significantly at a concentration of only 2 μg/ml (ppm). In the case of VII, IV, and III, higher concentrations reduced *C. zealandica* feeding very substantially.

Six of the isoflavones for which sufficient purified material was available

TABLE 2. EFFECT OF ISOLATED ISOFLAVONES ON INGESTION BY 3RD-INSTAR *Costelytra zealandica* AND *Heteronychus arator* LARVAE

	Feeding response (%)						
	C. zealandica concentration (μg/ml)				*H. arator* concentration (μg/ml)		
	2	20	100	200	10	100	200
Licoisoflavone B (VII)	45.6*[a]	28.6*	25.9*	−1.5*	58.4*	57.4*	50.9*
Luteone (IV)	37.4*	27.9*	4.0*		103.3	60.5*	64.7*
2′-Hydroxygenistein (III)	76.4*	72.6	38.7*	12.7*	100.4	54.6*	67.9*
Licoisoflavone A (V)	60.3*	69.8*	51.8*	68.5*	86.5	84.0	67.3*
Angustone A (VI)	93.9	85.9	76.2	35.9*			56.8*
Angustone B (VIII)	93.2	50.9	35.3*	63.5			
Angustone C (IX)	89.6	56.1					
Genistein (I)		85.6[b]	66.6	82.6	107.7	95.5	106.4
Wighteone (II)	77.4	63.9		67.1			

[a] Significant reduction in feeding: $P < 0.05$ Wilcoxon's rank sum test on untransformed data.
[b] 10 μg/ml.

TABLE 3. EFFECT OF ISOLATED ISOFLAVONES ON GROWTH OF *Colletrichum gloeosporioides* AND *Cladosporium cladosporioides* SPORELINGS

	ED_{95} (μg/ml)[a]	
	C. gloeosporioides	*C. cladosporioides*
Licoisoflavone B (VII)	20	20
Luteone (IV)	30	30
2′-Hydroxygenistein (III)	>100	>100
Licoisoflavone A (V)	30	30
Angustone A (VI)	>100[b]	>100
Angustone B (VIII)	>100	>100
Angustone C (IX)	>100	>100
Genistein (I)	>100	>100
Wighteone (II)	30	30

[a] See Methods and Materials.
[b] Complete inhibition of some sporelings and progressive inhibition of sporeling growth at concentrations >40 μg/ml.

were tested with *H. arator* (Table 2), and five of these showed significant feeding-deterrent activity. Each of these compounds was also active with *C. zealandica*. Only licoisoflavone B (VII) caused a significant reduction in feeding at concentration below 100 μg/ml, and in no case was the reduction in *H. arator* feeding with purified compounds comparable to that observed with the crude extract (Table 1).

All nine isoflavones were tested for antifungal activity against both *Colletotrichum gloeosporioides* and *Cladosporium cladosporioides* in the sporeling growth assay (Table 3) and showed a similar pattern of activity towards both fungi (Skipp and Bailey, 1977). Of the four compounds most active in the insect feeding-deterrent assays, three, licosoflavone B (VII), licosoflavone A (V), and luteone (IV), prevented growth of 95% of sporelings at 20, 30, and 30 μg/ml, respectively. No effect on sporeling growth was observed for 2′-hydroxygenistein (III) up to 100 μg/ml. Of the remaining five compounds, only wighteone (II) displayed distinct antifungal activity (ED_{95} 30 μg/ml), although some inhibition of the growth of *C. gloeosporioides* was observed with angustone A (VI).

DISCUSSION

The results show that, as in the case of *Lotus pedunculatus* (Russell et al., 1978), isoflavonoids with high insect feeding deterrent activity are present in crude root extracts of *Lupinus angustifolius* and suggest these compounds may play a role in the resistance of this plant to *C. zealandica* and *H. arator*.

The resistance of *Lupinus* spp. to foliar-feeding insects has, in several cases, been linked to the alkaloid complement (Bentley et al., 1984; Cantot and Papineau, 1983; Wink et al., 1982; and references cited). However, alkaloids do not appear to be implicated in the resistance of *L. angustifolius* to these root-feeding insects. Low-alkaloid cultivars such as "Uniharvest" (Barnard, 1972; Ruiz, 1978) as well as high-alkaloid cultivars such as "Borre" have been found to be resistant to *C. zealandica* (Farrell and Sweney, 1974; Farrell and Stufkens, 1977) and to show feeding-deterrent activity to *H. arator* (Sutherland and Greenfield, 1978, and this study), and this is reflected in the partitioning of the feeding-deterrent activity.

This study also provides further evidence for a dual defensive role for isoflavonoids in legume roots. Prenyl isoflavones are well-established antifungal components in *Lupinus* spp. Wighteone (II) and luteone (IV) have been reported as constitutive antifungal components from both the aerial parts and roots of a number of *Lupinus* spp., including *L. angustifolius* cv. "Beliak" (Fukui et al., 1973; Harborne et al., 1976; Ingham et al., 1977, 1983), often cooccurring with the parent isoflavones genistein (I) and 2′-hydroxygenistein

(III). In detailed studies of *L. albus* root extracts, licoisoflavone A (V), angustone A (VI) (= 2′-hydroxylupalbigenin), licoisoflavone B (VII), the chromenylisoflavones parvisoflavone B and alpinumisoflavone, the diprenylisoflavone lupalbigenin, and six related dihydrofuranoisoflavones have been isolated as antifungal components in addition to I–IV (Ingham et al., 1983; Tahara et al., 1984a). While all these compounds have been reported to show antifungal activity in TLC plate bioassays with *Cladosporium herbarum* (Ingham et al., 1983; Tahara et al., 1984a), the quantitative sporeling growth bioassay reported here was more discriminatory. Of the nine isoflavones tested, high activity (ED_{95} below 100 μg/ml) was limited to four (II, IV, V, and VII; Table 3). These compounds were also highly active in the TLC bioassay, although not with the same relative order of activities (Tahara et al., 1984a).

The isoflavonoid complement of *Lupinus* spp. roots thus appears to be implicated in resistance to both insects and fungi, and three isoflavones (IV, V, and VII) show the association of high antifungal activity with insect feeding deterrent activity we have observed with other isoflavonoids (Sutherland et al., 1980). However, for some compounds, the biological activity is more specific. Wighteone (II) shows high antifungal activity, but lacks significant feeding-deterrent activity at the concentrations tested, while 2′-hydroxygenistein (III) is an active feeding deterrent but is inactive in the sporeling growth bioassay. These results show the structural requirements for activity against insects and fungi are not identical, despite the strong association.

High antifungal activity of the lupin isoflavones is restricted to the monoprenylated compounds. The parent isoflavones show low activity, as do the polar dihydrofuranoisoflavones with hydroxylated side chains found in *L. albus* (Tahara et al., 1984a) and other products of prenyl side-chain oxidation formed as fungal detoxification products from luteone (IV) (Tahara et al., 1984b) and licoisoflavone A (V) (Tahara et al., 1985). The higher antifungal activity of the prenylated compounds has been attributed to their higher lipophilicity (Harborne et al., 1976; Ingham et al., 1977). However, the diprenyl isoflavones, although more lipophilic than the active monoprenyl compounds, show low antifungal activity (Tahara et al., 1984a, and this study). While the bioassay conditions may not adequately reflect the in vivo activity of the water-insoluble diprenyl compounds, or include the relevant test organisms, these findings do suggest there is an optimum lipophilicity for antifungal activity. It is also worth noting that the active compounds all possess an amphiphilic structure with distinct lipophilic and polar regions.

Insect feeding-deterrent activity in these isoflavones is associated with a 2′-hydroxygenistein (III) nucleus. Licoisoflavone B (VII), which showed activity towards both insects at low concentrations (Table 2), possesses a ring B-fused dimethylpyranyl ring as found in the highly active isoflavonoid feeding deterrents phaseollin and phaseollinisoflavan from bean roots (Sutherland et al.,

1980). While the parent compound (III) and the monoprenyl derivatives (IV, V, and VII) showed feeding-deterrent activity to *C. zealandica* at low concentrations, the additional prenylation found in angustones A (VI) and B (VIII) is association with reduced feeding-deterrent activity, as observed with antifungal activity. The relationship between isoflavonoid structure and feeding-deterrent activity towards *H. arator* is not clearly defined. The activity of isoflavonoids as *C. zealandica* feeding deterrents has been shown to be very dependent on their molecular shape and the arrangement of polar and lipophilic groups in the molecule (Lane et al., 1985).

From these considerations it may be seen that while the structural features for antifungal activity and feeding activity may not be identical, they coincide in IV, V, and VII. Although these compounds show activity against both types of test organism, this does not define a dual defensive role. A full assessment of the role of lupin isoflavonoids in the defense of the plant root system would require that the isoflavonoid complement be considered as a whole, taking into account the scope and level of biological activity of each component, its abundance and location in the plant (Barz and Hoesel, 1979), and possible induction by disease organisms (Ingham and Dewick, 1980) or insect feeding. As a first approximation, considering the concentrations at which insect feeding was reduced (Table 2), and the estimated total yields (Results), licoisoflavone B (VII) and, for *C. zealandica*, 2′-hydroxygenistein (III) and licoisoflavone A (VI) appear to be the most significant insect feeding deterrents isolated. On a similar basis, licoisoflavone A (VI) appears to be the most significant antifungal compound.

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ISOLATION AND IDENTIFICATION OF APOLAR METABOLITES OF INGESTED 20-HYDROXYECDYSONE IN FRASS OF *Heliothis virescens* LARVAE

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Abstract—A large amount of 20-hydroxyecdysone was orally administered to larvae of the tobacco budworm, *Heliothis virescens*, in order to investigate its detoxification mechanisms. Four major relatively nonpolar metabolites were isolated from their frass. These compounds were identified as the 22-linoleate, 22-palmitate, 22-oleate, and 22-stearate of 20-hydroxyecdysone using various forms of spectroscopy, including NMR. This is the first report of this type of metabolite from an insect.

Key Words—20-Hydroxyecdysone, feeding, insect, metabolites, conjugates, *Heliothis virescens*, feces.

INTRODUCTION

Current research in our laboratory is centered around the chemical basis of insect–plant relationships and particularly the physiological effects of phytoecdysteroids. We have isolated several phytoecdysteroids from various tropical plant species which are known to be resistant to insect attack (Kubo et al., 1983a, 1984). One of the most abundant and widespread phytoecdysteroids is 20-hydroxyecdysone (Bergamasco and Horn, 1983), which is also the moulting hormone in insects themselves. Recent studies in our laboratory demonstrated that small amounts of this ecdysteroid consumed with diet can inhibit growth and ecdysis in larvae of the lepidopterous species *Pectinophora gossypiella* (pink bollworm), *Spodoptera frugiperda* (fall armyworm), and *Bombyx mori* (silkworm) (Kubo et al., 1983b). The inhibition of ecdysis, which can occur through

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disruption of the normal titer of 20-hydroxecdysone, results in failure to complete ecdysis after normal apolysis and confines the insect in the pharate condition (Kubo and Klocke, 1986). This abnormal pharate condition prevents feeding, excretion, and locomotion and eventually results in death of the affected insect.

In contrast, certain insect species are apparently able to survive on plants containing large amounts of phytoecdysteroids, such as the moth *Milonia vasalis* which feeds on leaves of *Podocarpus macrophyllis* (Hikino and Takemoto, 1974). Kubo et al. (1981) found that larvae of the bollworm complex, *Heliothis zea* and *H. virescens*, are not affected by high concentrations of 20-hydroxyecdysone in their diet. Clearly, these insects must have a defensive mechanism which operates against disruption of the hormonal coordination. Therefore, we examined the physiological basis of this phytoecdysteroid resistance in one such insect, the tobacco budworm, *H. virescens*, by studying the metabolic fate of the ingested phytoecdysteroid.

We describe here the isolation and structural elucidation of several relatively nonpolar metabolites of ingested 20-hydroxyecdysone from frass of tobacco budworm larvae.

METHODS AND MATERIALS

Insects. Larvae of the tobacco budworm, *Heliothis virescens* (Fabricius) (Lepidoptera, Noctuidae) were reared from eggs obtained from a culture maintained at the U.S.D.A. at Stoneville, Mississippi. Upon hatching, larvae were reared individually on an artificial diet (Chan et al., 1978) in plastic containers at 28°C and at 16:8 light–dark cycle. The phytoecdysteroid-containing diet is prepared by adding a solution of a known concentration of ecdysteroid to the cellulose part of the diet. After evaporation of the solvent, the cellulose laced with the ecdysteroid is mixed with the other ingredients of the diet to form the test diet. Control diet is made similarly except for the addition of the ecdysteroid.

Newly hatched larvae ($N = 50$) were reared on an artificial diet containing 1000 ppm 20-hydroxyecdysone through the fifth instar. Fecal material of larvae fed on test and control diet was collected regularly and stored in methanol at 0°C. The fecal material of these larvae, as well as the diets themselves, were analyzed for the presence of metabolites of 20-hydroxyecdysone. These samples were homogenized in methanol, and the filtrate was evaporated to dryness and partitioned between *n*-hexane and water. The aqueous layer was extracted with ethyl ether. The ethyl ether layer was dried over Mg_2SO_4, filtered, and evaporated to dryness. This fraction was purified by C_{18} reversed-phase medium-pressure liquid chromatography on a Pharmacia SR10/50 column packed with hand-made 40 μm ODS. The mobile phase used was methanol–water (9:1),

delivered with a Pharmacia P-3 peristalic pump at a flow rate of 1 ml/min. Fractions (5 ml) were collected, and an aliquot of each fraction was injected into the HPLC system to check their purity.

Chemicals. 20-Hydroxyecdysone had previously been isolated from various tropical medicinal plants (Kubo et al., 1983a, 1984). Solvents used were of reagent or HPLC grade. A Sep-pak C_{18} cartridge (Waters Assoc., Milford, Massachusetts) was used for the preliminary purification of the HPLC samples.

Analysis. Analyses were carried out with an Eyela PLC-5 liquid chromatograph system (Tokyo Rikakikai, Tokyo, Japan). A YMC pack ODS column (15 cm × 6 mm) (Yamamura Chemical, Kyoto, Japan), equipped with Uptight precolumn (2 cm × 2 mm) (Upchurch Scientific, Oak Harbor, Washington) and packed with pellicular ODS (Pell, Whatman Co.), was used. The mobile phase used was methanol–water (9:1) at a flow rate of 1.25 ml/min. The effluent was monitored by built-in UV detector at 254 nm.

Identification. Isolated compounds were analyzed by several spectroscopic techniques. Ultraviolet (UV) spectra were recorded on a Hitachi 100-80 spectrophotometer. Infrared (IR) spectra were recorded on a Perkin-Elmer 1310 IR spectrophotometer. Secondary ionization mass spectra (SI-MS) were obtained on a Hitachi RMU-6MG apparatus. Proton nuclear magnetic resonance ([^{1}H]NMR) spectra were determined on a Nicolet NT-300 spectrometer.

RESULTS AND DISCUSSION

To examine the metabolic fate of ingested phytoecdysteroids in larvae of *H. virescens*, fecal material was collected from larvae reared on artificial diet containing 1000 ppm 20-hydroxyecdysone. The following four samples were chromatographically analyzed; I, control diet; II, test diet (containing 1000 ppm of 20-hydroxyecdysone); III, control feces (obtained from insects feeding on the control diet); and IV, test feces (obtained from insects feeding on the test diet). The resulting chromatograms are illustrated in Figure 1. Four compounds appearing in chromatogram IV, M-1, M-2, M-3, and M-4, were not found in the other three samples. 20-Hydroxyecdysone appeared in the chromatogram of II (retention time: 3.0 min). Chromatogram IV also showed 20-hydroxyecdysone. These results suggest that the four major components of sample IV, M-1, M-2, M-3 and M-4, are metabolites of 20-hydroxyecdysone. All four metabolites, M-1 (7.3 mg), M-2 (10.5 mg), M-3 (8.5 mg), and M-4 (3.4 mg), were obtained in large enough quantities to perform various spectroscopic studies.

Spectroscopic data of M-1: UV (EtOH) λ_{max} 242 nm; IR ($CHCl_3$) 1715, 1654 cm^{-1}; SI-MS *m/z* 743 $(M + 1)^+$, 725 (743-H_2O), 707 (743-2H_2O), 689 (743-3H_2O), 463 (743-fatty acid), 445 (463-H_2O), 427 (463-2H_2O), 409 (463-3H_2O), 353 (409-C_4H_8), and 301 (ecdysone nucleus); [^{1}H]NMR ($CDCl_3$) δ 0.85

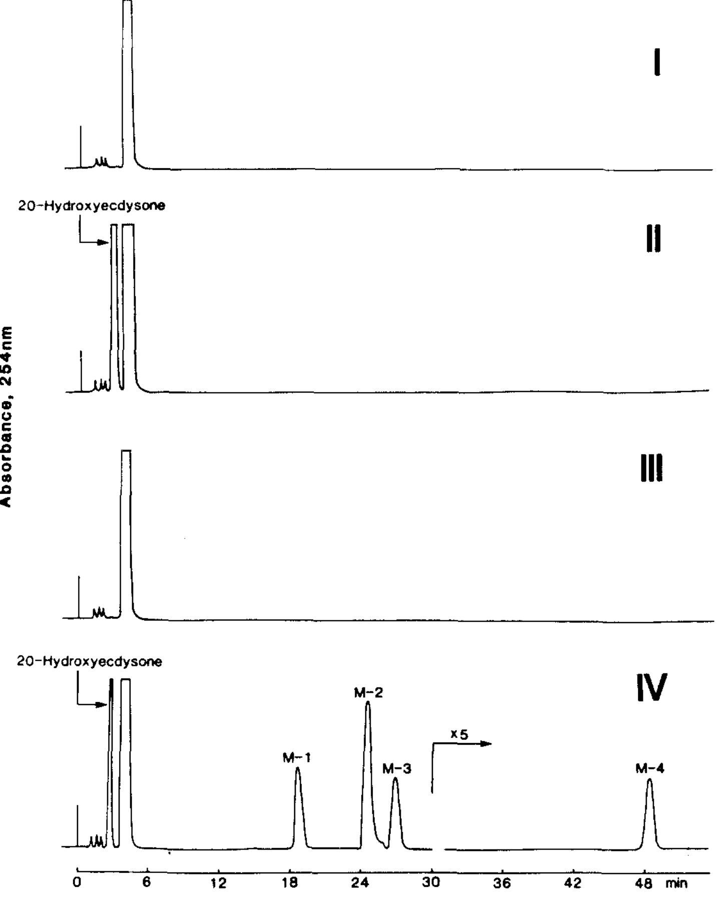


FIG. 1. HPLC of I, control diet; II, test diet (containing 1000 ppm of 20-hydroxyecdysone); III, control feces (obtained from insects feeding on the control diet); and IV, test feces (obtained from insects feeding on the test diet).

(3H, s, 18-CH_3), 0.89 (3H, t, J = 6.7, terminal-CH_3), 0.97 (3H, s, 19-CH_3), 1.20 (3H, s, 21-CH_3), 1.24–1.26 (6H, 2s, 26, 27-CH_3), 2.37 (2H, t, J = 7.0, —O—CO—CH_2—), 2.42 (1H, m, 5β-H), 2.77 (2H, t, J = 5.8, 11′-CH_2), 3.01 (1H, m, 9-H), 3.86 (1H, m, 2-H_{ax}), 4.02 (1H, m, 3-H_{eq}), 4.86 (1H, d, J = 9.4, 22-H), 5.34 (4H, m, 9′, 10′, 12′, 13′ olefinic Hs), 5.84 (1H, d, J = 1.6, 7-H).

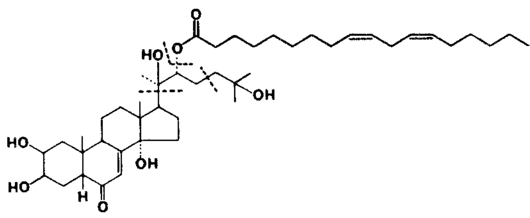
M-2: UV (EtOH) λ_{max} 242 nm; IR ($CHCl_3$) 1715, 1654 cm^{-1}; SI-MS m/z 719 $(M + 1)^+$, 701 (719-H_2O), 683 (719-2H_2O), 665 (719-3H_2O), 463 (719-fatty acid), 445 (463-H_2O), 427 (463-2H_2O), 353 (409-C_4H_8), and 301 (ecdysone nucleus); [^{1}H]NMR ($CDCl_3$) δ 0.86 (3H, s, 18-CH_3), 0.88 (3H, t,

J = 7.0, terminal-CH_3), 0.98 (3H, s, 19-CH_3) 1.24–1.25 (6H, 2s, 26, 27-CH_3), 2.37 (2H, t, J = 7.1, —O—CO—CH_2), 2.43 (1H, m, 5β-H), 3.00 (1H, m, 9-H), 3.88 (1H, m, 2-H_{ax}), 4.04 (1H, m, 3-H_{eq}), 4.89 (1H, d, J = 9.4, 22-H), 5.85 (1H, d, J = 1.7, 7-H).

M-3: UV (EtOH) λ_{max} 242 nm; IR ($CHCl_3$) 1715, 1654 cm^{-1}; SI-MS m/z 745 $(M + 1)^+$, 727 (745-H_2O), 709 (745-2H_2O), 691 (745-3H_2O), 463 (745-fatty acid), 445 (463-H_2O), 427 (463-2H_2O), 353 (409-C_4H_8), and 301 (ecdysone nucleus); [^{1}H]NMR ($CDCl_3$) δ 0.86 (3H, s, 18-CH_3), 0.88 (3H, t, J = 7.0, terminal-CH_3), 1.21 (3H, s, 21-CH_3), 1.24 (6H, s, 26, 27-CH_3), 2.37 (2H, t, J = 7.1, —O—CO—CH_2), 2.43 (1H, m, 5β-H), 3.00 (1H, m, 9-H), 3.88 (1H, m, 2-H_{ax}), 4.04 (1H, m, 3-H_{eq}), 4.89 (1H, d, J = 9.4, 22-H), 5.34 (2H, m, 9′, 10′ olefinic Hs), 5.85 (1H, d, J = 1.7, 7-H).

M-4: UV (EtOH) λ_{max} 242 nm; IR ($CHCl_3$) 1715, 1654 cm^{-1}; SI-MS m/z 747 $(M + 1)^+$, 729 (747-H_2O), 711 (747-2H_2O), 693 (747-3H_2O), 463 (747-fatty acid), 445 (463-H_2O), 427 (463-2H_2O), 409 (463-3H_2O), 353 (463-C_4H_8), and 301 (ecdysone nucleus); [^{1}H]NMR ($CDCl_3$) δ 0.86 (3H, s, 18-CH_3), 0.88 (3H, t, J = 6.7, terminal-CH_3), 0.99 (3H, s, 19-CH_3), 1.21 (3H, s, 21-CH_3), 1.23 (6H, s, 26, 27-CH_3), 2.37 (2H, t, J = 7.2, O—CO—CH_2—), 2.44 (1H, m, 5β-H), 3.00 (1H, m, 9-H), 3.89 (1H, m, 2-H_{ax}), 4.05 (1H, m, 3-H_{eq}), 4.88 (1H, d, J = 9.4, 22-H), 5.85 (1H, d, J = 1.6, 7-H). Typical SI-MS and [^{1}H]NMR spectra are illustrated in Figures 2 and 3.

The UV and IR spectra of all four compounds were quite similar. The presence of ester was suggested by the IR absorption at 1715 cm^{-1}. The SI-



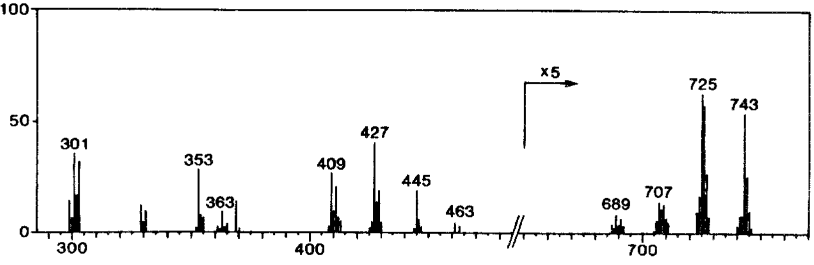


FIG. 2. Secondary ionization mass spectrum of M-1

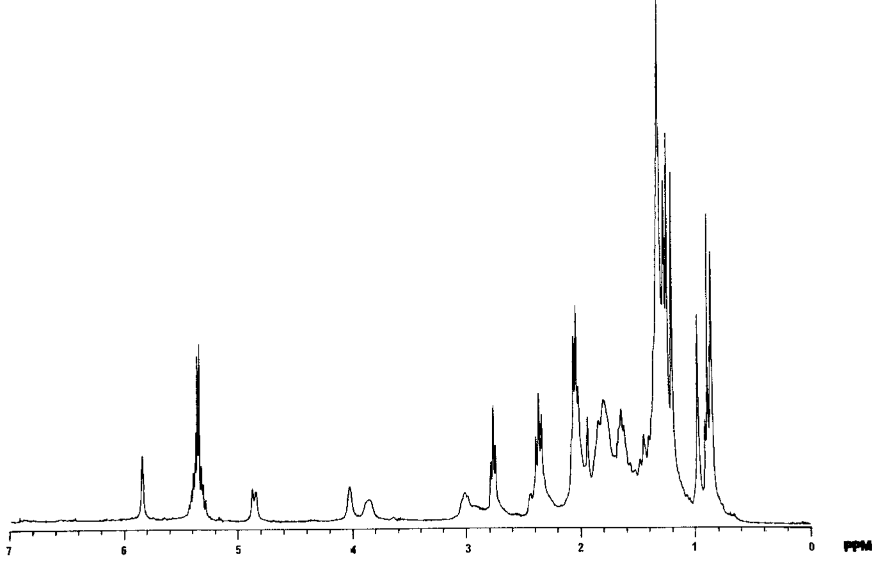


FIG. 3. 300-MHz [^{1}H]NMR spectrum of M-1 in $CDCl_3$.

MS fragment patterns for all four compounds were almost the same below *m/z* 463. The main fragmentations of 20-hydroxyecdysone usually occur at C-17/20 (*m/z* 301) and C-20/22 (*m/z* 363)(Nakanishi, 1971). An intense peak at *m/z* 301 could also be seen in the SI-MS of all four compounds. However, all SI-MS had a peak at *m/z* 363 of quite low intensity. It appeared to be replaced by an intense peak at *m/z* 353, which is not found in the SI-MS of 20-hydroxyecdysone. Since the retained *m/z* 301 peak is believed to result from cleavage of the C-17 to C-20 bond, we believe its presence suggested any ester group present must be on the side chain. The absence of the *m/z* 363, representing cleavage of the C-20 to C-22 bond, suggests that the ester group could be placed on the hydroxyl at C-22 in all four compounds. Each acyl group: M-1, $C_{18:2}$, M-2, C_{16}, M-3, $C_{18:1}$ and M-4, C_{18} fatty acids, could be conjectured due to each $(M + 1)^{+}$-463.

A comparison of the [^{1}H]NMR spectra of M-1, M-2, M-3, and M-4 with the previously assigned [^{1}H]NMR spectrum of 20-hydroxyecdysone showed strong similarities between the spectra (Kubo et al., 1985). However, there was a shift of the H-22 signal from the value in 20-hydroxyecdysone of δ 3.32 to a new value of δ 4.86 in all four compounds. The order of magnitude of this shift is consistent with an esterification of the hydroxyl group at C-22. Also, an HPLC analysis of metabolites hydrolyzed with 0.006% NaOH in ethanol coincided with 20-hydroxyecdysone. A long-range coupling between H-7 (δ 5.85) and H-9 (δ 3.00) was seen in the two-dimensional contour plot.

Typical proton signals of fatty acids, δ 0.88 and δ 2.37, appeared in all four [^{1}H]NMR spectra. These resonances were assigned to terminal methyl and 2′-methylene in their fatty acid ester side chains. The resonances at δ 2.77 and δ 5.35 in the spectra of M-1 and at δ 5.34 in the spectra of M-2 coincided with previously reported [^{1}H]NMR shifts and coupling patterns of the olefinic proton signals of methyl linoleate and methyl oleate respectively (Hashimoto et al., 1965).

Hence the structure of the metabolites were determined to be the 20-hydroxyecdysone-22-fatty acid esters; M-1: linoleate, M-2: palmitate, M-3: oleate, and M-4: stearate (Scheme 1).

Much of the metabolism of ecdysteroids in insects is well documented (Koolman, 1982). Several metabolic pathways have been reported in several

M-1

M-2

M-3

M-4

SCHEME 1. Structures of metabolites in the feces of *H. virescens*.

species of Lepidoptera including: *Antheraea polyphemus* (Cherbas and Cherbas, 1970), *Bombyx mori* (Moriyama et al., 1970; Hikino et al., 1975), *Hyalophora cecropia* (Gorell et al., 1972), *Prodenia eridania* (Yang and Wilkinson, 1972), *Manduca sexta* (King, 1972), *Choristoneura fumiferana* (Lagueux et al., 1976), and *Pieris brassicae* (Lafont et al., 1983). According to these investigations, the metabolism of ecdysteroids usually occurs by hydroxylation at C-20; epimerization at C-3; dehydration at C-3; acetylation at 3-OH; conjugation to sulfate esters, phosphate esters, glucosides, and glucuronides; and conversion to 26-oic acids. Recently, apolar metabolites of 20-hydroxyecdysone were found using ^{3}H-labeled 20-hydroxyecdysone in feeding experiments with ticks (Connat et al., 1984; Wigglesworth et al., 1985). The structures of apolar metabolites from tick whole-body extracts were identified by hydrolyzed fatty acid analysis, mass spectrometry, and by comparison to a synthetic 20-hydroxyecdysone-22-palmitate (Diehl et al., 1985). Apolar metabolites have been investigated from the cockroach *Periplaneta americana* (Slinger et al., 1986) and other arthropods (Connat and Diehl, 1986). However, their structures remain unidentified at this time.

The compounds isolated in the present study were the same as those previously reported in whole-body extracts of ticks (Diehl et al., 1985). However, this is the first report of this type of metabolite from an insect. These metabolites are found in fecal material at relatively high concentrations: approximately 16% of ingested 20-hydroxyecdysone.

Several metabolic studies of ecdysteroid metabolism in lepidopterous larvae have been carried out by injecting ^{3}H-labeled ecdysteroids into the insect body. However, the oral administration of ecdysteroids must be considered when examining phytoecdysteroids in insect–plant relationships. It has been pointed out that the hydroxy function at C-22 in ecdysteroids is essential for hormonal activity (Sorm, 1974). Synthetic 22-deoxy-20-hydroxyecdysone (Galbraith et al., 1969) and 2,22-dideoxy-20-hydroxyecdysone (Ikekawa et al., 1980) are about 10–100 times less active than 20-hydroxyecdysone. The presence of phosphate or adenosinemonophosphate esters on the 22-hydroxy of ecdysteroids also reduces the biological activity (Sall et al., 1983). This suggests the esterification at the 22-hydroxy group with fatty acids to be a detoxification mechanism in *H. virescens* against ingested 20-hydroxyecdysone. Research is in progress to examine the mode of action of this defensive mechanism.

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VARIATION IN HOST FRUIT VOLATILES ATTRACTIVE TO APPLE MAGGOT FLY, *Rhagoletis pomonella*

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Abstract—We conducted a comparative study of volatiles produced by whole *Crataegus* hawthorn fruit and four cultivars of apple (Royal Red Delicious, Red Astrachan, McIntosh, and Wealthy) and determined quantitative and qualitative changes of volatiles associated with fruit ripening. Within the approximate range of the GLC fraction known to elicit behavioral activity in the apple maggot fly, *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae), 52 esters were identified.

Key Words—Apple volatiles, apple maggot fly, *Rhagoletis pomonella*, Red Astrachan, Royal Red Delicious, Wealthy, McIntosh, hawthorn, *Crataegus*.

INTRODUCTION

Control of the apple maggot fly, *Rhagoletis pomonella* (Walsh), is currently accomplished by organophosphate applications three to five times a season (Neilson et al., 1976; Reissig et al., 1982). This is purely a preventive method, with no regard for the presence of flies in the orchard. Although insecticide applications keep indigenous populations low, commercial fruit often can be infested by flies that have immigrated from external sources, such as abandoned orchards, wild apple, or hawthorn (*Crataegus* spp.).

Development of a sensitive trap that allows detection of low numbers of immigrating flies could ultimately reduce the number of insecticide applications as well as enhance the timing of such applications. Additionally, because of the apple maggot's recent invasion of western states, especially California (Joos et al., 1984), sensitive traps may be crucial for detection of the fly's spread into uninfested and quarantine regions.

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To date, the most effective trap for detecting immigrating flies incorporates visual and olfactory stimuli from the fly's host fruit and is a sticky-coated red sphere combined with six esters characterized in volatiles collected from stored Red Delicious apples. The esters are hexyl acetate, butyl 2-methylbutyrate, propyl hexanoate, hexyl propionate, butyl hexanoate, and hexyl butyrate (Fein et al., 1982; Reissig et al., 1982). Field studies showed that there is an optimum range of release rates for these volatiles (Reissig et al., 1982).

Using these traps, Reissig et al. (1982) found in preliminary tests that the relative effectiveness of volatile-baited traps to unbaited traps varied not only among different orchards containing different cultivars, but also within orchards throughout the season as fruit matured. These results suggest that natural apple odor may compete with the synthetic blend due to greater attractiveness, concentration, or both. Indeed, early-ripening, sweet cultivars are frequently more heavily infested by *R. pomonella* than other cultivars, and for years researchers and orchardists have suggested that the type or amount of odor emitted by these apples is particularly attractive to the flies (O'Kane, 1914; Brittain and Good, 1917; Porter, 1928; Bush 1966; Prokopy et al., 1973).

Prompted by these anecdotal and experimental observations, we conducted a comparative study of volatiles produced by early (Red Astrachan), early-intermediate (Wealthy), intermediate (McIntosh), and late-ripening (Royal Red Delicious) apple cultivars. We also collected volatiles from a species of *Crataegus* because hawthorns are the native host fruit of *R. pomonella* and are very readily attacked by the fly (Prokopy et al., 1982). For each fruit type, we determined quantitative and qualitative changes of volatiles associated with fruit ripening. This work may provide a foundation for the development of more attractive blends for monitoring traps and, possibly, for development of optimum blends for use at different stages of fruit maturation.

METHODS AND MATERIALS

Collection of Fruit Volatiles. We collected apple volatiles in the manner described by Fein et al. (1982). For each sample, we picked 20–40 apples just prior to volatile collection, removed stems and leaves, and dried wet apples with a paper towel. Apples were placed in a foil-lined desiccator, which was washed and rinsed with acetone between collections. Using a vacuum pump, air was pulled through a purifying column of Porapak Q, then through the desiccator and onto a column (18 cm long, 0.7 cm diameter) of Ambersorb XE-348 beads (Rohm and Haas). Prior to use, the beads were washed in a Soxhlet extractor for 24 hr with a 3 : 1 chloroform–methanol wash, and then oven-dried.

Collections were made for 3 hr from June 11 to September 2, and for 24 hr from September 5 until November 19, 1983. Methyl decanoate was used as

an internal standard by injecting 35 μg in 2 μl of Skelly B onto the glass wool at the inlet of the column of beads. Air was pulled through the system for an additional 10 min. After this time, the column was attached to a 10-ml syringe and washed by pulling 8 ml of redistilled Skelly B back and forth, ten times, over the beads. The extracts were kept in Teflon-lined, screw-capped glass vials until analyzed. For volatile collections, we picked fruit from four apple cultivars that mature at different times: Red Astrachan (early), Wealthy (early-intermediate), McIntosh (intermediate), and Royal Red Delicious (late), and also from hawthorn (*Crataegus coccinea* L.). All fruit of like type were picked from a single tree. Hawthorn volatiles were collected and treated in the same manner as were the apple volatiles, except the mass of fruit was determined in grams and collections were made for 48 hr.

Sampling began on June 11, 1983, and continued every week until no apples remained on the trees. Hawthorns were collected from the middle of August 1984, again until no fruit remained on the tree. Fruit were randomly picked from all sections of the trees.

A separate test was made to determine if there were any qualitative or quantitative differences in volatile production by picked versus unpicked apples. To accomplish unpicked apple collections, the entire volatile collection apparatus was carried to the field. For both picked and unpicked apples, a glass funnel large enough to completely surround the apple was placed beneath the fruit and was attached to a pump and column of beads as described above. Volatile samples from picked apples were collected immediately following harvest. Within the examined range of volatiles, the samples collected did not vary substantially in volatile composition or concentration.

Analysis of Samples. From all of the samples collected and analyzed by GLC, we chose three time points from each fruit type for further analysis: a time well before fruit ripeness, an intermediate time, and when the fruit were ripe.

Two to three microliters of each sample were injected (splitless) onto a fused silica cross-linked methyl silicone capillary column that was programmed after a 1-min hold at 80°C to 180°C at 10°/min. At the beginning of each day, a hydrocarbon standard series (7–18 carbons) and a standard apple ester blend were run. In order to correct for day-to-day variations, all retention times were converted to Kovats' retention indices. A preliminary identity of each component was made by comparing the retention indices of the analyzed sample with those of the standard. Mass spectra were also obtained on each component with an HP-5985 GC-MS interfaced with a 20-m OV-101 glass capillary column.

Most synthetic samples were obtained from commercial sources, but if not available, were synthesized via established methods (Vogel, 1961) and purified by distillation. Mass spectral and GLC retention time data were collected on all synthetic esters.

Data Analyses. The absolute quantity of each component of a particular collection was obtained by dividing the peak area of the component with that of the internal standard and multiplying by the amount of the external standard (35 μg). For apple collections, each sum was standardized by converting all values to represent the amount released by 30 apples in 24 hr. For hawthorn, values represent the amount released by 500 g of fruit in 24 hr. The data were graphed as a simulated GLC tracing. If components came off as one peak, they were separated in the simulated tracing to show the relative amounts as determined by the GC-MS analyses.

RESULTS

Analysis was limited to the approximate range of esters found in the active GLC fraction of Fein et al. (1982) (approximately 5–12 carbon esters) because of the great number of volatiles present in the samples. The combined GLC and mass spectral data were used to identify 31 esters in the hawthorn extract and 48 in the apple extracts. Most identifications were straight-chain or 2-methyl esters that ranged from methyl to hexyl for the alcohol portion and propyl through octyl for the acid portion (Table 1).

Because few (Red Delicious) or no (all other cultivars) compounds were detected in the first volatile collections of the apple cultivars (taken well before fruit ripening), only analyses of the latter two collections are presented. Analyses of volatile collections taken one to two months prior to apple ripening are shown in Figure 1; analyses of collections from ripe apples are shown in Figures 2 and 3. For hawthorn, because no compounds were detected until the fruit were ripe in September, only analysis of the final collection is presented (Figure 3).

Except for the hexyl acetate peak in Wealthy, the ethyl hexanoate peak in Red Astrachan, and several minor peaks, the esters identified from unripe apple extracts were very similar (Figure 1). Each contained butyl hexanoate, hexyl butyrate, hexyl 2-methylbutyrate, and hexyl hexanoate. All but the Red Delicious extract contained butyl 2-methylbutyrate and butyl octanoate.

Regarding volatile composition of the extracts collected when the apples were ripe (Figures 2 and 3), all contained a butyrate and 2-methylbutyrate series. In comparison to the Wealthy extract, which contained a single dominant (60%) component (hexyl 2-methylbutyrate), or McIntosh, which contained only several major components, the Red Astrachan and Red Delicious extracts were more complex with an important series of hexanoates as well as a few octanoates.

In ripe hawthorn extracts, straight-chain hexanoates and octanoates were the major components (Figure 3). Hexyl hexanoate was the dominate (40%) ester. Unlike apples, 2-methyl esters were unimportant, but like most of the

TABLE 1. VOLATILES PRESENT IN RED ASTRACHAN, WEALTHY, RED DELICIOUS, AND MCINTOSH APPLE CULTIVARS AND *Crataegus coccinea* HAWTHORNS[a]

Peak number	Retention index	Compound	Peak number	Retention index	Compound
1	782	Ethyl butyrate	27	1135	Isobutyl hexanoate
2	800	Propyl propionate	28	1135	Hexyl isobutyrate
3	800	Butyl acetate	29	1177	Butyl hexanoate
4	833	Ethyl 2-methylbutyrate	30	1178	Hexyl butyrate
5	842	Propyl isobutyrate	31	1182	Ethyl octanoate
6	863	Isopentyl acetate	32	1222	Hexyl 2-methylbutyrate
7	885	Propyl butyrate	33	1235	2-methylbutyl hexanoate
8	890	Butyl propionate	34	1274	Heptyl butyrate
9	896	Ethyl pentanoate	35	1274	Butyl heptanoate
10	904	Pentyl acetate	36	1274	Pentyl hexanoate
11	911	Methyl hexanoate	37	1277	Propyl octanoate
12	934	Propyl 2-methylbutyrate	38	1278	Hexyl pentanoate
13	939	Butyl isobutyrate	39	1321	Heptyl 2-methylbutyrate
14	982	Butyl butyrate	40	1333	Isobutyl octanoate[b]
15	986	Ethyl hexanoate	41	1372	Heptyl pentanoate
16	992	Propyl pentanoate	42	1373	Butyl octanoate
17	996	Hexyl acetate	43	1374	Octyl butyrate
18	1028	Butyl 2-methylbutyrate	44	1374	Hexyl hexanoate
19	1040	Pentyl isobutyrate	45	1433	2-methylbutyl octanoate[b]
20	1078	Pentyl butyrate	46	1466	Pentyl octanoate
21	1081	Propyl hexanoate	47	1466	Heptyl hexanoate
22	1083	Ethyl heptanoate	48	1467	Hexyl heptanoate
23	1092	Butyl pentanoate	49	1473	Nonyl butyrate
24	1093	Hexyl propionate	50	1490	Octyl pentanoate
25	1108	Methyl octanoate	51	1564	Hexyl octanoate
26	1124	Pentyl 2-methylbutyrate	52	1574	Octyl hexanoate

[a] Column 1 refers to numbering of GC peaks in Figures 1–3.
[b] Tentative identification.

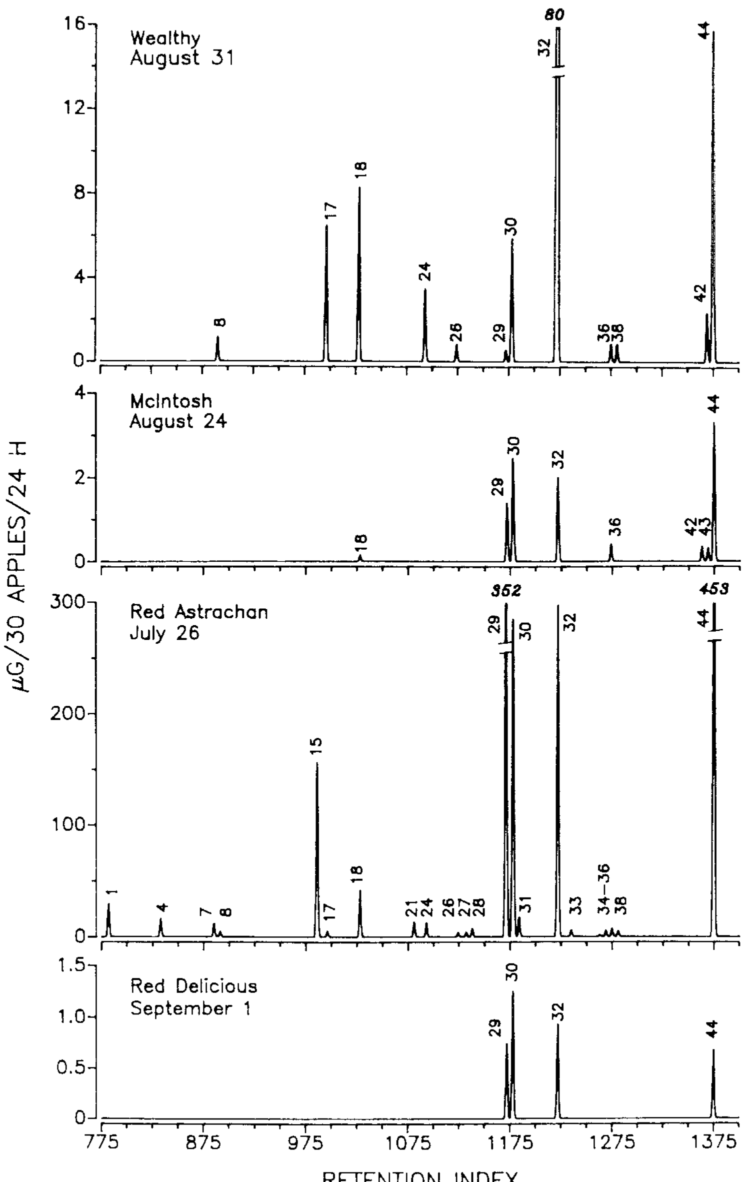

FIG. 1. Simulated GC traces of volatiles produced by Red Astrachan, McIntosh, Wealthy, and Red Delicious apples 1–2 months prior to fruit ripening. Vertical numbers near peaks refer to compound identifications, which are listed in Table 1. If compounds came off as one peak, they were separated in the tracing to show relative amounts. Horizontal numbers designate peak amounts that were off-scale.

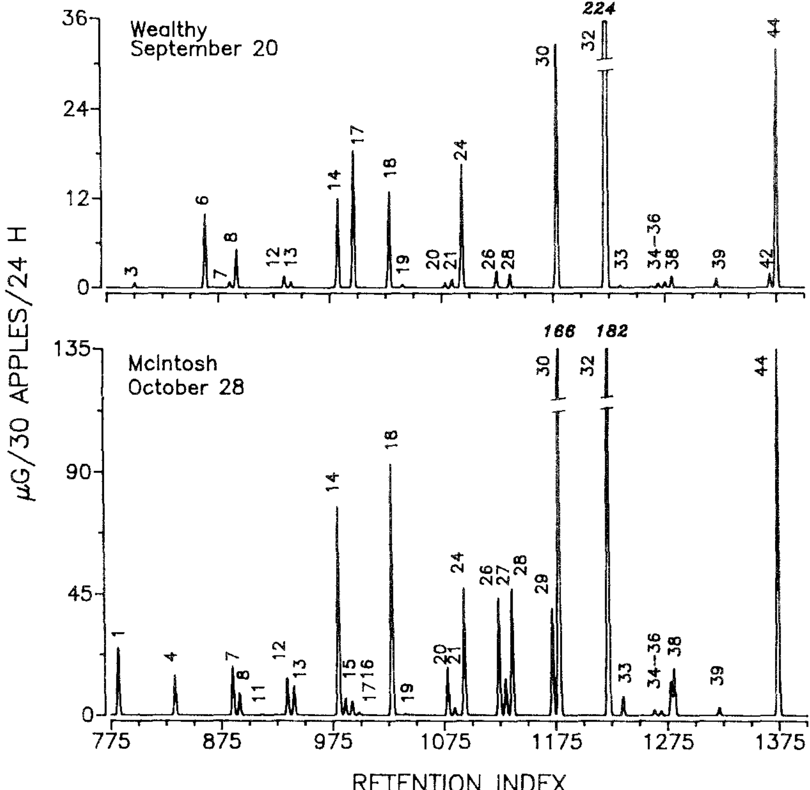

FIG. 2. Simulated GC traces of volatiles produced by ripe Wealthy and McIntosh apples. See Figure 1 for additional notes.

apples, especially unripe apples, the hawthorn extract contained butyl hexanoate, hexyl butyrate, and hexyl hexanoate. Greatest similarity appeared between Red Astrachan and hawthorn. In addition to the above compounds, large ethyl and butyl octanoate peaks appeared in both extracts.

For all apple cultivars, the number and quantity of esters was higher in ripe than in unripe apples. In McIntosh and Red Delicious extracts, where two months separated the volatile collections, appearance of new esters (especially short-chain esters) in the ripe apple collection was pronounced. When ripe, both produced a ''flush'' of esters that was hundreds of times more concentrated than that of unripe apples. In Red Astrachan and Wealthy extracts, where one month separated volatile collections, compounds already present in unripe apples appeared in greater concentration following fruit ripening. In Wealthy, ratios of the various compounds did not change much following ripening, but in Red

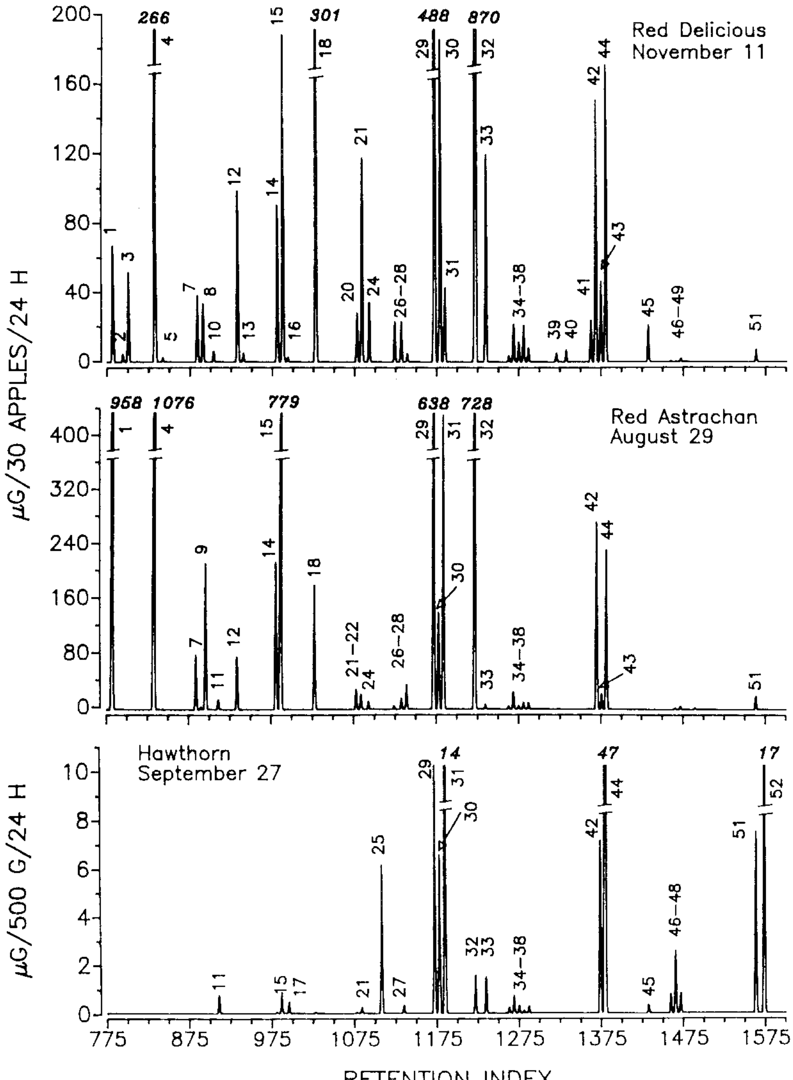

FIG. 3. Simulated GC traces of volatiles produced by ripe Red Astrachan and Red Delicious apples and *C. coccinea* hawthorns. See Figure 1 for additional notes.

Astrachan, the major peak in unripe extracts (hexyl hexanoate) was swamped by an increase of short-chain esters (ethyl butyrate, ethyl 2-methylbutyrate, and ethyl hexanoate) produced by ripe apples.

Because the time of greatest *R. pomonella* host-seeking and oviposition behavior occurs from late July to September, foraging flies would likely en-

counter ripe Red Astrachan fruit, almost-ripe Wealthy and hawthorn fruit, and unripe McIntosh and Red Delicious fruit. During this interval of peak fly activity, only Red Astrachan and, to a lesser extent, Wealthy were producing a sizable quantity of volatiles. Comparison among the apple extracts collected at this time revealed that the total volatile production by Red Astrachan was ca. 60 times greater than Wealthy, 600 times greater than McIntosh, and 1000 times greater than Red Delicious.

DISCUSSION

Volatiles produced by many apple cultivars have been thoroughly investigated numerous times (Dimick and Hoskin, 1983). Similar to our findings, these studies have often shown similarity in the compounds identified, but differences in the ratio of these compounds from cultivar to cultivar (Flath et al., 1969; Dimick and Hoskin, 1983). Further, all of the major apple components identified in our study have previously been reported. However, these studies seldom report analyses of volatiles produced by whole fruit immediately following harvest and do not allow comparison of volatiles produced by the particular fruit types of interest to us.

During the interval when apple maggot fly activity occurs (July–September) and within the volatile region found to be most attractive to the apple maggot (Fein et al., 1982), there was surprising similarity among the apple cultivars as well as between apple and *C. coccinea* hawthorns. This latter finding was of particular interest because *R. pomonella* originally infested hawthorn and has only recently expanded its host range to include apple as well as several other introduced and domesticated fruits, such as cherry and pear. Because olfactory cues appear to play a role in initial orientation to the host plant by *R. pomonella* and also influence oviposition-site selection (Bush, 1966; Prokopy et al., 1973, unpublished data), perhaps this similarity in volatile composition has facilitated the apparently easy shift of *R. pomonella* from hawthorn to apple.

Among the apple cultivars, similarity in ester composition and ratio was most apparent in Red Delicious and Red Astrachan. Such a similarity was previously reported by Fein et al. (1982), but in their study, the largest peak reported for both cultivars was hexyl acetate. Although our collection and analysis techniques were consistent with those used by Fein and coworkers, we did not isolate any hexyl acetate from either cultivar. The previous study, however, was conducted with apples kept in storage. Studies of Red Delicious and other cultivars have shown that changes in volatile profile occur rapidly following even short storage intervals and fluctuate throughout the storage period (Brown et al., 1966; Guadagni et al., 1971). In general, but depending on apple ripeness and storage conditions, certain short-chain esters build up to a maximum within

a few weeks after harvest (Hatfield and Patterson, 1977; Dimick and Hoskin, 1983).

All of the apple cultivars we examined are moderately to highly susceptible to attack by *R. pomonella*, but if ranked, Red Astrachan and, to a lesser extent, Wealthy, would likely be considered most susceptible. While numerous factors, such as changes in fruit acidity and skin toughness, would be associated with the early ripening of these cultivars (Dean and Chapman, 1973) and probably would contribute to the susceptibility of these cultivars, the higher level of volatiles produced may also be important. On the other hand, the apple maggot fly may be selectively tuned to reception of hawthorn volatiles. Perhaps the remarkable similarity between the volatile profiles of Red Astrachan and hawthorn, combined with Red Astrachan's exceptionally high rate of volatile release, may in part account for the extreme susceptibility of this particular cultivar. In the future, comparative studies of volatiles produced by cultivars that are seldom attacked by *R. pomonella* might prove interesting.

Although there is little evidence to support such a contention (Reissig et al., 1985), we question whether the increasing concentration of natural odor from ripening apples may compete with the synthetic blend. Indeed, within the range of volatiles that we analyzed, we calculated that a ripe Red Astrachan apple produces over 8 μg of esters per hour. At this rate, it would take only 60 apples to produce an amount comparable to the release rate of synthetic blend (500 μg/hr). For the other cultivars we investigated, it would require ca. 950, 330, or 95 ripe Wealthy, McIntosh, or Red Delicious apples, respectively, to equal the volatile release of a trap. Prior to fruit ripening, it would require 200 Red Astrachan apples to equal the volatile release of a trap or 2000, 35,000, or 100,000 Wealthy, McIntosh, or Red Delicious apples, respectively, to equal trap release. However, whether trap efficacy declines over the season, or if this occurs as a result of competition with ripe apple aroma, remain to be tested. No doubt, numerous other factors associated with fruit ripening may influence trap efficacy. For example, changes may occur in the physiological state of the flies. It is very likely that early in the season, fruit are hard and acidic, and thus, unsuitable to a *R. pomonella* female foraging for oviposition sites. Under such circumstances, the probability that a female would respond to the synthetic blend may be much higher than later in the season, when abundant, suitable fruit are available.

We are currently testing apple maggot fly response (throughout the growing season) to synthetic blends based on the volatile profiles determined from hawthorn and the four apple cultivars. Because dissimilarity in the profiles was due to differences in ratio of the various components rather than to the appearance of any one or a series of novel components, at the superrelease rates of volatiles normally utilized in field tests, differences in the level of attractiveness among the blends may not be apparent. For this reason, and because variation in overall concentration of volatiles produced was the most salient difference

observed among the cultivars, controlled release rates of the volatile blends will be a key focus in future studies.

Acknowledgments—We thank Dr. Terry Acree and Mr. Robert Butts of the Food Science Department, NYS Agric. Expt. Station, for their help with the gas chromatographic and mass spectrometric analyses; Dr. Lou Bjostad for the graphics program and assistance with mass spectrometric analyses; Dr. Walter Wolf for advice on topics too numerous to list; and Dr. Robert Seem of the Plant Pathology Department, NYS Agric. Expt. Station, for use of his computer facilities.

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QUANTITATIVE AND QUALITATIVE VARIATION IN MALE PHEROMONES OF *Phragmatobia fuliginosa* and *Pyrrharctia isabella* (LEPIDOPTERA: ARCTIIDAE)

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Abstract—The dihydropyrrolizine pheromones, hydroxydanaidal and danaidal, were identified from the scent organs of male *Phragmatobia fuliginosa* (L.) and *Pyrrharctia isabella* (J.E. Smith). Qualitative and quantitative GLC analyses were conducted on ca. 80 field-collected males of each species. The total pheromone titer was distributed bimodally in each species with most males having either a small amount (< 10 ng) of pheromone or a large amount (1–10 μg in *Pyrrharctia* and 0.3–3 μg in *Phragmatobia*). *Pyrrharctia* males in the 1- to 10-μg range had a predominance of hydroxydanaidal, with little if any danaidal. Most *Phragmatobia* males in the 0.3- to 3-μg range had danaidal with little if any hydroxydanaidal. These compounds elicited a courtship response in sexually receptive females of both species. A bioassay based on this response was used to measure the thresholds of female response to these compounds. *Pyrrharctia* females were more sensitive to (*R*)-(−)-hydroxydanaidal than to danaidal. *Phragmatobia* females were more sensitive to danaidal then to (*R*)-(−)-hydroxydanaidal.

Key Words—*Phragmatobia fuliginosa, Pyrrharctia isabella,* male pheromones, sex pheromones, Lepidoptera, Arctiidae, pyrrolizidine alkaloids, hydroxydanaidal, danaidal.

INTRODUCTION

Dihydropyrrolizines have been identified from the scent organs of males of numerous danaine butterfly species (Meinwald and Meinwald, 1966; Meinwald et

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al., 1971, 1974; Edgar et al., 1971, 1973; Edgar, 1982; Komae et al., 1983). In several cases these compounds were shown to be derived from pyrrolizidine alkaloids (PAs) contained in plants that were fed upon by adult males (Edgar et al., 1973; Schneider et al., 1975). The pheromonal function of one of these compounds, danaidone, has been established for only one of these butterflies (Pliske and Eisner, 1969; Schneider and Seibt, 1969).

The male scent organs (coremata) of arctiid moths of the genus *Utetheisa* are inflatable sacs covered with scent scales that emerge from slits in the sides of the genital valves. The dihydropyrrolizine hydroxydanaidal (Figure 1: II) was identified from *Utetheisa pulchelloides* (Hamps.) and *U. lotrix* (Cram.), and the related compound, danaidal (Figure 1: I), was identifed from the latter species only (Culvenor and Edgar, 1972). Conner et al. (1981) identified hydroxydanaidal from *U. ornatrix* (L.), showed that it is derived from PA precursors obtained during larval feeding, and established the role of the compound as a close-range courtship pheromone in that species.

Moths of the Asian arctiid genus *Creatonotos* also have coremata, but these are tubular structures arising from the 7th abdominal sternite and so are not homologous to those found in *Utetheisa*. Schneider et al. (1982) showed that larval ingestion of PAs is necessary for both the presence of hydroxydanaidal in adult male coremata as well as the full development of the organs themselves in *Creatonotos gangis* L. and *C. transiens* (Walker). The behavioral role of hydroxydanaidal as an attractant of females, and possibly males, to mating aggregations in these two species has been referred to in several reports (Wunderer et al., unpublished, cited in the following: Schneider, 1983; Boppré and Schneider, 1985; Boppré, 1986).

We studied the pheromone biology and mating behavior of two north temperate arctiids, *Phragmatobia fuliginosa* (L.) (hereinafter referred to as *Phragmatobia*) and *Pyrrharctia isabella* J.E. Smith (hereinafter referred to as *Pyrrharctia*). Like *Creatonotos*, males of these species have eversible tubular

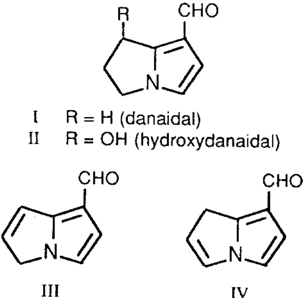


FIG. 1. I, II: dihydropyrrolizine pheromones of arctiid moths; III, IV: GLC degradation products of II.

coremata (Nielsen, 1979, 1982). However, in contrast to the protracted static display of the coremata exhibited by *Estigmene acrea* (Drury) (Willis and Birch, 1982) and *Creatonotos* spp. (Schneider et al., 1982), *Phragmatobia* and *Pyrrharctia* males evert their coremata only briefly during the courtship flight while approaching a calling female (Nielsen, 1982; Krasnoff, 1987). In this paper we document the presence of hydroxydanaidal and danaidal in the coremata of both of these species, as well as the behavioral activity of the two compounds. We show that pheromone quantity of individual wild males varies widely and in a characteristic way within each species. We also show that pheromone composition differs between the two species and that this difference is reflected in differences in female response.

METHODS AND MATERIALS

Insects. All of the male moths used in this study were captured in the field at sites in Ontario, Schuyler, Seneca, and Tompkins counties, New York State, between June and November, 1984. Most of the males were collected at lights, but some were collected as mature larvae and allowed to pupate and eclose in the laboratory following a modification of the methods of Goettel and Philogène (1978).

Colonies of each species were derived from eggs of field-collected females. Larvae were reared in the laboratory on either leaf cuttings of wild plants (*Plantago* spp., *Taraxacum* spp.) or on an artificial diet (Shorey and Hale, 1965). Larvae were held at 25° ± 2°C under a 16:8 light-dark photoperiod. Females used in bioassays were either exposed to a reversed light cycle as pupae and used during working hours or were exposed to natural light and used during the natural mating activity period (ca. 0.5–2.0 hr after dark for *Pyrrharctia* and ca. 2.0–4.0 hr after dark for *Phragmatobia*).

Bioassay. During courtship, a *Phragmatobia* or *Pyrrharctia* female often responds to the corematal display of an approaching conspecific male with a characteristic wing-fluttering and clicking behavior (Krasnoff, 1987). Preliminary experiments showed that male coremata artificially extruded by squeezing, solvent extracts of male coremata, or synthetic hydroxydanaidal held close to the antennae of calling *Pyrrharctia* or *Phragmatobia* females elicited this response, whereas solvent blanks presented in this manner never did. This response provided the basis for a pheromone bioassay.

During the mating period, calling females were coaxed to perch on wooden dowels. Materials to be tested were applied to the tip of a glass or metal rod in a measured amount of solvent. The solvent was allowed to evaporate, the tip of the rod was held ca. 2 mm from the tips of a female's antennae for 3 sec and the female's response noted. The rod was never allowed to make physical contact with the antennae of the test subject (cf. Conner et al., 1981). This method

of presentation was designed to approximate the exposure of a calling female to the pheromone of an aproaching male and to assure that only olfactory stimuli were affecting the female. Although responses ranged from short bursts of wing-fluttering and clicking lasting less than 1 sec to sustained responses lasting more than 5 sec, any response involving wing-fluttering and/or clicking was scored as positive in these experiments. If neither of these behaviors was observed a negative score was recorded.

Chemical Identifications. Coremata of individual males were manually everted by gently squeezing the abdomen. They were then gripped with forceps, removed with a razor blade, placed in ca. 0.5 ml redistilled dichloromethane in a tightly capped 4-ml vial, and allowed to stand for at least 1 hr at ca. 22°C. Samples were stored at −20°C until they were analyzed.

Preparative GLC was carried out at 150°C on a 2-m glass column (ID 4 mm) packed with 1% (w/w) OV-101 (methyl silicone) on 100- to 120-mesh Gas-Chrom Q. Fractions were collected from this column by cold-trapping in 30-cm glass capillary tubes. Capillary GLC was conducted with a 25-m cross-linked methyl silicone column (ID 0.31 mm) and a 5% phenylmethyl silicone column (ID 0.20 mm) used with splitless injection and programmed from 80° to 200°C at 10°/min after an initial hold of 1 min, or a 30-m Supelcowax 10 column (ID 0.25 mm) programmed from 80° to 230° at 15°/min after an initial hold of 1 min. Quantification was achieved by comparing electronically integrated pheromone peak areas with that of a known amount of an internal standard (hexadecane). As little as 10 pg of material could be detected by this method. Electron ionization and chemical ionization (isobutane) mass spectra were obtained with a Hewlett-Packard 5985 GC-MS system using methyl silicone, 5% phenylmethyl silicone, and Supelcowax columns identical to those described above.

Preliminary analyses and bioassays indicated that some males of both species appeared to have relatively large amounts of pheromone, whereas others appeared to have little or none at all. Therefore a screening procedure was developed to permit a refinement in the quantification system. Before quantitative analysis of a large number of field samples, ca. 1–2 μl out of 200–500 μl of each sample was tested for behavioral activity. Samples producing a positive response were given 500 ng of the internal standard (hexadecane), whereas only 5 ng were added to samples that produced no response. Samples in the positive category were analyzed by injecting 1–2 μl out of a total of 300–500 μl. Samples in the negative category were concentrated under a stream of dry nitrogen to ca. 20 μl volume and 3–4 μl were injected.

Synthetic Sources. Synthetic (*R*)-(−)-hydroxydanaidal, obtained from Dr. J. Meinwald for use in bioassays and in chromatographic and spectroscopic standards, was shown by GLC to be >99% pure. Synthetic danaidal, obtained from Dr. J.A. Edgar and used similarly, was determined by GLC to be >99%

pure. Serial dilutions for bioassays were made in redistilled dichloromethane. The control for bioassays was a 1% solution of hexadecane in dichloromethane.

Threshold Studies. With the bioassay described above, the threshold of response of *Pyrrharctia* and *Phragmatobia* females to both danaidal and (*R*)-(−)-hydroxydanaidal was measured for ca. 20 insects in each of four separate experiments. Only the (*R*)-(−) enantiomer of hydroxydanaidal was used because preliminary tests showed that it had much greater activity than is antipode. Moreover (*R*)-(−)-hydroxydanaidal was identified, by a behavioral bioassay, as the pheromone in *Utetheisa ornatrix* (Conner et al., 1981) and as the predominant constituent of the corematal secretions of *Creatonotos gangis* and *C. transiens* (Bell et al., 1984; Bell and Meinwald, 1986).

Calling females, never previously exposed to synthetic or male-derived odors, were presented with a control and then a series of doses of pheromone increasing in potency in logarithmic steps. Preliminary trials established a starting dose that could be assured of getting no positive response. Making certain that a female would not respond to the first dose in a series eliminated doubt as to whether she would have responded to a lower dose. The first dose (i.e., the lowest) in the series that elicited a response for each individual female was noted. After a female's threshold of response was established in this way, she was not tested with higher doses. Testing continued until all females had responded.

A single application was never used for more than six females in succession, so no more than 30 sec elapsed from the first presentation to the last for any dose. Illumination in all four experiments was provided by a 6-V lantern with red acetate covering the lens. *Pyrrharctia* female response to danaidal was assayed during the natural activity period. Temperatures during this experiment ranged from 16 to 18°C. Also metal rods (7-cm finishing nails) were used in place of glass in this experiment only. All of the other tests were conducted with clock-shifted insects at 20–25° C. In the test of *Pyrrharctia* female response to hydroxydanaidal, doses were delivered in either 1 μl or 10 μl of solvent, and some of the females used were derived from the laboratory colony while some were from field-collected larvae. In all other tests, all doses were delivered in 10 μl of solvent, and all females used were from laboratory colonies.

RESULTS

Chemistry. Preliminary packed-column GLC analyses on OV-101 showed that synthetic hydroxydanaidal chromatographed poorly, producing a broad peak with considerable tailing. Fractionation of behaviorally active extracts of *Pyrrharctia* males gave activity in all fractions collected after the retention time of hydroxydanaidal. Subsequently, both hydroxydanaidal and danaidal were iden-

tified from individual field-collected *Phragmatobia* and *Pyrrharctia* males by comparisons of GLC retention times on polar and nonpolar capillary columns and mass spectra with those of authentic samples of synthetic material.

We encountered a peak that always appeared with hydroxydanaidal and eluted at the same time as danaidal on methyl silicone and 5% phenyl methyl silicone (Figure 2). This compound was present as ca. 10% of total hydroxy-

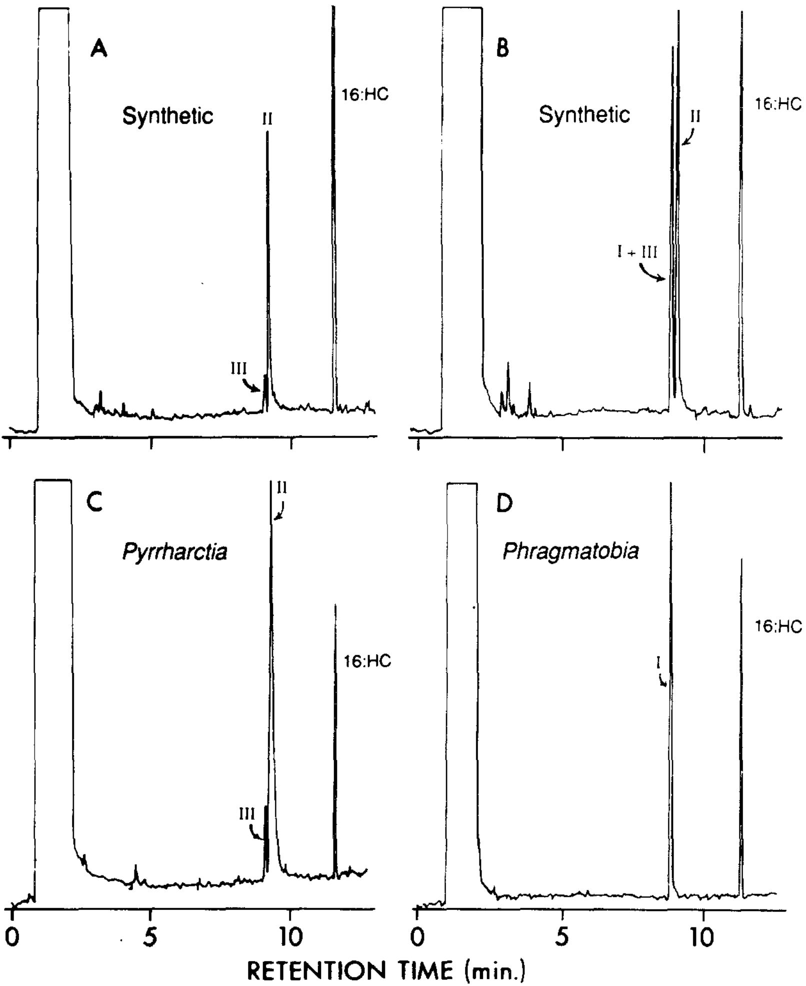


FIG. 2. Chromatograms of (A) synthetic hydroxydanaidal, (B) synthetic hydroxydanaidal + synthetic danaidal, (C) a *Pyrrharctia* male corematal extract, and (D) a *Phragmatobia* male corematal extract. Compound numbers correspond to those in Figure 1. 16:HC is hexadecane, the internal standard.

danaidal in chromatographic analyses (N = 15) of synthetic hydroxydanaidal. The CI mass spectrum of this peak indicated a molecular weight of 133, 18 mass units lower than hydroxydanaidal. The EI mass spectrum showed peaks two mass units lower than danaidal throughout the spectrum (Figure 3). These spectra are consistent with expectations for a dehydration product (Figure 1: III) that might arise readily from hydroxydanaidal.

A virtually identical EI mass spectrum was obtained for a peak with a retention time of 0.90 relative to the later peak on methyl silicone and 5% phenylmethyl silicone. This earlier peak was always smaller than the later peak (range: 8–15%). On this basis we suggest that it is rearrangement of the dehydration product of hydroxydanaidal (Figure 1: IV). Because these compounds appear in chromatograms of synthetic hydroxydanaidal shown to be >95% pure on the basis of [^{1}H] NMR analysis (G. Zarilli and J. Meinwald, personal communication), it is most likely that they are being generated under the severe conditions (250°C) encountered in the GLC injector.

Samples showing microgram quantities of compound III on methyl silicone were analyzed on Supelcowax. A mass spectral survey indicated that compound III did not elute from Supelcowax. Although Supelcowax separated danaidal and hydroxydanaidal, it gave lower quantitative estimates than methyl silicone for both compounds, suggesting that active sites were not allowing substantial amounts of these compounds to elute. Consequently, quantitative data were derived from analyses performed on methyl silicone. By adding together the

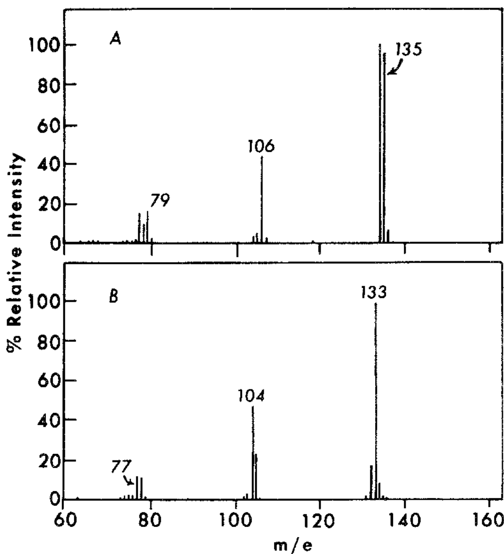
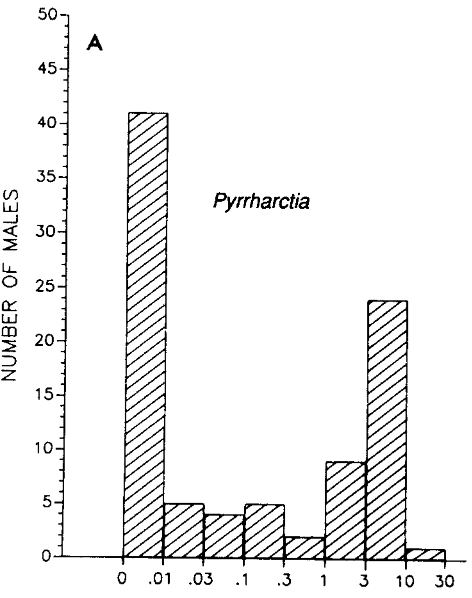


FIG. 3. Mass spectra of (A) danaidal from a *Phragmatobia* male corematal extract and (B) compound III from an analysis of synthetic hydroxydanaidal.



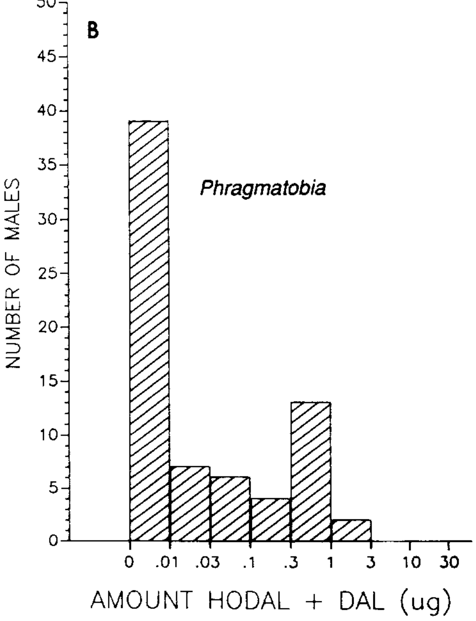


FIG. 4. Frequency distributions of total male pheromone content (hydroxydanaidal + danaidal). A. *Pyrrharctia* ($N = 91$). B. *Phragmatobia* ($N = 71$). Bars represent number of males within the indicated range.

estimates of danaidal and hydroxydanaidal for each individual male, and using the totals to provide an overall picture of the variability of the amount of PA-derived pheromone for males of each species, the confounding effect of compound III on the quantification system was avoided.

Pyrrharctia males had from < 10 pg to 12.3 μg of total pheromone. In *Phragmatobia* males, the measurements ranged from < 10 pg to 1.5 μg. Figure 4A shows a distinct bimodality in the amount of PA-derived pheromone found in *Pyrrharctia* males, with most males in either the 0–10 ng range (45%) or the 1–10 μg range (35%). There is a similar bimodality in *Phragmatobia* (Figure 4B) but with the higher mode ca. one order of magnitude lower than that found in *Pyrrharctia*. Here we found 55% of all males in the sample in the 0 to 10-ng range and 21% in the 300- to 1500-ng range.

Of the 15 *Phragmatobia* males with >0.3 μg of total pheromone, most had >99% danaidal, although one had as little as 12% (Figure 5). On the other hand, although three *Pyrrharctia* males had more danaidal than hydroxydanaidal (260 ng danaidal: 1 ng hydroxydanaidal, 170 ng danaidal: 50 ng hydroxydanaidal, and 68 ng danaidal: 4 ng hydroxydanaidal), all of the 33 *Pyrrharctia* males with > 1 μg of total pheromone showed > 85% hydroxydanaidal (Figure 5). The breakdown product of hydroxydanaidal (III) accounts for some or all of the "danaidal" in the samples showing microgram quantities of hydroxydanaidal. Therefore, ratios of hydroxydanaidal to danaidal represent minima. GLC analysis on Supelcowax firmly established the presence of danaidal in

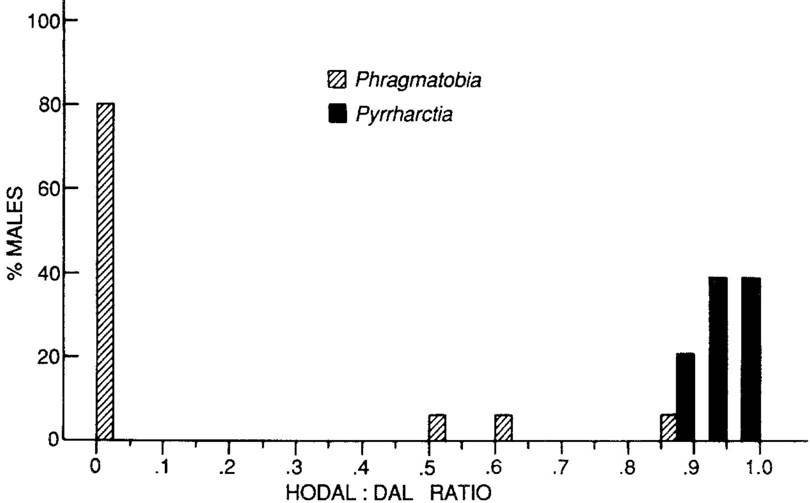


FIG. 5. Frequency distributions of hydroxydanaidal-danaidal ratio for *Pyrrharctia* males with > 1 μg of total pheromone (N = 33) and *Phragmatobia* males with >0.3 μg total pheromone (N = 15). Bars represent number of males within the indicated range.

several of these higher mode *Pyrrharctia* males, and, as expected, the estimate of the proportion of danaidal in the total pheromone blend was always smaller than the estimate obtained from methyl silicone. However, underestimating the percentage of hydroxydanaidal in the total blend for *Pyrrharctia* males only minimizes the difference between the ''typical'' blends of the two species, which is still highly significant ($P < 0.0001$, Mann-Whitney U test).

Threshold Studies. Some female *Pyrrharctia* responded to as little as 50 fg of (*R*)-(−)-hydroxydanaidal, most responded to 5 pg or less, and in no case were more than 50 pg required to elicit a response (Figure 6A). The response profile moves up two orders of magnitude in this species when danaidal is substituted for hydroxydanaidal (Figure 6B). To be certain that the apparent lower sensitivity of *Pyrrharctia* females to danaidal relative to hydroxydanaidal was not due to the lower temperatures obtaining during the danaidal test, we assayed several of the subjects in the danaidal test with hydroxydanaidal and found that they did indeed respond to lower doses of hydroxydanaidal than danaidal under the same conditions.

We observed the reverse situation in *Phragmatobia*, with most females responding to less than 50 pg of danaidal and all responding to 5 ng or less (Figure 7A). Up to 50 ng of hydroxydanaidal were required for many of the *Phragmatobia* females to respond (Figure 7B) and one female (not respresented in Figure 7B) failed to respond to 500 ng, the highest dose tested.

DISCUSSION

The samples analyzed in this study exposed a pattern of quantitative variation among individual wild males of both species that is, as far as we know, unique in the pheromone literature. Boppré et al. (1978) reported greater absolute variation in danaidone titers of 19 field-collected *Danaus chrysippus* (31–420 μg), but proportionally the differences are much greater in *Phragmatobia* and *Pyrrharctia*, with differences of three to four orders of magnitude between the lowest and the highest measurements compared to the 15-fold difference seen in *D. chrysippus*. Boppré and Schneider (in press, referred to in Boppré and Schneider, 1985) also refer to a ''drastic'' variation in hydroxydanaidal titers in field-collected *Creatonotos* males. In light of the differences in hydroxydanaidal titers between *Creatonotos* males reared in the laboratory on PA-deficient plants (<5 ng) and those reared on PA-containing plants (up to 450 μg) (Schneider et al., 1982), and the polyphagous nature of *Creatonotos* in the field (Boppré and Schneider, 1985), it seems possible that the extent of variation could be greater than that seen here by one to two orders of magnitude. However, the distinct bimodal distribution of pheromone titer observed in *Phragmatobia* and *Pyrrharctia* was reported in neither *Danaus* nor *Creatonotos*.

From laboratory feeding experiments, we have evidence that the pher-

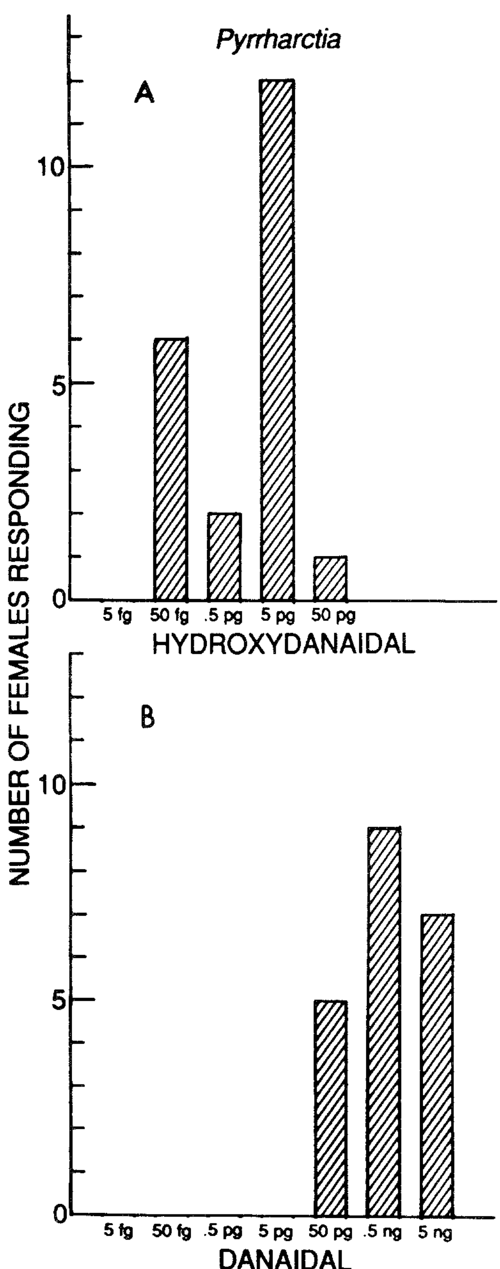


FIG. 6. Number of *Pyrrharctia* females responding first to given dose of (A) (*R*)-(−)-hydroxydanaidal (N = 21 females) and (B) danaidal (N = 21 females). Dosage series were present sequentially from lowest to highest. Highest dose labeled was highest necessary to elicit a response in any female in test.

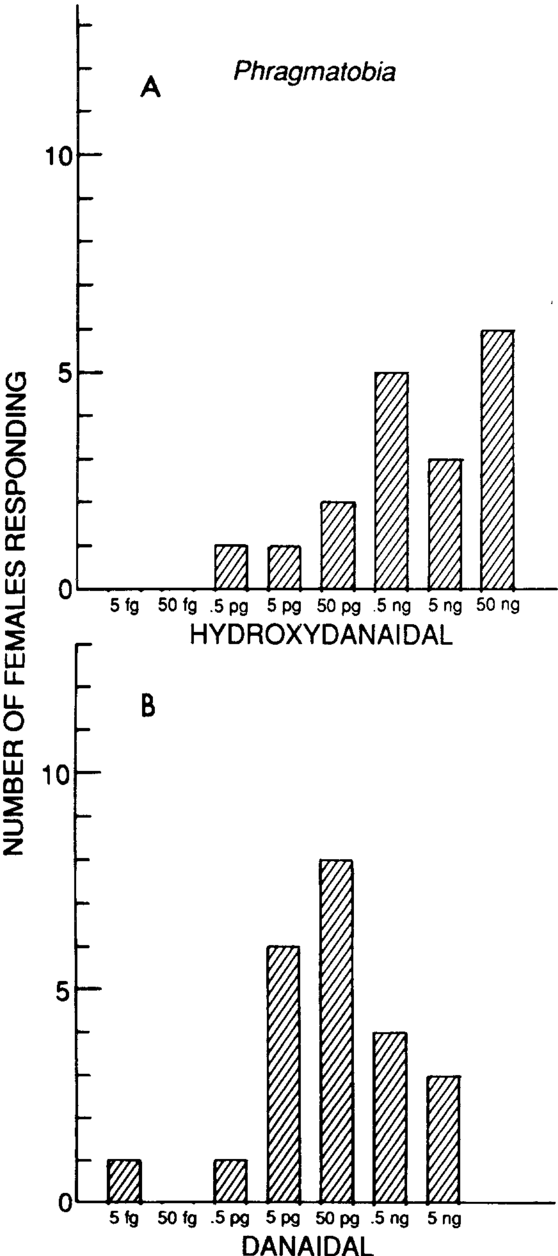


FIG. 7. Number of *Phragmatobia* females responding first to given dose of (A) (*R*)-(−)-hydroxydanaidal (N = 18 females) and (B) danaidal (N = 23 females). Dosage series were presented sequentially from lowest to highest. Highest dose labeled was highest necessary to elicit a response in any female in test.

omones identified here are derived from PA-precursors in the larval diet, and although *Phragmatobia* and *Pyrrharctia* are polyphagous as larvae, we have observed them feeding on PA-containing plants in the field (Krasnoff, 1987). In this light, the variation in pheromone content in field-collected *Phragmatobia* and *Pyrrharctia* males reported here can be seen as indicative of variation in larval feeding history in the field. Males with the higher amounts of pheromone are, we believe, those that gained access, as larvae, to some source of PAs. Those with no pheromone at all clearly did not feed on any PA plants during larval development. Inasmuch as female arctiids are capable of passing PAs into their eggs (Benn et al., 1979; Dussourd et al., 1984; Boppré, unpublished data cited in Boppré and Schneider, 1985), it is possible that *Phragmatobia* and *Pyrrharctia* males with small amounts of dihydropyrrolizines inherited the precursors for them from their mothers without themselves feeding on any PA-containing plants as larvae. Males with intermediate amounts of pheromone, say 50–100 ng, may have either inherited a lot of precursor or fed only minimally on PA-laden food.

Some notable exceptions notwithstanding, a "typical" *Pyrrharctia* male in the higher categories for the species had hydroxydanaidal with little if any danaidal, while a "typical" *Phragmatobia* male had danaidal with little if any hydroxydanaidal. The few *Pyrrharctia* males with more danaidal than hydroxydanaidal and *Phragmatobia* males with more hydroxydanaidal than danaidal, recall the case reported by Culvenor and Edgar (1972), in which *Utetheisa lotrix* males collected at different sites yielded different ratios of hydroxydanaidal and danaidal. We were intrigued by the fact that two of the three *Pyrrharctia* males in our sample with more danaidal than hydroxydanaidal came from the same site. In accordance with the speculations of Culvenor and Edgar, we propose that any atypical pheromone profiles in *Phragmatobia* and *Pyrrharctia* males are due either to genetic differences or to differences in the PA plants, and hence the alkaloids themselves, ingested by the larvae in different regions. We are currently investigating these possibilities.

Overall, our qualititative pheromone data depict an association of *Pyrrharctia* males with hydroxydanaidal and *Phragmatobia* males with danaidal that is supported by the data on the behavioral response profiles of the females of each species. We do not interpret these differences as having evolved in a context of reproductive isolation between these two species because they are neither seasonally cosynchronous nor cross-attractive due to major differences in the female sex attractants (Roleofs and Cardé, 1971; Descoins and Frerot, 1984; Meyer, 1984; Krasnoff and Roelofs, unpublished).

Although numerous compounds have been identified from male lepidopteran scent organs, reports of overt female behavioral responses to male pheromones are still relatively few (Nishida et al., 1982 and references cited; Krasnoff and Vick, 1984, and references cited). Fewer still are quantitative behavioral data on the level of female sensitivity to male pheromones (Jacobson et

al., 1976). Preliminary tests showing that nanogram quantities of hydroxydanaidal elicited virtually 100% response from *Pyrrharctia* females suggested that the response thresholds could be very low and this, indeed, proved to be the case. Females proved sensitive enough to male pheromone that even a male in the lower mode of the distribution, with as little as 1 ng of total pheromone, could possibly evoke a response in almost any female, assuming that a male's coremata release pheromone as efficiently as a glass rod.

The quantitative relationship between male pheromone titer and female sensitivity has broader implications for understanding the significance of these pheromones in the mating behavior of *Phragmatobia* and *Pyrrharctia*, as well as the arctiids that have been under investigation by other workers. In *Utetheisa ornatrix*, the absence of the PA-derived male pheromone has been shown experimentally to reduce a male's mating success (Conner et al., 1981). However, *U. ornatrix* has a larval host-range limited to plants of the genus *Crotalaria*, all of which contain PAs. Consequently, it is unlikely that females in nature will encounter a male without the pheromone. In contrast, *Phragmatobia* and *Pyrrharctia*, as well as the *Creatonotos* spp. referred to above (Boppré and Schneider, 1985; Boppré, 1986), apparently have a looser relationship to PA-containing plants, i.e., they are essentially polyphagous as larvae, but will feed on PAs if the opportunity arises. The resulting variation in male pheromone titer raises questions: What effect, if any, does the amount of male pheromone have on mating success of males in these polyphagous species? In the case of *Phragmatobia* and *Pyrrharctia*, do more than half of all the males in the field suffer a disadvantage due to their failure to feed on PA-containing plants during larval development? We anticipate that current investigations of the courtship of these species and the effects of pheromone dose on the intensity and duration of the female response, as well as the distance at which it can be elicited, will provide insights into the relationship between variation in male pheromone titer and variation in male mating success.

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SEMIOCHEMICALS PRODUCED BY WESTERN BALSAM BARK BEETLE, *Dryocoetes confusus* SWAINE (COLEOPTERA: SCOLYTIDAE)[1]

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Abstract—The most prominent beetle-produced volatiles identified in the abdominal extracts of male *Dryocoetes confusus* Swaine after they had bored for 24 hr in logs of subalpine fir, *Abies lasiocarpa* (Hook.) Nutt. were: *exo*- and *endo*-brevicomin, *trans*-verbenol, verbenone, myrtenol, *trans*-pinocarveol, *cis*- and *trans*-*p*-menthen-7-ol, 3-caren-10-ol, and several monoterpenes and sesquiterpenes. Myrtenol was the only conspicuous compound in extracts from males that had been exposed to *A. lasiocarpa* resin volatiles for 24 hr. Laboratory bioassays indicated that both (±)- and (+)-*exo*-brevicomin were attractive to female *D. confusus*, and that the (−) enantiomer did not inhibit response to its antipode. Results from field trapping experiments indicated that both *exo*-brevicomin and myrtenol are aggregation pheromones for *D. confusus*. *exo*-Brevicomin baits were effective in causing attack by *D. confusus* on baited and surrounding trees, suggesting that this pheromone may have utility in manipulating populations of the beetle.

Key Words—Semiochemicals, pheromones, enantiomers, western balsam bark beetle, *Dryocoetes confusus*, Coleoptera, Scolytidae, *exo*- and *endo*-brevicomin, *trans*-verbenol, verbenone, myrtenol, *trans*-pinocarveol, *cis*- and *trans*-*p*-menthen-7-ol, 3-caren-10-ol.

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INTRODUCTION

The western balsam bark beetle, *Dryocoetes confusus* Swaine, is a major pest of mature subalpine fir, *Abies lasiocarpa* (Hook.) Nutt., in North America (Furniss and Carolin, 1977). It acts in association with a pathogenic fungus, *Ceratocystis dryocoetoides* Kendrick and Molnar (Kendrick and Molnar, 1965), which it introduces into living trees. This symbiotic invasion of beetles and fungus is ultimately lethal to the trees. From 1948 to 1975 the volume of trees killed in British Columbia comprised a reported 15 million cubic meters of wood (Stock, 1981) enough to build 375,475 houses.

Stock and Borden (1983) found that *D. confusus* males produce a pheromone-based secondary attraction. Abdominal extracts of males allowed to bore into fresh host logs or exposed to the vapors of subalpine fir blister resin for 24 hr were attractive in laboratory bioassays. These discoveries suggest that, as for other scolytids (Borden, 1984a), attractive semiochemicals could be used to manipulate *D. confusus* if the identity of the chemicals were known.

A preliminary analysis (Schurig et al., 1983) has disclosed that male *D. confusus* excised from subalpine fir logs after 24 hr contained *exo*- and *endo*-brevicomin in approximately an 18:1 ratio, and that *exo*-brevicomin was present as 99.2% (+)-enantiomer, while the *endo*-brevicomin was 81.6% (+)-enantiomer. Males of a European species, *D. autographus* Ratz., produce *exo*- and *endo*-brevicomin, which have proven to be attractive in field tests although the role of *exo*-brevicomin is unclear (Kohnle and Vité, 1984).

Our objectives were: (1) to isolate and identify the major volatiles found in abdominal extracts of *D. confusus* males that were allowed to bore into host logs, and females that were allowed to feed or join males in nuptial chambers, (2) to isolate and identify the volatiles in abdominal extracts of *D. confusus* males that were exposed to *A. lasiocarpa* blister resin, and (3) to test the major male-produced volatiles and host monoterpenes for their ability to attract beetles in the field to baited traps and to induce attack on baited trees.

METHODS AND MATERIALS

Collection and Maintenance of Beetles. A. lasiocarpa logs infested with *D. confusus* were obtained from trees felled at various locations in the B.C. interior and held at 0–5°C until used. When beetles were required, the logs were placed in screened cages held at approximately 28°C. The emergent beetles were collected daily, their sex was determined by the presence (females) or absence (males) of the setal brush on the frons (Bright, 1976), and the beetles were stored at 4°C on moistened paper.

Preparation of Extracts. Male beetles were placed in preformed entrance holes in the bark of fresh *A. lasiocarpa* logs, restrained by gelatin capsules

affixed outside the holes, allowed to bore into the phloem tissue for 24 hr, and then removed from the bark. Their abdomens were excised and extracted immediately in double-distilled pentane held over Dry Ice. Approximately 1200 male abdomens were extracted in this manner, and the extracts were stored at −40°C.

Female beetles were allowed to either feed on host bark or to join male beetles by placing them singly inside the gelatin capsules over the holes into which the males had bored. After 24 hr, they were excised from the bark and their abdomens extracted in the same manner as for males.

In two experiments, groups of 11 and 24 emerged male *D. confusus* were placed on moistened filter paper in individual 1.85-ml shell vials in sealed glass jars in which was placed an open, 2-ml vial containing 1.8 g (experiment. 1) or 0.8 g (experiment 2) of fresh blister resin from *A. lasiocarpa*. After 24 hr in the dark in an atmosphere saturated with the resin vapors, the beetles were removed, and their abdomens individually extracted as above. There were groups of 10 and 24 control males, respectively, for the two experiments. They were treated in the same manner as experimental beetles, except that they were not exposed to *A. lasiocarpa* resin volatiles.

Analysis of Extracts. Hewlett-Packard 5830A and 5880A gas chromatographs equipped with capillary inlet systems and flame-ionization detectors (FID) were employed for analyses by gas chromatography (GC). Samples were analyzed on open-tubular glass columns coated with SP-1000 (Supelco, Bellefonte, Pennsylvania). The temperature program for analytical GC was 70°C for 2 min, then 4°C/min to 180°C, holding for 20 min. A Varian 1200 gas chromatograph equipped with a 10:1 effluent splitter, FID, and thermal gradient collector (Brownlee and Silverstein, 1968) was used for micropreparative separation of volatiles. The column used was a 3.05-m × 3.18-mm-OD stainless-steel tube packed with 10% SP-1000 on Supelcoport (100-120 mesh). The temperature program was 70°C for 2 min, then 4°C/min to 210°C, holding for 5 min. For the micropreparative separation, the crude pentane extracts from the abdomens of 1200 males were combined and concentrated to 3 ml by evaporation under a gentle stream of N_2 at −10°C. After removal of 0.5 ml for analysis as an unfractionated control, the remaining 2.5 ml was further concentrated to ~70 μl. For both chromatographs, helium was the carrier gas, and the injection port and detector temperatures were 260°C and 270°C, respectively.

Mass spectra were determined on a Hewlett-Packard 5895A GC-MS-DS fitted with a 30-m × 0.32-mm-ID fused silica column coated with SP-1000 (J & W Scientific, Inc., Rancho Cordoba, California) using helium as the carrier gas. Peak identities were established by comparison of their mass spectra to those of authentic samples.

Chemicals for Laboratory and Field Tests. The sources and purities of

(+)-3-carene, myrcene, (−)-β-phellandrene, (±)-α-pinene, (−)-β-pinene and (±)-*exo*-brevicomin have been reported elsewhere (Conn et al., 1983). (±)-*endo*-Brevicomin (99.7%) and (±)-limonene (95.0%) were obtained from Albany International Co. (Columbus, Ohio) and Matheson, Coleman & Bell (East Rutherford, New Jersey), respectively. (±)-Myrtenol was prepared by selenium dioxide oxidation of (±)-α-pinene, and after purification via the *p*-nitrobenzoate derivative was 99.7% pure. The (+)- and (−)-*exo*-brevicomin were synthesized by Johnston and Oehlschlager (1982). Compounds synthesized by us as authentic samples for mass spectral analysis were *trans*-verbenol, verbenone *cis*- and *trans*-*p*-methen-7-ol, and 3-caren-10-ol. *trans*-Pinocarveol was a gift from L.C. Ryker, Oregon State University, Corvallis, Oregon.

Laboratory Bioassays. Tests for response to the fractions of the abdominal extracts (experiment 1) as well as (±)-*exo*-brevicomin and its enantiomers (experiment 2) (Table 1) were done with a walking bioassay in an arena olfactometer, using Stock and Borden's (1983) modifications of the basic design and procedures used by Wood and Bushing (1963). Each stimulus was tested with 5 groups of 15 beetles each. In each experiment, pentane solvent controls were used, as were attractive controls comprising the crude abdominal extracts.

Field Trapping Experiments. Two randomized-block trapping experiments were conducted in 1984 in an overmature stand of spruce and subalpine fir at Prospect Creek, approximately 40 km northwest of Merritt, B.C. Both experiments utilized eight-funnel multiple-funnel traps (Lindgren, 1983) placed at least 30 m apart on isolated lines through the forest. The first experiment tested (±)-*exo*-brevicomin alone, in combination with one of the six major monoterpenes in *A. lasiocarpa* (Zavarin et al., 1971) (myrcene, 3-carene, α-pinene, β-pinene, β-phellandrene, or limonene), or in combination with an equal part mixture of all six monoterpenes. The second tested myrtenol alone, in combination with two monoterpenes (myrcene and 3-carene), or in combination with *exo*-brevicomin and the two monoterpenes. Numbers of replicates, dates, types of bait receptacles and release rates are given in Table 2.

Field Tests with Baited Trees. On June 12, 1983, a 10-replicate experiment was set up in an overmature stand of white spruce, *Picea glauca* (Moench) Voss, and subalpine fir at McKendrick Pass, approximately 30 km northwest of Smithers, B.C. Ten *A. lasiocarpa* [$\bar{X}$ diameter at breast height (dbh) ± SE = 42.1 ± 2.7 cm] were baited with *exo*-brevicomin (Table 3) and 10 trees ($\bar{X}$ dbh ± SE = 44.3 ± 3.0 cm) were marked as controls. The trees were on a line, with a minimum distance of 50 m between trees. The treatments for each two-tree block (one baited and one control tree) were assigned randomly.

On October 6, 1983, each baited tree was checked for attack by *D. confusus*. Trees with loose, granular frass in the bark crevices or around the base of the bole were judged to be successfully mass-attacked. Those with no gran-

TABLE 1. RESPONSE OF FEMALE *D. confusus* IN LABORATORY BIOASSAYS TO ABDOMINAL EXTRACT OF MALES THAT BORED INTO HOST LOGS FOR 24 HR AND FRACTIONS OF EXTRACT (EXPERIMENT 1), AS WELL AS TO RACEMIC *exo*-BREVICOMIN AND ITS ENANTIOMERS (EXPERIMENT 2) (75 FEMALES TESTED PER STIMULUS)

Exp. No.	Stimulus	Dose	Responders (%)[a]
1	Pentane, solvent control	20 μl	12.0 a
	Abdominal extract of males in log 24 hr,		36.0 bc
	attractive control	2 beetle equiv.	
	Fractions (with major constituents)		
	1, solvent	2 beetle equiv.	10.7 a
	2, monoterpenes	2 beetle equiv.	17.3 ab
	3, *exo*- and *endo*-brevicomin, monoterpenes	2 beetle equiv.	48.0 c
	4, sesquiterpene	2 beetle equiv.	26.7 abc
	5, terpene alcohols, sesquiterpenes	2 beetle equiv.	28.0 abc
	6, trace unkowns	2 beetle equiv.	14.7 ab
	7, terpene alcohols, sesquiterpenes	2 beetle equiv.	18.7 ab
	8, trace unknowns	2 beetle equiv.	14.7 ab
2	Pentane, solvent control	20 μl	5.3 a
	Abdominal extract of males in log 24 hr,		
	attractive control	2 beetle equiv.	36.0 bcd
	(±)-*exo*-brevicomin	500 ng	40.0 cd
		50 ng	38.7 bcd
		5 ng	58.7 d
	(−)-*exo*-brevicomin[b]	500 ng	38.7 bcd
		50 ng	36.0 bcd
		5 ng	16.0 b
	(+)-*exo*-brevicomin	500 ng	54.7 cd
		50 ng	38.7 bcd
		5 ng	32.0 bc

[a] Percents within an experiment followed by same letter not significantly different, Newman-Keuls test modified for comparing proportions (Miller, 1981), $P < 0.05$.
[b] (−)-*exo*-Brevicomin 96% enantiomerically enhanced (ee), (+)-*exo*-brevicomin 95% ee.

ular frass, but with clear resin streaming from points at which the beetles had penetrated to the sapwood were judged to be unsuccessfully attacked. Every tree >20 cm dbh within a 10-m radius of the baited or control trees was also assessed for attack.

On June 8 and 27, 1984, a 12-replicate, nine-treatment, randomized-block experiment was set up with 10 replicates at McKendrick Pass, and two replicates at Prospect Creek. The trees were at least 25 m apart and had a $\bar{X}$ dbh (±SE) of 39.1 ± 0.8 cm. They were baited with the same baits as in the first

TABLE 2. RESPONSE OF *D. confusus* AND RED-BELLIED CLERIDS, *Enoclerus sphegeus*, AT PROSPECT CREEK TO MULTIPLE-FUNNEL TRAPS BAITED WITH *exo*-BREVICOMIN ALONE OR WITH REPRESENTATIVE MONOTERPENES FROM *A. lasiocarpa* (EXPERIMENT 1), AND WITH MYRTENOL ALONE AND IN COMBINATION WITH SELECTED MONOTERPENES AND *exo*-BREVICOMIN (EXPERIMENT 2)

xperiment, :plicates ıd dates	Stimulus[a]	No. *D. confusus* captured ($\bar{X} \pm$ SE)[b] Males	Females	Total	No. *E. sphegeus* captured ($\bar{X} \pm$ SE)[b]
10 replicates,	Unbaited control	1.1 ± 0.7 a	1.3 ± 0.9 a	2.4 ± 1.7 a	0.0 a
5 from June 27	eB	5.3 ± 1.5 b	2.8 ± 1.4 a	8.1 ± 2.7 b	1.7 ± 0.8 b
to July 20 and	eB, M	8.9 ± 3.7 b	6.6 ± 4.1 a	15.5 ± 2.7 b	2.2 ± 0.5 b
5 from July,	eB, 3C	7.1 ± 2.4 b	5.2 ± 2.4 a	12.3 ± 4.5 b	2.1 ± 0.8 b
20 to 27, 1984	eB, αP	8.3 ± 5.2 b	5.7 ± 4.1 a	14.0 ± 9.3 b	3.6 ± 1.2 b
	eB, βP	5.1 ± 1.3 b	3.8 ± 1.7 a	8.9 ± 2.8 b	5.0 ± 2.0 b
	eB, L	4.2 ± 1.4 b	2.6 ± 0.7 a	6.8 ± 1.8 b	2.3 ± 1.1 b
	eB, βPh	4.7 ± 1.4 b	1.8 ± 1.1 a	6.5 ± 2.4 b	2.2 ± 1.2 b
	eB, Tm	6.4 ± 2.5 b	4.0 ± 2.1 a	10.4 ± 4.5 b	3.6 ± 1.2 b
9 replicates,	Unbaited control	0.3 ± 0.2 a	0.9 ± 0.5 ab	1.2 ± 0.8 a	
July 27–	Mr	0.2 ± 0.2 a	0.2 ± 0.2 a	0.4 ± 0.2 a	
August 13,	eB, M, 3C	1.2 ± 0.3 ab	1.2 ± 0.6 ab	2.4 ± 0.7 ab	
1984	M, 3C, Mr	2.5 ± 1.6 ab	3.6 ± 2.1 ab	6.1 ± 3.5 ab	
	eB, M, 3C, Mr	6.2 ± 2.4 b	6.3 ± 2.8 b	12.6 ± 5.0 b	

ɔB = *exo*-brevicomin, Mr = myrtenol, M = myrcene, 3C = 3-carene, αP = α-pinene, βP = β-pinene, L = limonene, βPh = β-phellandrene, and Tm = terpene mix (equal parts, all six). Release devices and rates ıs determined at approx. 21°C in the laboratory as follows: eB, 1 bubble cap and 1 glass capillary (1.0 mm nternal diameter), 1.0 mg/24 hr; Mr, single, 1.9 ml, open microcentrifuge tube, 0.9 mg/24 hr; monoterɔenes, two closed, low-density polyethylene microcentrifuge tubes, 4–20 mg/24 hr.
Means within a column for each experiment followed by same letter are not significantly different, three-way ANOVA on data transformed by $\log_{10}(x + 1)$ followed by Newman-Keuls test, $P < 0.05$. *E. sphegeus* not collected in experiment 2.

trapping experiment (experiment 1, Table 2). Twelve trees were left unbaited as controls. On September 11, 1984, the trees were assessed as in 1983 for attack by *D. confusus*.

RESULTS

Chemical Identification and Laboratory Bioassays. The most prominent potential pheromone in the abdominal extract of males that had bored into *A. lasiocarpa* logs for 24 hr was the bicyclic ketal, *exo*-brevicomin (Figure 1). Other compounds present in these male abdominal extracts were: *endo*-brevicomin, *trans*-verbenol, verbenone, myrtenol, *trans*-pinocarveol, *cis*- and *trans*-

TABLE 3. ATTACK BY *D. confusus* ON AND AROUND SUBALPINE FIR TREES BAITED WITH *exo*-BREVICOMIN[a] (MCKENDRICK PASS, B.C., 1983)

	Experimental trees					Surrounding trees >20 cm dbh within 10 m					
		Total attacked		Successfully mass-attacked				Total attacked[b]		Successfully mass-attacked	
Treatment	No. Trees	No.	%	No.	%	No. dead trees	No. living trees	No.	%	No.	%
Baited	10	10	100	6	60	3	187	45	24.1	5	2.3
Control	10	1	10	0	0	6	153	12	7.8	1	0.7

[a] Released from two glass capillaries (1.0 mm internal diameter) at rate of 1.0 mg/24 hr determined in the laboratory at 21 °C.
[b] Significant difference between numbers of attacked trees, χ^2 test, $P < 0.01$.

p-menthen-7-ol, 3-caren-10-ol, plus several monoterpenes and sesquiterpenes (Figure 1). *exo*-Brevicomin was present in highest levels in unmated males in host bark, and declined in amount after they were joined by females. There was no *exo*-brevicomin in emergent males (Figure 2), but all males (emergent, fed, or fed and mated) contained *trans*-verbenol. Only *trans*-verbenol was detectable in females that were either fed on *A. lasiocarpa* bark or allowed to join males in new galleries.

Bioassay results indicated that male abdominal extracts were attractive to females, as was the gas chromatographic fraction containing *exo*- and *endo*-brevicomin (Table 1). Significant responses were obtained to racemic *exo*-brevicomin, and both of its enantiomers (Table 1). However, the observed attraction to (−)-*exo*-brevicomin was probably due to the presence of a small amount (approx. 2%) of the (+)-enantiomer. This result also indicates that the (−)-enantiomer does not inhibit the response of the females to naturally occurring (+)-*exo*-brevicomin.

There was no significant response to any fraction other than that containing *exo*-brevicomin (Table 1). Moreover, there was no increase in response over that to the *exo*-brevicomin fraction when all fractions were combined. To investigate the possibility that, as in other scolytids, there was a terpene alcohol pheromone that was disclosed in males allowed to bore into logs, the resin exposure experiments were conducted.

Abdominal extracts of males that had been exposed to *A. lasiocarpa* blister resin contained very few compounds. However, the terpene alcohol, myrtenol, a derivative of α-pinene, was detectable in male abdominal extracts in both experiments (Figure 2).

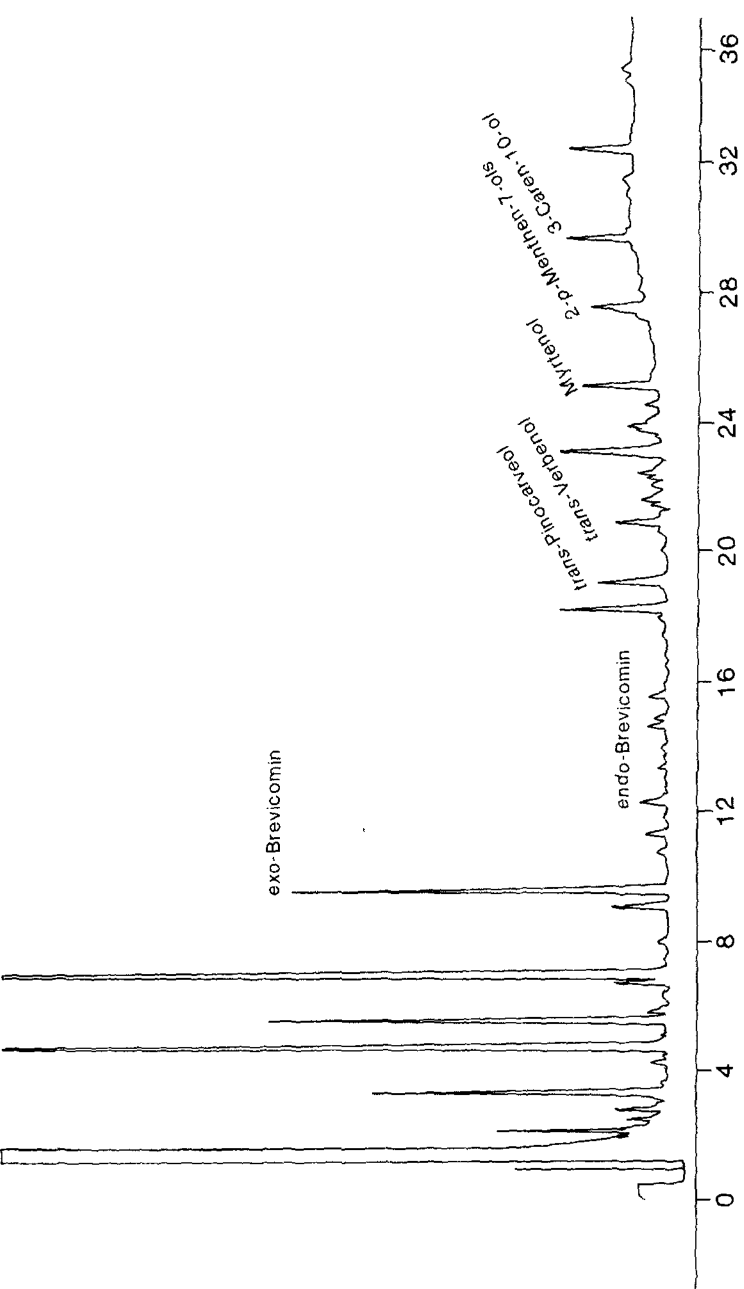

FIG. 1. Gas–liquid chromatogram of volatiles from male *D. confusus* allowed to bore into *A. lasiocarpa* logs for 24 hr. Volatiles were isolated from crushed abdomens by steam distillation (Godefroot et al., 1981). Peaks eluting earlier than *exo*-brevicomin are monoterpenes. Peaks with retention times of 18, 23, and 32.3 min are unknown sesquiterpenes. A 28-m × 0.66-mm-ID glass column coated with SP-1000 was employed for analysis.

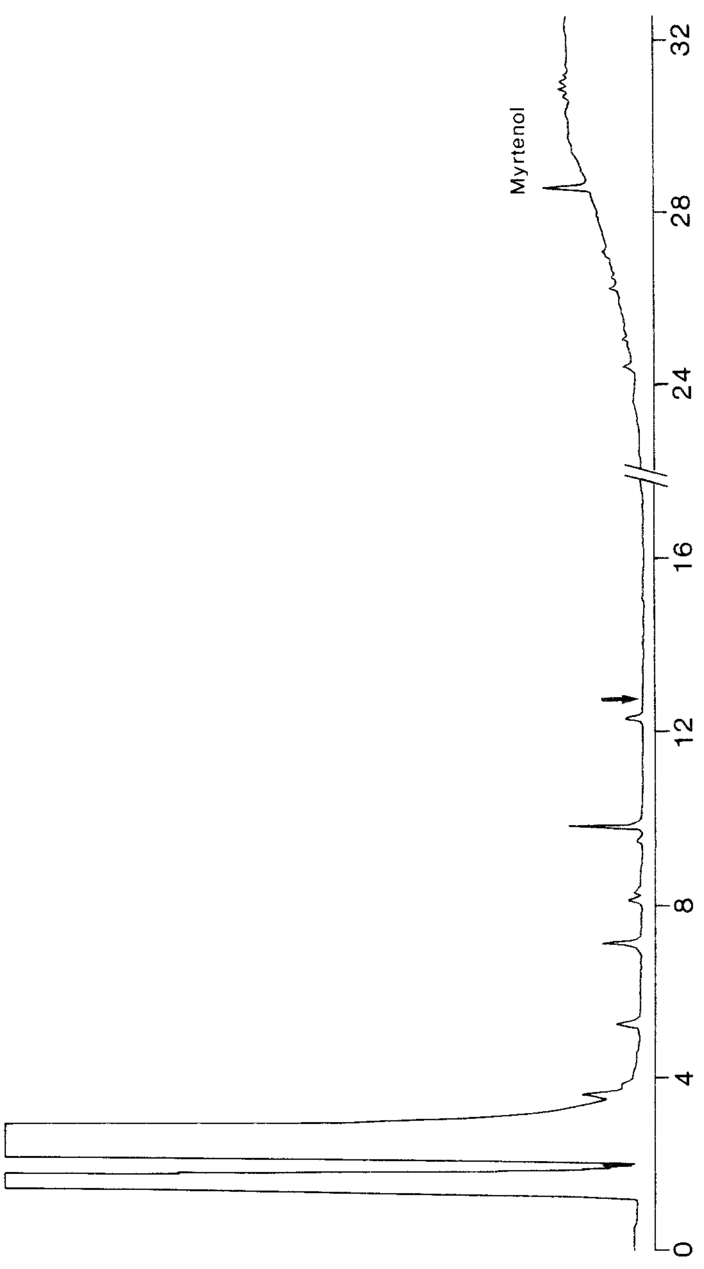

FIG. 2. Gas–liquid chromatogram of the pentane abdominal extract of a representative male *D. confusus* exposed to the volatiles of *A. lasiocarpa* blister resin for 24 hr. Arrow denotes retention time of *exo*-brevicomin. A 30-m × 0.5-mm-ID glass column coated with SP-1000 was employed for analysis.

Field Trapping Experiments. Male beetles were attracted to racemic *exo*-brevicomin alone or in combination with selected monoterpenes (experiment 1, Table 2). The monoterpenes caused neither a significant increase nor a decrease in response. However, as responses to *exo*-brevicomin mixed with myrcene or 3-carene were consistently high, the two nonoterpenes were included as baits in subsequent experiments.

The experiment with myrtenol was done late in the season when so few beetles were present that the responses to *exo*-brevicomin with the two monoterpenes were not significantly higher than to the unbaited control traps (experiment 2, Table 2). However, when myrtenol was added to the baits, the total response was over five times higher than to *exo*-brevicomin and monoterpenes, and the responses by both sexes were the only ones to stand alone as significantly higher than those to the unbaited control traps (experiment 2, Table 2).

Field Tests with Baited Trees. All 10 trees baited with *exo*-brevicomin in 1983 were attacked by *D. confusus*, and six of these were successfully mass attacked (Table 3). Moreover, there was a significantly higher number of attacked trees surrounding the baited trees than the unbaited control trees. As there were 4.3 and 4.7 attacked trees/plot surrounding unsuccessfully and successfully attacked baited trees, respectively, a successful attack does not appear to be necessary to induce attacks on trees around a baited tree. In comparison to the control plots, the baited trees caused an unnatural shift in the diameter distribution of surrounding trees attacked (Figure 3). Many trees in the 20 to 25-cm-dbh class were attacked around the baited trees, probably because they were simply in the path of beetles orienting upwind in the odor plume. No trees

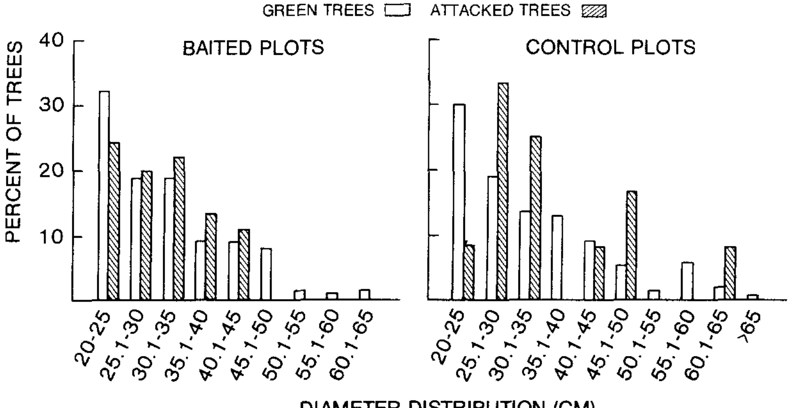

FIG. 3. Comparison of percent of attacked and unattacked *A. lasiocarpa* by diameter class in 10-m-radius plots surrounding 10 trees baited with *exo*-brevicomin and 10 unbaited, control trees. McKendrick Pass, B.C. 1983. Release devices and rates as in Table 3.

TABLE 4. ATTACK BY *D. confusus* ON SUBALPINE FIR TREES BAITED WITH *exo*-BREVICOMIN ALONE OR IN COMBINATION WITH SELECTED MONOTERPENES (TEN REPLICATES AT MCKENDRICK PASS, B.C., AND TWO REPLICATES AT PROSPECT CREEK, B.C., 1984)

Treatment[a]	No. Trees	Total attacked No.	Total attacked %[b]	Successfully mass-attacked No.	Successfully mass-attacked %[b]
Unbaited control	12	2	16.7 a	0	0.0 a
eB	11	11	100.0 b	6	54.5 b
eB, M	12	12	100.0 b	6	50.0 b
eB, 3C	12	12	100.0 b	9	75.0 b
eB, αP	11	11	100.0 b	8	72.7 b
eb, βP	12	11	91.7 b	8	66.7 b
eb, βPh	12	12	100.0 b	9	75.0 b
eB, L	12	10	83.3 b	7	58.3 b
eB, TM	12	12	100.0 b	6	50.0 b

[a] Abbreviations of volatiles, release devices, and rates of release as in Table 2 except that eB was released from two bubble caps.
[b] Percents within a column followed by same letter not significantly different, Newman-Keuls test modified for comparing proportions (Miller, 1981), $P < 0.05$.

>45 cm dbh were attacked. In contrast, only one of the 12 attacked trees in the control plots were in this diameter class, and three attacked trees (25%) were >45 cm dbh.

Addition of monoterpenes to the *exo*-brevicomin baits, at an approximate rate of 20 mg/24 hr release, had no evident effect on attack by *D. confusus* (Table 4). Almost all of the baited trees were attacked. From 50 to 75% were successfully attacked, and there were no significant differences in attack frequencies. The 25% variation in attack frequency was probably due more to local availability of beetles than to the attractiveness of the baits.

Response by Red-Bellied Clerid. Significant numbers of red-bellied clerids, *Enoclerus sphegeus* (F.), responded to traps baited with *exo*-brevicomin alone (Table 2). Their response was not significantly increased by addition of single monoterpenes or the monoterpene mixture to *exo*-brevicomin.

DISCUSSION

Until the discovery of *exo*-brevicomin in *D. confusus* (Schurig et al., 1983) and the demonstration that it is attractive to both sexes (Tables 1 and 2), it had been

reported as an aggregation pheromone exclusively in *Dendroctonus* spp. (Borden, 1984b). It has also been detected in male *Leperisinus varius* (F.) for which it, in combination with two other male-produced volatiles, had an antiaggregative effect (Francke et al., 1979). Thus, as investigations proceed in the Scolytidae, it is likely that restriction of certain pheromones to only a few taxa will prove to be unfounded, supporting Blum's (1970) concept of pheromonal parsimony between diverse taxa.

The report by Kohnle and Vité (1984) that *exo*- and *endo*-brevicomin were equally attractive to *D. autographus* in field tests is unusual, as this is the first report of attraction to both isomers in the Scolytidae. As (+)-, (−)-, and (±)-*endo*-brevicomin are now available in our laboratory and as (+)-*endo*-brevicomin has also proven to be a potent aggregation pheromone for the Southern pine beetle *Dendroctonus frontalis* Zimmerman (Vité et al., 1985), we plan to test these materials in combination with *exo*-brevicomin as potential attractants for *D. confusus.*

The disclosure of myrtenol in extracts of males exposed to *A. lasiocarpa* blister resin for 24 hr (Figure 2) suggests that it is a major derivative of α-pinene inhaled by the beetles. Both sexes of *Ips paraconfusus* (Lanier) and *Dendroctonus brevicomis* LeConte produced (−)-myrtenol following exposure to vapors of (−)-α-pinene (Byers, 1983). Myrtenol is also a common constituent of volatiles produced by other scolytids, such as the Douglas fir beetle, *Dendroctonus psendotsugae* Hopkins (Rudinsky, 1976) and *Ips typographus* (Birgersson et al., 1984), and has been identified as a multifunctional pheromone in the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Rudinsky et al., 1974). It is probable that allowing males to bore into host logs in the laboratory does not accurately duplicate conditions present in a living host attacked by aggessive bark beetles. Therefore, a practice of exposing beetles of the pheromone-producing sex to host volatiles as well as allowing them to bore into host logs should probably be followed in order to reconstitute conditions that occur in living host trees in the field.

The finding that *exo*-brevicomin and myrtenol are attractive to *D. confusus* in the field (Table 2) suggests that they are the principal aggregation pheromones in this species. However, additional research should test *endo*-brevicomin and the other terpene alcohols found in male beetles for attraction in the field and should evaluate myrtenol as a bait on trees.

Although several clerids are known to use bark beetle pheromones as host-finding kairomones (Borden, 1982), the response by *E. sphegeus* to *exo*-brevicomin is the first recorded instance of such a phenomenon for this species.

The reliability with which *exo*-brevicomin induced attack on baited trees with or without monoterpenes (Tables 3 and 4) and concentrated attack on adjacent trees (Figure 3) suggests that it may have utility in manipulating populations of *D. confusus.*

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ACTIVITY OF VOLATILE COMPOUNDS IN GLANDULAR TRICHOMES OF *Lycopersicon* SPECIES AGAINST TWO INSECT HERBIVORES

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Abstract—Several major chemicals in the glandular heads of type VI trichomes of *Lycopersicon* species were identified and quantified by gas chromatography and mass spectrometry. Two normal odd-chained ketones, 2-undecanone (47 ng) and 2-tridecanone (146 ng), and one unknown sesquiterpene (5 ng), comprised approximately 95% of the contents of a gland of *L. hirsutum* f. *glabratum* Mull. In a closely related plant, *L. hirsutum* Humb. & Bonpl. (LA 361), two unknown insecticidal sesquiterpenes accounted for 6% of the gland contents. Additionally, small amounts of one unknown monoterpene and another unknown sesquiterpene were found in type VI glands of a commercial tomato variety, *L. esculentum* Mill. Bioassays comparing the gland exudate (by direct contact) and isooctane extracts of glands to neonate larvae of *Keiferia lycopersicella* (Walsingham) (Lepidoptera: Gelechiidae) and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) indicated that: (1) 2-tridecanone and 2-undecanone were the major insecticidal compounds in *L. hirsutum* f. *glabratum*, (2) the two unknown sesquiterpenes in *L. hirsutum* were acutely toxic to both species, and (3) gland contents in the commercial tomato variety provided only a physical barrier to *K. lycopersicella*, and were not detrimental to *S. exigua*. In topical bioassay trials, synthetic mixtures of 2-tridecanone and 2-undecanone (3 : 1) demonstrated potentiation. Concentrations of these chemicals decreased as trichomes aged. Quantities of insecticidal chemicals and density of type VI trichomes varied with plant age and location within plants.

Key Words—*Lycopersicon*, *Keiferia lycopersicella*, *Spodoptera exigua*, Lepidoptera, Gelechiidae, Noctuidae, leaf trichomes, 2-tridecanone, 2-undecanone, terpenoids, toxicity.

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INTRODUCTION

Glandular trichomes of wild tomatoes in the genus *Lypersicon* have recently become the subject of considerable study because of their effectiveness in protecting tomato plants from insect damage (Rodriguez et al., 1972; Schuster, 1977; Dimock and Kennedy, 1983; Schwartz and Snyder, 1983; Snyder and Carter, 1984; Kennedy and Sorenson, 1985) and the possibility of breeding this character into the commercial tomato *L. esculentum* (Kennedy and Henderson, 1978; Fery and Kennedy, 1983; Fery et al., 1984). The secretion of glandular trichomes from one tomato accession, *L. hirsutum* Humb. & Bonpl. (PL 251301), has also proven toxic to the spider mite *Tetranychus urticae* Koch (Aina et al., 1972), suggesting that trichome-based resistance could be an effective tool in a pest management program against a wide variety of arthropod pests.

Relatively few chemical analyses of trichome contents or exudates in tomato species have been reported. Studies by Beckman et al. (1972) found phenolics in capitate cells of four-lobed trichomes of *L. esculentum*, and Duffey and Isman (1981) determined that rutin (a flavonoid glycoside) was a major constituent of the trichomes in commercial tomatoes. More recently, Kennedy and Dimock (1983) reported that 2-tridecanone (a methyl ketone) was the dominant compound in the type VI glands (characterized by Luckwill, 1943) of *L. hirustum* f. *glabratum* (PL 134417) which was acutely toxic to *Manduca sexta* (L.), *Heliothis zea* (Boddie), and *Leptinotarsa decemlineata* (Say).

Our previous studies on resistance in wild and commercial tomatoes to a specialist herbivore, the tomato pinworm (TPW), *Keiferia lycopersicella*, demonstrated that type VI glandular trichomes were the primary cause of larval mortality (Lin and Trumble, 1986). In this paper we: (1) quantify the constituents of glandular heads of type VI trichomes from selected wild and commercial tomato accessions, (2) document effects of trichome age and location within plants on chemical composition of type VI trichomes, and (3) report a bioassay procedure designed to quantify the insecticidal activity of these compounds for both *K. lycopersicella*, and a generalist herbivore, the beet armyworm (BAW), *Spodoptera exigua*.

METHODS AND MATERIALS

All plants used in the following tests were grown from seed in a glasshouse in 15-cm pots containing UC soil mix (Matkin and Chandler, 1957) and a slow-release fertilizer. Day length was maintained at 16 hr using artificial illumination. Seeds of *L. hirsutum* (LA 361) were obtained from Dr. C. Rick (University of California, Davis) and *L. hirsutum* f. *glabratum* (PL 134417) were obtained from Dr. G. Kennedy (North Carolina State University). Commercial *L.*

esculentum cv. VFN 7718 were purchased from Champion Seed Co. (529 Mercury Lane, Brea, California 92621).

Identification and Quantification of Chemicals in Type VI Glands. All gland samples were analyzed for chemical content using a capillary gas chromatograph (GC) and GC-mass spectrometer. The GC was a Packard gas chromatograph equipped with hydrogen flame ionization detector and a DB-5 60-m × 0.25-mm-ID capillary column. Temperature was programmed from 45 to 210°C at 1°C/min. The head pressure for helium was 20 pounds per square inch. The flow rates for hydrogen and compressed air were 30 and 200 ml/min, respectively. Internal standards for quantitative analyses were 2-decanone and 2-pentadecanone peak areas and retention time(s) were calculated using a Spectra-Physics Autolab System I program integrator (333 N. First St., San Jose, California). Electron-impact mass spectra were recorded from samples at 70 eV with a VG ZAB-HF mass spectrometer. Comparisons of chemical quantities in glandular trichomes were evaluated with the Student's t test.

Identification of the chemical contents in glandular heads of type VI trichomes was based on three samples of 100 intact mature glands which were collected from the adaxial, interveinal areas of three leaflets per plant (8th–9th leaves from the bottom) for five plants of each variety ($N = 45$ samples of 100 glands/variety; plants were 123 days postgermination). With the aid of a dissecting microscope, a glass probe was used to rupture the glands and transfer their contents directly into isooctane. The probe was rinsed in ethanol and isooctane after collecting each 100-gland sample to avoid any contamination of subsequent samples. Additional samples of 200–4500 glands were also collected for identification of other possible minor constituents. All glands were collected between 11 AM and 2 PM to minimize potential diel variation in chemical production (Turner et al., 1980).

Effect of Trichome Age and Location within Plants on Chemical Composition of Type VI Glands. Fully developed type VI trichomes exhibited two stages which were clearly discernible using a dissecting microscope. In the initial mature stage, the trichomes possessed glandular heads with a clear liquid content. The subsequent stage, referred to as aged, had yellow-orange glandular heads which appeared to have a more viscous content. In order to determine if gland age affected the chemical content, three 200 gland samples of each maturity stage were collected from four plants each of *L. hirsutum* and *L. hirsutum* f. *glabratum*, and prepared for analysis as described previously ($N = 12$ samples of 200 glands/maturity stage). Mature and aged trichomes were collected from the same leaves.

The influence of location within plants on chemical content of type VI trichomes was evaluated by collecting 200 gland samples from three locations per plant on five plants each of *L. hirsutum* and *L. hirsutum* f. *glabratum*. To standardize the test material, glands were collected weekly from 2–5-week post-emergence-old plants on: (1) the terminal leaflet of the third leaf from the bot-

tom, (2) the stem between the second and third lowest nodes, and (3) terminal leaflet of 1-week-old leaf at the plant apex. This plant stage was chosen because the tomatoes were in the critical developmental period following germination, and the specific leaves tested began to senesce at five weeks. In addition, number of type VI glands per unit leaf area was determined using a micrometer grid reticule and a dissecting microscope.

Bioassays of Gland Exudate, Gland Extracts, and Pure Compounds. TPW and BAW used in these trials were obtained from cultures initiated with larvae collected in Orange County, California and maintained on tomato plants (VFN 7718) and artificial diet (Patana, 1969), respectively. Insects were maintained at 27 ± 1°C and a photoperiod of 12:12 light–dark. Mature type VI glands were collected from terminal leaflets (*N* = 12 leaflets/variety) of the eighth and ninth leaves from the base of 2-month-old *L. hirsutum*, *L. hirsutum* f. *glabratum*, and *L. esculentum*. Additional type VI glands (*N* = 12 samples of 200/leaflet) were collected for GC quantitative analysis in order to document concentrations of key compounds in fresh plant material for comparison of isooctane gland extracts with pure compounds. Data on the toxicity resulting from topical applications of gland exudate, isooctane extracts of glands, and pure compounds to BAW and TPW were standardized by transforming all data to nanograms of chemicals per larva to allow direct comparisons between treatments. Data were then analyzed by probit analysis with the Proc Probit procedure of the Statistical Analysis System (Helwig and Council, 1979) after correction for control mortality with Abbott's (1925) formula.

First-instar larvae of BAW and TPW were exposed directly to gland exudate on fresh plant material by adhering individuals to a water-moistened 000 paint brush, and with the aid of a dissecting microscope, breaking type VI glands against the dorsal thorax. Four replicates of 15 larvae of TPW were treated at the following gland "dosage" (*N* = 60 larvae/dose) for the two wild tomato accessions tested: *L. hirsutum* = 1, 2, 4, 6, 8 glands/larva, and control (moistened brush treatment only); *L. hirsutum* f. *glabratum* = 1, 2, 3, 4, 5, and control. Similarly, BAW were exposed as follows: *L. hirsutum* = 20, 30, 40, 50, 60, and control; *L. hirsutum* f. *glabratum* = 12, 15, 18, 21, 24, and control. Mortality was also observed in trials with *L. esculentum*, where TPW or BAW were exposed to 20 or 60 glands, respectively, the maximum possible for the size of the larvae. Larvae were held at 26 ± 1°C, and mortality was evaluated at 24 hr. Larvae were considered dead if they were unable to move within 30 sec of being prodded.

In a related experiment, neonate larvae of both insect species were exposed to an extract of glands dissolved in isooctane. Isooctane was selected as a carrier because this solvent was used for all GC analyses. Gland contents were collected with a glass probe as described earlier, and serial dilutions of concentrate were topically applied to neonate larvae using a 0.5-μl Hamilton syringe. Be-

cause of the small size of the larvae, dosages were applied to the dorsum of the thorax in a 0.01-μl droplet for TPW, and in a 0.2-μl droplet for BAW. Larvae were treated on a glass surface instead of filter paper, since our preliminary trials indicated that efficacy was artificially reduced on filter paper as some test material was lost to absorption into the substrate. Four replicates of 15 larvae of each species were exposed to the extract of each of the five dosages and isooctane controls evaluated, and mortality was assessed at 24 hr. Dosages tested for TPW were 60, 70, 80, 90, and 100 glands of *L. hirsutum* f. *glabratum*/μl of isooctane and 30, 40, 50, 60, and 70 glands of *L. hirsutum*/μl of isooctane. Dosages evaluated for BAW were 60, 70, 80, 90, and 100 glands of *L. hirsutum* f. *glabratum*/μl of isooctane and 10, 20, 30, 40, and 50 glands of *L. hirsutum*/μl of isooctane.

The contact toxicities of the two commercially available pure chemicals, 2-undecanone and 2-tridecanone (purity 97.0%, Pfaltz & Bauer, Inc., Waterbury, Connecticut), were also evaluated. Each compound was dissolved in isooctane to a concentration of 50 mg/ml, as was a 3 : 1 mixture of 2-tridecanone and 2-undecanone. Neonate larvae of TPW and BAW were treated with five concentrations (dosages) of the test chemicals and a control (isooctane). Dosages tested for TPW were 3.6, 4.0, 4.8, 5.6, and 6.4 mg of 2-tridecanone/ml of isooctane; 2.0, 2.4, 3.2, 4.0, and 4.8 mg of 2-undecanone/ml of isooctane; and 0.1, 0.3, 0.5, 0.7, and 0.8 mg of mixture/ml of isooctane. Dosages evaluated for BAW were 12, 14, 16, 18, and 20 mg of 2-tridecanone/ml of isooctane; 12, 14, 16, 18, and 20 mg of 2-undecanone/ml of isooctane; and 6, 8, 10, 12, and 14 mg of mixture/ml of isooctane. Data were analyzed for potentiation after Finney (1971). All topical applications were made with a 0.5-μl Hamilton syringe, using the same equipment and procedures as previously described.

RESULTS AND DISCUSSION

Identification and Quantification of Chemicals in Individual Glands. GC analyses of isooctane extracts of the type VI glands of *L. hirsutum* f. *glabratum* detected three major components: 2-tridecanone, 2-undecanone, and one unknown compound in the relative proportion of 30 : 10 : 1, respectively, (Figure 1). The predominance of 2-tridecanone was consistent with previous reports documenting the presence of this chemical in foliage of *L. hirsutum* f. *glabratum* (Soost et al., 1968; Dimock and Kennedy, 1983). The relatively high proportion of 2-undecanone was unexpected. Mass spectrometric analysis of the unknown compounds produced different fragmentation patterns with identical molecular ions at *m/e* 204 (Table 1), which are consistent with the assignment of the molecular formula of a sesquiterpene (SES) ($C_{15}H_{24}$) (Silverstein et al., 1974).

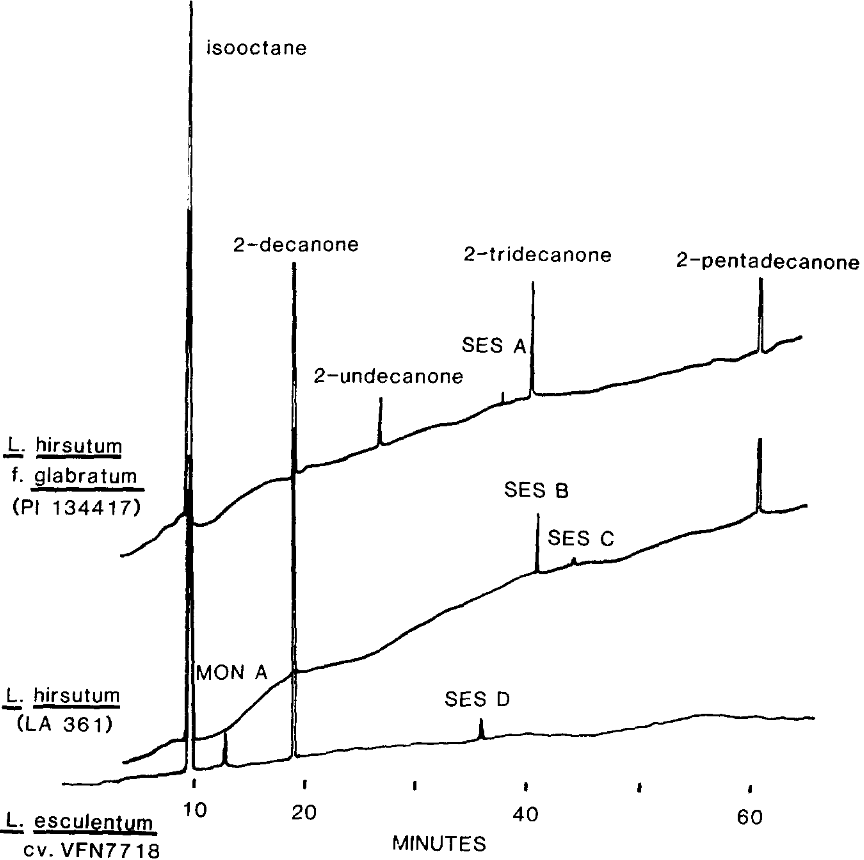


FIG. 1. Chromatograms of isooctane extracts of glandular heads of type VI trichomes from *L. hirsutum* (LA 361), *L. hirsutum* f. *glabratum* (PI 134417), and *L. esculentum* (VFN 7718) using a DB-5 0.25-mm-ID × 60-m long capillary column on a Packard gas chromatograph.

In *L. hirsutum* (LA 361), two unknown sesquiterpenes (SES B and SES C) were detected by GC-MS analysis (Table 1, Figure 1). The retention time of the unknown SES B was very similar to 2-tridecanone, but augmentation with 2-tridecanone confirmed that SES B was different. In *L. esculentum*, one unknown monoterpene and one unknown sesquiterpene (SES D, which was different from SES A, B, and C; Figure 1) were found to be the major volatile components. Although Soost et al. (1968) reported several ketone groups as the major volatile compounds in whole foliage of *L. hirsutum* and *L. esculentum* cv. Ace, our experiments demonstrated that there were neither 2-tridecanone nor other methyl ketones in the glandular heads of type VI trichomes of *L hir-*

TABLE 1. MASS SPECTRA[a] OF MAJOR UNKNOWN COMPONENTS IN GLANDULAR HEADS OF TYPE VI TRICHOMES FROM *L. hirsutum* (LA 361), *L. hirsutum* f. *glabratum* (PI 134417), AND *L. esculentum* CV. VFN 7718

Variety	Compound	*m/e* (% relative intensity)				
L. hirsutum	Sesquiterpene A[b]	204(13)	161(34)	148(31)	133(91)	120(42)
f. *glabratum*	($C_{15}H_{24}$)	119(39)	107(46)	105(60)	93(99)	91(84)
		81(41)	79(81)	77(48)	69(75)	67(41)
		53(38)	41(100)	39(42)		
L. hirsutum	Sesquiterpene B[b]	204(13)	120(12)	119(97)	105(15)	93(100)
	($C_{15}H_{24}$)	92(17)	91(34)	77(30)	69(36)	56(15)
		55(14)	41(36)	39(10)		
	Sesquiterpene C[b]	204(22)	121(100)	107(61)	105(65)	93(79)
	($C_{15}H_{24}$)	91(66)	79(65)	53(63)	41(94)	39(55)
L. esculentum	Monoterpene A[b]	136(30)	121(10)	93(100)	77(48)	68(22)
	($C_{10}H_{16}$)	53(20)	41(59)			
	Sesquiterpene D[b]	204(20)	189(11)	161(28)	148(20)	133(68)
	($C_{15}H_{24}$)	120(32)	105(43)	93(100)	79(80)	69(72)
		53(36)	41(100)			

[a] See text for GC-MS conditions. The *m/e* values for the unknowns are arranged in order of descending fragment size.
[b] Tentative compound identification based on GC and MS data.

sutum (LA 361) and *L. esculentum* cv. VFN 7718. Even though the GC retention time of the major unknown monoterpene in *L. esculentum* cv. VFN 7718 was close to limonene, which was reported as the major volatile on cv. Ace (Soost et al., 1968), the mass spectrum demonstrated that these compounds were different. The identification of these unknown terpenoids in *L. hirsutum* and *L. esculentum* is currently under investigation. To date, the quantities of glandular material have not been sufficient for qualitative analysis.

Gas chromatography revealed an average of 146 ng of 2-tridecanone, 49 ng of 2-undecanone, and 5 ng of sesquiterpene A per type VI gland of *L. hirsutum* f. *glabratum*, constituting approximately 93% of the estimated volume (Table 2). These results are in contrast to those obtained earlier by Kennedy and Dimock (1983), who reported only ca. 6.3 ng of 2-tridecanone in a glandular head of *L. hirsutum* f. *glabratum*. Such differences might occur in response to method of extraction, the GC sensitivity level, photoperiod during plant growth or, as noted in the following section, gland age, plant developmental status, or location within pl?

The relative concentrations ca .ne volatile compounds in the other *Lycopersicon* accessions tested were considerably less than for *L. hirsutum* f. *glabratum*. The quantity of the two unknown sesquiterpenes in *L. hirsutum* was

TABLE 2. QUANTIFICATION OF CHEMICALS IN ISOOCTANE EXTRACTS OF GLANDULAR HEADS OF TYPE VI TRICHOMES FROM *L. hirsutum* (LA 361), *L. hirsutum* f. *glabratum* (PI 134417), AND *L. esculetum* CV. VFN 7718

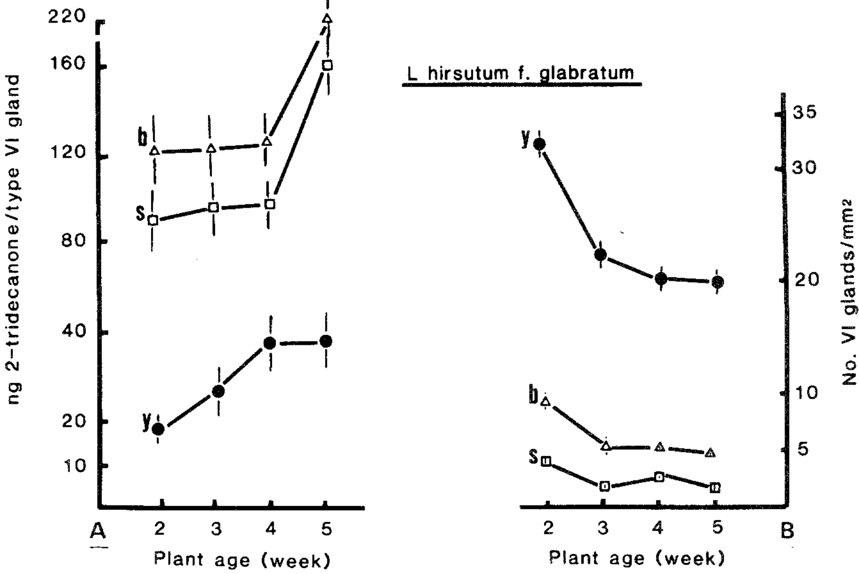
Accession	Chemical	Amount (ng ± SD/gland[a])	% of gland content
L. hirsutum	2-Tridecanone	145.7 ± 7.6	68.1
f. *glabratum*	2-Undecanone	48.6 ± 2.5	22.1
	Sesquiterpene A	4.9 ± 0.3[b]	2.3
L. hirsutum	Sesquiterpene B	7.2 ± 2.0	5.0–6.3
	Sesquiterpene C	0.8 ± 0.2[c]	0.5–0.6
L. esculentum	Monoterpene A	3.0 ± 1.2[d]	2.1–2.6
	Sesquiterpene D	0.5 ± 0.1[d]	0.4–0.5

[a] Unless noted, 200 glands per sample, 6 samples/plant, 5 plants/variety, all plants were 123 days old; radius of type VI glands was 0.04 mm in *L. hirsutum* f. *glabratum*, 0.035 mm in *L. hirsutum*, and 0.05 mm in *L. esculentum* [density assumed to range between 0.8 g/cm^3 (Buckingham, 1982, for high tridecanone content) to 1.0 g/cm^3 for water].
[b] 3000 glands per sample.
[c] 1000 glands per sample.
[d] 4500 glands per sample.

only 8 ng/gland, while the two unknown terpenoids in *L. esculentum* accounted for only 3.5 ng of the contents of a type VI glandular head.

Effect of Trichome Age and Location within Plants on Chemical Composition of Type VI Glands. The chemical contents of type VI glands varied with trichome age in *L. hirsutum* and *L. hirsutum* f. *glabratum*: all chemical concentrations decreased significantly as the glands aged ($P \leq 0.05$, t test). In mature glands from *L. hirsutum* f. *glabratum*, 149.2 ng/gland of 2-tridecanone was detected versus 31.9 ng in an aged gland. In mature glands, the ratio of 2-tridecanone to 2-undecanone in glands of *L. hirsutum* f. *glabratum* was constant (ca. 3.4). However, neither 2-undecanone in *L. hirsutum* f. *glabratum* nor SES C in *L. hirsutum* could be detected in samples of 200 aged glands. Thus, contents of glands vary with the age of the gland as well as accession.

Chemical content of glandular heads in the type VI trichomes of *L. hirsutum* and *L. hirsutum* f. *glabratum* also varied with plant age and location within plants (Figure 2). In the glands of *L. hirsutum* f. *glabratum*, 2-tridecanone concentration remained relatively constant for leaves reaching maturity, then increased sharply when leaves became senescent (Figure 2A). A similar trend was evident for aging stems. Concentration of 2-tridecanone in glands on 1-week-old foliage increased with plant age, but was four to six times lower than in glands on mature leaves or stems. However, since the density of type



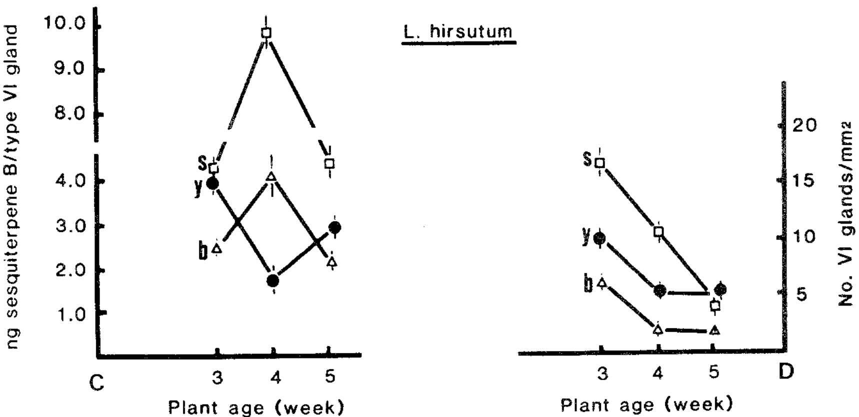


FIG. 2. Chemical contents of glandular heads of type VI trichomes as affected by age and location within plants: y = 1-week-old leaves, b = third true leaves from the bottom, and s = internodal stem between second and third nodes in *L. hirsutum* f. *glabratum* (PI 134417) and *L. hirsutum* (LA 361). Brackets on data points delineate standard errors.

VI trichomes on the young leaves was approximately four times that of the older foilage (Figure 2B), the young leaves were similarly protected.

In the mature glands of *L. hirsutum*, SES B content varied regardless of location within plants (Figure 2C). Concentrations of SES B in glands from both stems and mature leaves fluctuated in a similar pattern: SES B increased until decreasing sharply as the leaves became senescent and stems grew tougher. This pattern is consistent with studies by Croteau and Loomis (1972) documenting that terpenoid synthesis was promoted by the availability of sucrose and other photosynthates, although this pattern could also occur as a result of increased catabolism without any change in the rate of synthesis. The function of sesquiterpenes as insecticides, repellents, and insect feeding deterrents has been previously reported (Kelsey et al., 1984). Sesquiterpene content in trichome heads from immature leaves was highest when plants were youngest. Since density of type VI trichomes was highest at this time as well (Figure 2D), the youngest leaves of young plants were also well protected. The importance of this protection was demonstrated in our previous experiments, where TPW survival was greatly improved by removal of the glandular heads of type VI trichomes (Lin and Trumble, 1986).

Bioassays of Gland Exudate, Gland Extracts, and Pure Compounds. Chemicals in the glandular heads of type VI trichomes from either wild tomato accession were toxic to larvae of both herbivore species. However, BAW was approximately 20 times less susceptible than TPW, despite weighing only three times more than TPW (23.6 μg/BAW; 7.6 μg/TPW). Such differences might occur in response to either differential detoxification systems or variations in cuticular penetrability.

No acute toxicity was observed at 24 hr for either TPW or BAW exposed to glandular heads from *L. esculentum*. However, a previous study reported that TPW would ultimately starve as a result of entrapment, whereas BAW was not affected (Lin and Trumble, 1986). Thus, chemical contents in glandular heads of type VI trichomes on this commercial variety provide only a physical barrier to TPW and are not detrimental to BAW.

Toxicities of equivalent concentrations of the two volatiles from gland exudate or isooctane extracts of glandular heads of *L. hirsutum* f. *glabratum* were not significantly different within each species of herbivore (Table 3). This suggests that not only did the isooctane successfully extract key volatiles, but that the estimates of 2-tridecanone and 2-undecanone concentration determined by GC analyses of isooctane extracts were close to the actual chemical content in type VI glandular heads.

Even with the relatively small amounts found in glandular heads, the two unknown sesquiterpenes provided considerable insecticidal activity. However, the isooctane extract from glandular heads of *L. hirsutum* was more toxic than glandular exudate to both TPW and BAW. If increased toxicity of the extract is due to the isooctane acting as a more efficient carrier, this suggests that a

TABLE 3. TOXICITY OF GLANDULAR HEADS OF TYPE VI TRICHOMES, ISOOCTANE EXTRACTS OF GLANDULAR HEADS FROM WILD TOMATOES, AND PURE COMPOUNDS TO *K. lycopersicella* (TPW) AND *S. exigua* (BAW)[a]

	LD_{50} (ng/larva)/95% confidence interval	
Chemical source	TPW	BAW
L. hirsutum f. *glabratum*		
Exudate	186.9/—[b]	3056.3/2924.7-3180.4
Extract	171.9/67.3-261.7	3374.7/2941.2-3748.5
L. hirsutum		
Exudate	28.5/23.4-46.7	306.2/283.9-332.0
Extract	3.7/2.6-5.8	43.8/33.6-51.8
Pure compounds		
2-Tridecanone ($2C_{13}O$)	51.0/32.0-79.9	3440.0/2820.0-4160.1
2-Undecanone ($2C_{11}O$)	37.0/26.3-57.1	3480.0/3380.1-3619.8
Mixture (3:1) of $2C_{13}O/2C_{11}O$	4.0[c]/2.2-9.6	2080.0[c]/2000.1-2440.5

[a] LD_{50} values and 95% confidence (Helwig and Council, 1979); larvae were directly contacted with glandular heads containing 149.5 ng of 2-tridecanone and 37.4 ng of 2-undecanone per gland of *L. hirsutum* f. *glabratum* (PI 134417), and 7.3 ng of unknown sesquiterpene B per gland of *L. hirsutum* (LA 361) (from concurrent GC analyses). Four replicates of 15 larvae were tested per dose, with five doses per treatment.
[b] A single gland caused in excess of 50% mortality.
[c] Significantly more toxic than expected from an additive effect ($P < 0.05$, chi-square test).

large portion of the unidentified content of the type VI glandular heads on *L. hirsutum* might be water or some other isooctane-insoluble materials.

In bioassays with pure compounds, 2-undecanone was at least as toxic to TPW and BAW as 2-tridecanone ($P > 0.05$, chi-square) (Table 3). In a study comparing the relative toxicity of analogs of 2-tridecanone, Dimock et al. (1982) found 2-undecanone to be less acutely toxic than 2-tridecanone to *H. zea*. A comparison of their results with our own suggests that the toxicity of the two chemicals varies with insect species. The LD_{50} values for the chemical reagents were consistent with the LD_{50} values produced by gland exudates and isooctane extracts for BAW. Similar comparisons were not possible for TPW, because even a single gland caused 100% mortality. Gland extracts were less toxic than the chemical reagents to TPW. This difference may be due, in part, to the ratio of 2-tridecanone and 2-undecanone. There was more 2-tridecanone (4:1) in the extract than in the pure compounds (3:1), which might have influenced penetration of the cuticle.

Mortalities resulting from combinations of both chemicals in a ratio ap-

proximating that found in mature glands (e.g., 3:1) demonstrated significant potentiation [expected additive effect for TPW, LD_{50} = 20.0 ng/larva, observed LD_{50} = 4.0 ng/larva, $P < 0.01$; expected additive effect for BAW = 2800.0 ng/larva, observed LD_{50} = 2080 ng/larva, $P < 0.05$ (chi-square tests)]. This potentiation provides a clear advantage for those plants with glandular heads of type VI trichomes synthesizing or sequestering both chemicals. Additionally, since in a natural state 2-tridecanone is a solid (Dean, 1979), and since 2-undecanone is a liquid which evaporates readily (Dean, 1979), we suspect that the 2-undecanone serves to keep 2-tridecanone dissolved in a liquid form (and therefore more suitable for contact toxicity), while the mixture helps retard evaporation of 2-undecanone from the glands. This hypothesis is supported, in part, by our earlier observation that 2-undecanone could not be detected in aged type VI glands. This combination of chemicals probably plays a key role in physical defense (entrapment) as well. Following release of the liquid content, the rapid evaporation of 2-undecanone would leave an increasingly viscid residue attached to the insect.

CONCLUSION

The major volatile chemicals produced and/or stored in glandular heads of type VI trichomes produce acute insecticidal activities against the specialist and generalist herbivores tested. Although these chemicals proved to be more toxic to the specialist, TPW feeds for the first two instars as a leafminer (Lin and Trumble, 1985) and may thereby behaviorally avoid contact with trichome exudates. In contrast, the generalist BAW feeds on whole foliage and is therefore forced to contact and consume the glandular heads. Thus, the potential defense offered by the glandular heads of type VI trichomes may be greater for the early instars of BAW than for TPW. However, additional studies on the effects of chemicals in glandular heads on oviposition behavior are needed before the importance of chemical activities against larvae can be determined.

Since the concentrations of these volatiles and density of type VI trichomes varied with gland age, plant age, and location within plants, variations in "investment" in specific tissues may occur. Information on such chemical variation could not only offer new avenues of research for plant systematists interpreting relationships difficult to clarify by strictly morphological means, but may assist plant breeders in selecting accessions exhibiting specific chemical traits as well as morphological characteristics which impact TPW and BAW populations. Additional research on the chemical content of type VI glands will be necessary, since the onset and rate of chemical production appears to be dependent on a variety of environmental factors (Langenheim et al., 1984).

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INCREASED SCENT MARKING IN MALE MONGOLIAN GERBILS BY URINARY POLYPEPTIDES OF FEMALE CONSPECIFICS

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Abstract—Male Mongolian gerbils scent mark during a standardized open-field test on an individually different but more-or-less constant level in the absence of females. The presence of females in the same room but without direct contact to the males increases the marking frequencies of the males to an individually different higher level. A similar increase in marking frequency is obtained by application of female urine directly to the nostrils of the males, but not by male gerbil urine or female laboratory mouse urine. This indicates that substances present in female gerbil urine are responsible for the increase in male marking behavior. Using this bioassay, various fractions of female urine were tested for their influence on marking behavior. Our results to date show that the component increasing male scent-marking behavior is hydrophilic and nonvolatile and is contained in the nondialyzable, high-molecular-weight polypeptide fraction of female gerbil urine.

Key Words—Mongolian gerbil, *Meriones unguiculatus*, scent marking behavior, urinary polypeptides, primer pheromones.

INTRODUCTION

Mongolian gerbils (*Meriones unguiculatus*) scent mark their environment with the secretions of a specialized ventral scent gland. The hormonal regulation of this scent-marking behavior is quite well known in males. Castration almost eliminates scent marking (Thiessen et al., 1968), while testosterone treatment of castrates can prevent and even reverse these changes (Blum and Thiessen, 1971; Probst, 1985a).

The effects of chemical signals on scent-marking behavior in gerbils are little known to date. Similar to castration, bilateral olfactory bulb ablation re-

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duces scent marking (Baran and Glickman, 1970; Lumia et al., 1975). However, direct effects of olfactory signals from scent-gland secretions and urine of males could not be demonstrated using open-field tests (Baran, 1973; Blum and Thiessen, 1970; Blum et al., 1975; Fullenkamp et al., 1985).

The observation that, when females are present, males scent mark at an increased level compared with that in the absence of females (Probst and von Holst, 1982) led to the identification of female urine as the source of chemical signals increasing scent marking to the individual's maximum levels (Probst, 1985b). The experiments reported here constitute the analysis of the active fraction of female urine capable of stimulating scent marking in male Mongolian gerbils.

METHODS AND MATERIALS

Animals and Housing. Mongolian gerbils (*Meriones unguiculatus*) were obtained from our breeding colony. After weaning at 28 days of age, males were housed singly in macrolon cages with wire mesh tops, while females were grouped two to three per cage (cage size: 37 × 20 × 15 cm). All gerbils were exposed to a 12:12-hr light–dark cycle (lights on from 12 AM to 12 PM) with constant room temperature of 22 ± 1°C and relative humidity of 55%. The air inside the room (volume 50 m^3) was exchanged 16 times per hour. All animals had free access to gerbil food pellets (Altromin, Lage, FRG) and water. Males and females were at least 6 months of age at the beginning of the experiments.

Behavioral Observation. The experimental males were kept in single cages in a separate room where only males were held, unless not otherwise required during the experiments. The scent-marking behavior was observed inside this room in an open-field apparatus (Thiessen et al., 1968), modified as described previously (Probst, 1985a). For the marking tests, the males were placed individually inside this open-field and during 5 min the occurrences of scent marking were counted. All observations were made during the third hour of the dark phase of the light cycle with a dimmed fluorescent bulb providing sufficient illumination for observation of the animals.

The experimental procedure consisted of three phases for every test situation. During the first phase, the basal marking activity for each male was determined (5–8 tests/animal). The average marking activity of each individual was then used as its basal marking activity. During the second phase, the conditions were changed according to the required test situation while scent-marking tests were carried on. Provided adequate stimulus is present, at least three days of stimulation are required to increase the males' marking activities from the basal level to the higher, stimulated level (Probst, 1985b.) Therefore, for calculation of the test situation marking activity, only the values from the day 5 to 12 of stimulation were used. To prevent interactions between different tests using the same individuals, a control phase was included, during which the

decline of marking activities to the original basal level was monitored after the stimulus had been withdrawn (7–14 tests/animal).

For statistical analysis, the individuals' average scent-marking response during the basal period was compared with the respective response during the different tests using Wilcoxon's matched-pairs signed-rank test. A treatment was considered to cause a significant influence on scent-marking activity when $P < 0.05$ (two-tailed).

Urine Preparations. Urine was collected from 20 female gerbils, 10 male gerbils, and 10 female laboratory mice. The animals were individually placed for 5 min, or less if they urinated earlier, in a beaker. The urine was pooled without considering the estrous state of the females and stored frozen at −25°C. For the bioassay, 10 μl of urine and urine fractions were pipetted directly into each nostril of the males twice per day, resulting in a total of 40 μl applied per day. Since pervious studies had shown that application of demineralized water as a control stimulus does not affect scent marking (Probst, 1985b), this test was not repeated during the present study.

Head-Space Distillation. Head-space distillation (Lee et al., 1980) of female urine was performed to examine the influence of volatile and nonvolatile chemical cues on scent marking. Briefly, about 2 ml nitrogen per minute were passed over urine at 37°C and the head space was trapped on a charcoal filter (von Stralendorff, 1982). The distillation was finished when phenylacetic acid broke through the filter and could be identified by its characteristic smell. The trapped volatiles were then extracted from the filter by small volumes of chilled physiological saline. This "volatile fraction" was stored frozen at −25°C until used.

The residue of the head-space distillation was dialyzed against distilled water at 10°C for 12 hr with two exchanges of water (Spectapor, Spectrum Medical Industries, Los Angeles, California; molecular weight cutoff at 8000 daltons). The "nondialyzable fraction" was freeze dried and reconstituted to the original volume with physiological saline.

Separation of Lipids. The separation of lipids contained in female urine was performed by liquid–liquid extraction of urine using Extrelut® columns (Merck, Darmstadt, FRG). Acidified urine (pH 2.0) was first extracted with diethylether, which yields the acidic lipophilic fraction. Subsequently, the column was alkalized to pH 10.0 by passing ammonia gas through the column, then extracted with dichlormethane to yield the basic lipophilic fraction. The aqueous residue was then eluted with saturated saline. (Wimmer et al., 1978).

For the biotest, the solvents of the acidic and basic lipophilic fractions were evaporated in the cold, and the residue was dissolved in physiological saline to the original volume. The aqueous fraction was dialyzed for 24 hr against distilled water at 10°C with four interchanges of water to remove the saline. The nondialyzable fraction was freeze dried and reconstituted to the original volume with physiological saline.

Polypeptide Fraction. To determine the effects of polypeptide fraction of female urine, polypeptides were reversibly precipitated with ammonium sulfate (Vandenberg et al., 1975). The precipitate was prepared by adding ammonium sulfate to chilled urine to give a final saturation of 80% at 4°C. After centrifugation, the precipitate was dialyzed against water and redissolved in the original volume of physiological saline.

RESULTS

During the presence of females in the experimental room—but without direct contact to the males—the scent-marking activity (number of scent marks per 5 min as determined in a standardized open-field test) is significantly increased by about 100% (Figure 1). This increase is mediated by chemical cues contained in the urine of females. Application of 40 μl urine per day to the nostrils of the males caused an increase in scent-marking activity comparable to that obtained by the presence of females inside the room (Figure 1). In contrast to female gerbil urine, male gerbil and female laboratory mouse urine did not significantly influence the marking activity of males when applied to their nostrils (Figure 1).

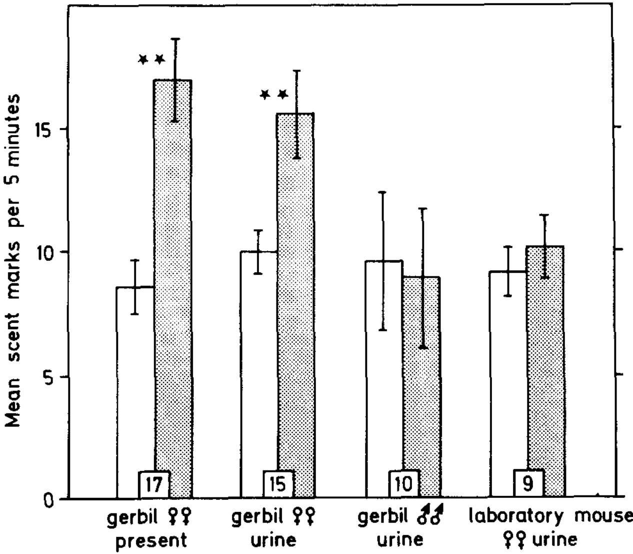

FIG. 1 Influence of the presence of female gerbils and application of female gerbil urine, male gerbil urine, and female laboratory mouse urine on open-field marking behavior of male gerbils. Mean ± SEM; given are the basal values (□) and the respective stimulated values (▩); number of males as indicated at the bottom of the bars; *: $P < 0.01$; **: $P < 0.001$.

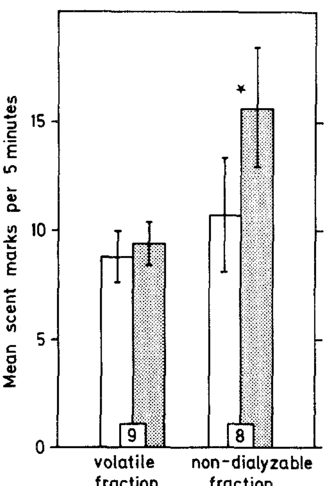

FIG. 2. Mean number of ventral scent gland marks before (□) and after application of the volatile and nondialyzable fractions (▩) of head-space distilled female urine. Mean ± SEM, number of males as indicated at the bottom of the bars; *: $P< 0.01$; **: $P < 0.001$.

For discrimination between volatile and nonvolatile stimuli, female urine was processed by head-space distillation. The volatile fraction was immediately redissolved in water (adjusted to the original volume) and applied to the males' nostrils. Although smelling intensely of phenylacetic acid, this volatile fraction proved to be inactive during the behavior tests (Figure 2), even when applied three times per day. The residue of the head-space distillation was dialyzed before application to remove low-moleulcar-weight substances and the remaining volatiles. This preparation still retained its scent-mark stimulating potency (Figure 2) and significantly increased scent-marking activity above prestimulation levels.

The separation of lipophilic and hydrophilic substances was achieved by liquid–liquid extraction of urine. Only the dialyzed aqueous extract of female urine significantly increased the males' scent-marking activity above the prestimulation level, while both the acidic and basic lipophilic extract were ineffective (Figure 3).

These tests excluded almost all substance classes except polypeptides. Therefore, urinary polypeptides were reversibly precipitated with 80% ammonium sulfate. The dialyzed precipitate then significantly stimulated scent marking (Figure 4). Surprisingly, the dialyzed supernatant significantly inhibited scent marking (Figure 4). A possible explanation could be that the marking activity was still increased from the preceding experiment and continued to decrease during these tests. Since the urinary protein concentration was higher

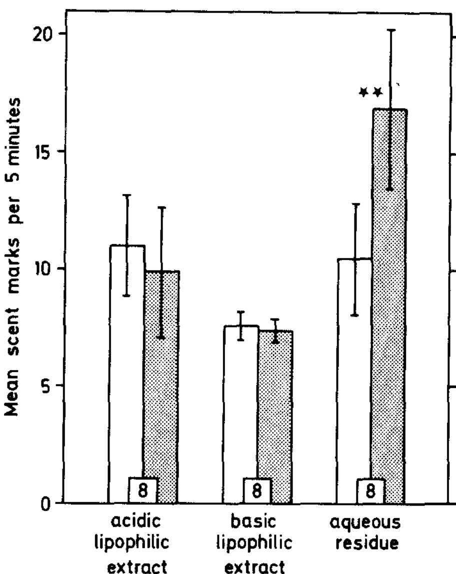

FIG. 3. Mean number of ventral scent gland marks before (▭) and after application of acidic and basic lipophilic extracts and the aqueous residue of female urine (▭). Mean $\pm$ SEM, number of males as indicated at the bottom of the bars; *: $P < 0.01$, **: $P < 0.001$.

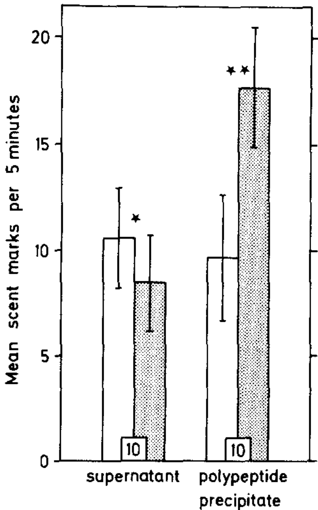

FIG. 4. Mean number of ventral scent gland marks before (▭) and after (▦) application of the supernatant and the ammonium sulfate-precipitated urinary polypeptides, respectively. Mean $\pm$ SEM, number of males as indicated at the bottom of the bars; *: $P < 0.01$; **: $P < 0.001$.

TABLE 1. MEAN MARKING ACTIVITIES OF MALE MONGOLIAN GERBILS AFTER EXPOSURE TO FEMALES AND FRACTIONS OF URINE

Condition	Number of males	Marking activity (mean ± SEM)
Females present	17	17.00 ± 1.56[b]
Female urine	15	15.05 ± 1.81[b]
Female lab. mouse urine	9	10.38 ± 1.39
Male gerbil urine	10	9.05 ± 2.83
Head-space volatiles	9	9.90 ± 1.00
Dialyzed residue	8	15.66 ± 2.64[b]
Acidic lipophilic extract	8	9.97 ± 2.19
Basic lipophilic extract	7	7.53 ± 0.52
Aqueous residue	8	16.93 ± 3.48[b]
Peptide precipitate	10	17.68 ± 2.34[b]
Supernatant	10	8.51 ± 2.34
Controls (females present)	17	8.66 ± 1.10
Controls (female urine)	16	10.08 ± 0.95
ANOVA[a]		$P < 0.001$

[a] Within experiment analysis of variance based on $\log_{10}$ transformed data.
[b] Significantly different from either baseline control at $P < 0.05$; determined using Duncan's multiple-range procedure.

in the male urine pool than in the female urine pool (male: 0.23 mg/ml; female: 0.20 mg/ml; determined using Coomassie blue and bovine serum albumin as standard), sex differences in protein concentration cannot account for the differential action of male and female urine in the bioassay.

The overall analysis of variance across all treatments comparing the controls of females present and female urine application with the various extracted fractions confirmed the significant effects found by using nonparametric statistics [$F(12,131) = 4.6$; $P < 0.001$; Table 1].

DISCUSSION

Urinary chemosignals exert a wide range of influences on reproduction in mammals (for summaries see: Whitten and Bronson, 1970; Milligan, 1980; Vandenberg, 1983). Best investigated are the effects of so-called "primer pheromones" on reproductive development and function in mice (reviewed by Marchlewska-Koj, 1983). Vandenberg et al. (1975) partially isolated from male mouse urine a compound accelerating puberty in juvenile females. In addition to sexual maturation, estrous cycles of adult females become more regular and can be synchronous in a group of females following the exposure to male urine

(Whitten, 1959; McClintock, 1983). Also, pregnancy in a newly mated female can be blocked by exposure to urine from a strange male (Bruce, 1959).

While the chemical nature of some signaling pheromones has been identified (e.g., von Stralendorff, 1982; Jemiolo et al., 1985), comparatively little is known about the chemical constitution of primer pheromones. Vandenberg et al. (1976) isolated a low-molecular-weight fraction from male urine accelerating puberty in female mice, which was further purified by Novotny et al. (1980). The failure to identify the biologically active chemical components may largely be due to the lack of sufficiently discriminating bioassays to compare the biological activity of different urinary fractions.

The bioassay presented here for the scent-mark-stimulating effect in male gerbils represents a relatively fast and very sensitive test. Similar to mouse primer pheromones, the scent marking increase requires continuous stimulation for at least three days until the higher marking level is achieved and, after withdrawing the stimulus, it takes several days until the males mark at their respective lower level. The female urinary chemosignal selectively influences male scent-marking behavior. Neither locomotor activity (number of open-field squares entered) nor serum testosterone concentrations were significantly different comparing basal and female-stimulated test phases (Probst, 1985b). Other open-field behaviors were apparently not changed, although no further quantitative evaluation was performed. Since the scent-marking increase is reversible and the procedure is noninvasive, one animal can be tested with different urinary fractions. The response obtained during stimulation can then be compared with the respective unstimulated marking activity of the same individual, thus largely increasing the precision of this bioassay.

The chemical signals responsible for this increased marking effect in male Mongolian gerbils is probably sex- and species-specific, since neither male gerbil urine nor female mouse urine stimulated scent-marking activity compared with female gerbil urine. The head-space distillation experiment demonstrated that the scent-mark-stimulating effect is not mediated by volatile substances. It is contained in the higher molecular weight fraction of female urine, since dialysis removes most lower-molecular-weight substances. Earlier results from Thiessen et al. (1974) showed that phenylacetic acid was the main chemical cue emitted from the scent gland of gerbils. The present results, however, indicate that phenylacetic acid might primarily be a behaviorally ineffective urinary component that is transfered to the scent gland during marking on urinated substrate rather than a component produced by the scent gland itself. Moreover, Thiessen et al. (1974) did not demonstrate the influence of phenylacetic acid on scent-marking behavior, but showed that gerbils can discriminate between phenylacetic acid and a control stimulus. During our experiments, no influence of phenylacetic acid on scent marking in the open field tests was found. Neither chronic stimulation for eight days with the phenylacetic acid containing acidic

lipophilic extract of female urine (Figure 3) nor direct application of phenylacetic acid (1% solution, 50 μl per marking peg) into the open field during the marking tests (control: 13.7 ± 3.6; phenylacetic acid: 11.7 ± 3.1; $n = 9$) caused a significant change in scent-marking behavior.

The stimulation of scent marking in male gerbils by chemical signals from females also provides an explanation for contrary results on the effect of testosterone on scent marking in castrated males. While Thiessen et al. (1968) observed an increase in scent marking of more than 100% above the respective precastration level during postcastration testosterone treatment, Swanson (1980) could not confirm this "supermarking." One reason for this might be that Thiessen et al. (1968) used males held without females present. These would scent mark at a reduced level prior to castration. However, there are no details of holding conditions given in this respect. During testosterone treatment, scent marking would then increase to the individual maximum level (Probst, 1985a) that female stimulated males show before castration, as in the experiments by Swanson (1980). As the present results show, testosterone-induced "supermarking" in castrated male gerbils (Thiessen et al., 1968) might be caused by comparing submaximal marking levels before castration with testosterone-induced maximally stimulated scent marking after castration, but not by a specific action of testosterone. This submaximal scent marking before castration can be prevented by the presence of sociochemical cues contained in the polypeptide fraction of female urine. After castration, testosterone treatment increases scent-marking activity above this submaximal level; however, even very high testosterone concentrations will not increase scent marking above the individual's maximum marking activity (Probst, 1985a).

Further research is necessary to investigate the influence of the gonadal state of females on their capability to increase male scent marking as well as the role of the dual olfactory pathways (Estes, 1972; Keverne, 1979; O'Connell and Meredith, 1984) involved in the perception of chemical signals in gerbils.

Acknowledgments—We would like to thank Drs. von Holst and von Stralendorff for critical discussions of this paper.

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SYNTHESIS AND FIELD BIOASSAY OF SOME ANALOGS OF SEX PHEROMONE OF CITRUS MEALYBUG, *Planococcus citri* (RISSO)[1,2]

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Abstract—A series of structural analogs of (+)-*cis*-(1*R*)-3-isopropenyl-2,2-dimethylcyclobutanemethanol acetate, sex pheromone of the citrus mealybug, *Planococcus citri* (Risso), was synthesized. The analogs were tested in a field bioassay in order to determine the structure–activity relationships of the pheromone. All changes in structure reduced the activity of the test compounds, to various degrees. The most active analog tested was the homolog (+)-*cis*-(1*R*)-3-isopropenyl-2,2-dimethylcyclobutaneethanol acetate (IV), whose activity, at a higher dosage, was comparable to that of the pheromone. The alcohol (+)-*cis*-(1*R*)-3-isopropenyl-2,2-dimethylcyclobutanemethanol was tested in mixtures with the pheromone and found to be neither an inhibitor nor a synergist. The results show that all functional groups of the pheromone molecule are essential for optimal biological activity.

Key Words—Citrus mealybug, *Planococcus citri*, Homoptera, Pseudococcidae, sex pheromone, synthetic analogs, structure–activity relationship, field bioassay.

INTRODUCTION

The citrus mealybug, *Planococcus citri* (Risso), is a cosmopolitan pest of numerous crops. It is, in particular, a serious economic problem in citrus groves (Bartlett, 1978; Bodenheimer, 1951).

[1]Homoptera: Coccoidea: Pseudococcidae.
[2]Contribution from the Agricultural Research Organization (ARO), The Volcani Center, Bet Dagan, Israel, No. 1667-E, 1986 series.
[5]Levi Eshkol Postdoctoral Fellow, 1984–1986.

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The female sex pheromone of this pest was identified (Bierl-Leonhardt et al., 1981) as (+)-(1*R*)-3-isopropenyl-2,2-dimethylcyclobutanemethanol acetate (I). The pheromone has been synthesized by several groups (Bierl-Leonhardt et al., 1981; Gaoni, 1982; Carlsen and Odden, 1984; Odinkov et al., 1984; Wolk et al., 1986) and shown to be highly attractive to males in field tests (Bierl-Leonhardt et al., 1981; Ortu and Delrio, 1982; Rotundo and Tremblay, 1982; Dunkelblum et al., 1983; Moreno et al., 1984; Tauber et al., 1985). These results demonstrate the potential of the synthetic pheromone for use in monitoring and possibly for control of the citrus mealybug.

This paper describes the synthesis of some structural pheromone analogs, shown in Figure 1, and reports on the biological activity of these compounds in field tests. The aim of the present study was to probe the structure–activity relationship in the *P. citri* sex pheromone and to find potential attractive analogs for practical use.

METHODS AND MATERIALS

Chemicals. The pheromone (I), $[\alpha]_D^{19}$: +20.7° (c 0.93, $CHCl_3$), and the keto analog (III), $[\alpha]_D^{22}$: +103.2° (c 6.20, $CHCl_3$), were prepared from (+)-α-pinene by a short synthesis developed recently by our group (Wolk et al., 1986). The alcohol (II), $[\alpha]_D^{22}$: +16.2° (c 3.64, $CHCl_3$), free of contaminating pheromone I, was obtained from the latter by alkaline hydrolysis. The trifluoroacetate (VII), $[\alpha]_D^{26}$: 12.1° (c 0.78, $CHCl_3$), formate (VIII), $[\alpha]_D^{26}$: +19.6° (c 0.52, $CHCl_3$), and propionate (IX), $[\alpha]_D^{26}$: +14.2° (c 1.96, $CHCl_3$), were prepared from the alcohol with trifluoroacetic anhydride, formic acid–acetic anhydride, and propionic anhydride, respectively.

Selective reduction of the aldehyde group of (+)-*cis*-pinonaldehyde, $[\alpha]_D^{19}$: +79.5° (c 1.33, $CHCl_3$), with sodium borohydride in acetic acid (Saksena and Mangiaracina, 1983) gave the corresponding ketoalcohol, $[\alpha]_D^{22}$: +73.3° (c 1.16, $CHCl_3$) (Gora et al., 1982), which was acetylated to the respective ketoacetate, $[\alpha]_D^{22}$: +58.1° (c 1.16, $CHCl_3$). Wittig methylenation of the latter afforded the homolog (IV), $[\alpha]_D^{22}$: −22.5° (c 1.00, $CHCl_3$), along with some of the corresponding alcohol.

The acyclic analog V was prepared by the following route (Scheme 1). Michael addition of dimethyl malonate to mesityl oxide gave the diester X, decarbomethoxylation of which in moist dimethyl sulfoxide (Krapcho, 1982) gave methyl 3,3-dimethyl-5-oxohexanoate (XI) (Andersen et al., 1981). Wittig methylenation of XI to the unsaturated ester XII (Jellal and Santelli, 1980), followed by lithium aluminum hydride reduction gave the alcohol XIII (Blume et al., 1965). Acetylation of XIII gave the desired analog V.

Hydrogenation of the pheromone I over the catalyst prepared from nickel acetate and sodium borohydride (Brown and Brown, 1963) gave exclusively the

SCHEME 1. Synthesis of acyclic analog V. a. NaH b. NaCl, H_2O/DMSO c. $Ph_3P{=}CH_2$ d. $LiAlH_4$ e. Ac_2O/Pyr.

cis-dihydro analog (VI), $[\alpha]_D^{26}$: +3.8° (c 0.84, $CHCl_3$), along with some of the isopropylidene isomer of the pheromone. The two products were easily separated by preparative gas-liquid chromatography. When the hydrogenation was performed over 5% palladium on carbon, considerable amounts of the *trans* isomer of VI were also obtained.

All synthetic analogs were purified by column chromatography and/or bulb-to-bulb distillation and were homogeneous, as verified by capillary GLC (DB225, 30 m × 0.25 mm). Satisfactory NMR and mass spectra were obtained for all analogs. The purity of all compounds was 95–98%.

Field Tests. The attractancy of the analogs (II–IX) was compared with that of the synthetic pheromone (I) in field bioassays. Most of the tests were performed in a grapefruit orchard in the experimental farm at Zerifin (coastal plain), Israel, during 1985. Triangular plastic traps (10 × 10 × 10 × 15 cm), equipped with a replaceable sticky board at the bottom, were hung in the canopy of two adjacent rows of trees at intervals of about 10 m. Rubber septa, impregnated with a hexane solution of the test chemicals, were used as baits. Some tests were conducted at the Biological Control Institute in Rehovot (coastal plain) Israel, in a shaded hut (15 × 4 m), where squashes infested with the citrus mealybug were stored. Traps were hung above the shelves, at a distance of 2–3 m between traps. The sticky boards were exchanged every 4–8 days and the trapped males were counted in the laboratory using a stereoscope microscope. Traps were rotated by one position on every check.

RESULTS AND DISCUSSION

In the pioneering paper on the identification and synthesis of the sex pheromone of *P. citri* (Bierl-Leonhardt et al., 1981), it was reported that only the (*cis*)-(+) enantiomer of 3-isopropenyl-2,2-dimethylcyclobutanemethanol acetate was significantly active as sex attractant to males. In view of this finding,

we decided to preserve the same relative configuration in all the cyclobutyl analogs, with the sole exception of the acyclic analog (V).

A series of analogs (Figure 1) was prepared and field bioassayed in groups with the synthetic pheromone (I) as standard for comparison. A control trap baited with a septum impregnated with pure hexane, was included in all the tests. The control traps caught no *P. citri* males, except for a few isolated instances of single males.

The first group of chemicals included the alcohol (II) alone and mixtures containing 2, 5, 10, 20, and 100% of II with the pheromone (I). The alcohol (II) is a side product in our recently developed synthesis of the pheromone (I) (Wolk et al., 1986); therefore it was of practical importance to assess the activity of II. The field tests indicated that the alcohol (II) was ineffective as a sex attractant. Only the addition of the equal amount of II to the pheromone (I) led

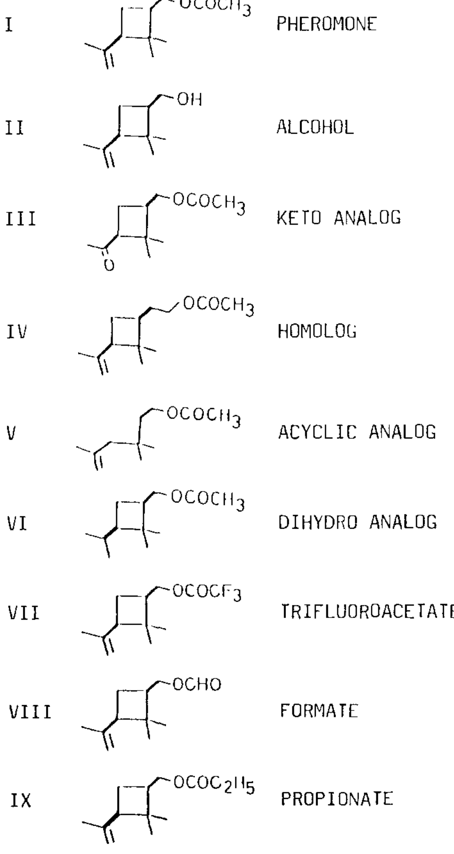

FIG. 1. List of compounds.

TABLE 1. FIELD BIOASSAY OF PHEROMONE (I), ALCOHOL (II), AND MIXTURES

Test	Alcohol (μg)			Pheromone (μg)	Total number of males trapped[a]
1[b]				500	332 x
	25	(5%)	+	500	289 x
	500				17 y
2[c]				250	978 x
	12.5	(5%)	+	250	818 xy
	250	(100%)	+	250	650 y
3[d]				500	176 x
	10	(2%)	+	500	156 x
	50	(10%)	+	500	260 x
	100	(20%)	+	500	212 x

[a]Four traps per treatment (Zerifin farm). Means followed by the same letter are not significantly different (Duncan 5%).
[b]April 18–May 5, 1985 (18 days).
[c]May 6–May 20, 1985 (14 days).
[d]October 24–December 10, 1985 (47 days).

to a significant reduction of the activity of the latter. Addition of smaller amounts of II had no statistically significant effect on the attractiveness of I, although the addition of 10% of II enhanced slightly the activity of the pheromone (Table 1). A very similar effect of the corresponding alcohol on the activity of an acetate pheromone was found in field tests with the sex pheromone of the Comstock mealybug, *Pseudococcus comstocki* (Bierl-Leonhardt et al., 1982).

The second group of compounds included the keto analog (III), the homolog (IV), the acyclic analog (V), and the dihydro analog (VI) (Table 2). These compounds incorporate various alterations in the pheromone skeleton, including the opening of the cyclobutyl ring in V. Compounds III, V, and VI gave negligible trap catches. The homolog (IV) had significant activity, and therefore it was decided to test this compound at several concentrations. Periodical trap catches of IV at three concentrations are recorded in Table 3. Increase of the amount of IV from 500 μg to 2000 μg enhanced the attractiveness substantially, in particular in the first four weeks, almost equaling the activity of the pheromone at the concentration of 500 μg.

The last group of analogs included three esters: the trifluoroacetate (VII), formate (VIII), and propionate (IX), which differ from the pheromone only in the ester function. These esters had low activity, in particular the trifluoroacetate which performed similarly to a control bait (Table 2).

So far all attempts to design analog compounds which would be as active as the corresponding pheromones failed, although useful analogs have been

TABLE 2. FIELD BIOASSAY OF ANALOGS III, IV, V, VI, VII, VIII, AND IX

Test	Attractant (500 μg)	Total number of males trapped[a]
1[b]	Pheromone (I)	2604 x
	Keto analog (III)	38 z
	Homolog (IV)	1007 y
	Acyclic analog (V)	14 z
2[c]	Pheromone (I)	305 x
	Dihydro analog (VI)	2 y
3[d]	Pheromone (I)	108 x
	Trifluoroacetate (VII)	0 z
	Formate (VIII)	15 y
	Propionate (IX)	35 y
4[e]	Pheromone (I)	104 x
	Propionate (IX)	26 y

[a] Four traps per treatment, except test 4 which consisted of five traps. Means followed by the same letter are not significantly different (Duncan 5%).
[b] May 20–June 6, 1985 (17 days), Zerifin farm.
[c] November 27–December 15, 1985 (18 days), Biological Control Institute, Rehovot.
[d] September 10–October 17, 1985 (37 days), Zerifin farm.
[e] December 15, 1985–January 10, 1986 (26 days), Biological Control Institute, Rehovot.

TABLE 3. FIELD BIOASSAY OF HOMOLOG (IV) WITH PERIODICAL RECORDING

Attractant[a]	Recording dates							Total males
	June			July				
Start (June 6, 1985)	10	17	23	1	7	14	22	(June 6–July 22, 46 days)
Pheromone (I)								
500 μg	203	334	135	182	132	159	92	1237 x
Homolog (IV)								
500 μg	87	152	51	70	22	21	8	411 z
1000 μg	129	195	42	66	52	42	24	550 z
2000 μg	135	262	115	148	86	47	38	831 y

[a] Four traps per treatment. (Zerifin farm). Means followed by the same letter are not significantly different (Duncan 5%).

prepared in a few cases of Lepidoptera (Beevor et al., 1977; Carlson and McLaughlin, 1982; Mitchell et al., 1975; Silk et al., 1985; Tatsuki and Kanno, 1981). Studies of analogs of a coccoid sex pheromone have been reported only for that of the Comstock mealybug, *P. comstocki.* All compounds tested had significantly lower activity than the respective pheromone (Bierl-Leonhardt et al., 1982; Uchida et al., 1981). The results of EAG studies and field tests carried out in all these studies on analogs indicated that all active sites in a pheromone are essential for optimal substrate–receptor interaction. Although various models of this interaction have been proposed (Bestmann and Vostrovsky, 1982; Kafka and Neuwirth, 1975; Roelofs and Comeau, 1971), the basic phenomenon is still not completely understood.

The pheromone of the citrus mealybug (I) has a unique structure with a relatively large number of active sites: a polar acetate group, a cyclobutyl ring, a double bond, a specific chain length, and two chiral centers which constrain the total stereochemistry of the molecule. With the exception of the achiral acyclic analog V, the two chiral centers were preserved in all the compounds in this study. The acetate group was hydrolyzed to the alcohol or replaced by a trifluoroacetate, formate, and propionate ester. The carbon–carbon double bond was either reduced or replaced by a carbon–oxygen double bond, and the chain length was increased by one carbon. All changes in the molecular structure of I (Figure 2) reduced the activity of the corresponding analogs. Reduction of the double bond, replacing it by a carbonyl function and opening of the cyclobutyl ring eliminated almost all activity (Table 2). These results establish the essential role of the ring and the olefinic function in the pheromone. Manipulation of the methanol acetate group led to interesting results. Hydrolysis of the acetate function or its replacement by another ester group reduced activity drastically. However, there was a considerable difference between the trifluoroacetate and the propionate esters (Table 2). The former had no activity at all, whereas the latter retained some attractiveness to the males. This result reflects the difference in the polarity between these two groups. The propionate has almost the same polarity as the acetate, while that of the trifluoroacetate is much greater (Liljefors et al., 1984). Addition of one carbon to the methanol side chain to form homolog IV gave the most active, nonpheromone compound tested (Table 3).

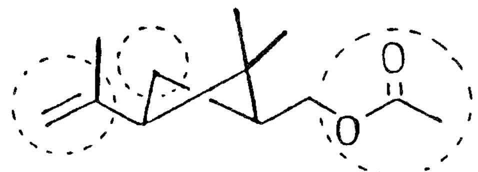

FIG. 2. Pheromone molecule with sites of alterations.

CONCLUSIONS

All changes (II–IX) in the shape and electron distribution of the sex pheromone molecule of *P. citri* (I) resulted in a significant reduction of its biological activity. All functional groups seem to be essential for full activity in the field. Lengthening of the cyclobutanemethanol moiety to cyclobutaneethanol, in the homolog IV, was the only modification which yielded an analog with a relatively high attractiveness to *P. citri* males. This analog retained all functional groups having the same polarity as the pheromone, with only a slight change in the molecular shape.

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RESPONSES OF PLANT-PARASITIC NEMATODE *Meloidogyne incognita* TO CARBON DIOXIDE DETERMINED BY VIDEO CAMERA–COMPUTER TRACKING

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Abstract—A computer tracking system was used to quantify the responses of infective second-stage juveniles of the plant-parasitic nematode *Meloidogyne incognita* to carbon dioxide. A sudden increase in concentration caused an increase in the rate of locomotion and a decrease in the frequency of changes of direction. The threshold was about 0.01% vol CO_2/vol gas when the baseline concentration was very low and 0.05% CO_2 when the baseline concentration was 1% CO_2. The latter value represents a relative change of 5%. Concentrations above 10% CO_2 caused a general decrease in movement. In a second type of experiment, a constant concentration gradient of CO_2 was established, and the net movement of the nematodes along the gradient was determined. At low concentrations, the threshold was about 0.02% CO_2/cm. At higher concentrations, the threshold gradient was below 0.01% CO_2/cm or a relative gradient of less than 1% change/cm. At all concentrations to which nematodes responded they were attracted. The degree of orientation was estimated to be approximately 10% under most conditions. The rate of migration under the most favorable conditions was about 0.7 cm/hr. Three possible functions of the response are discussed: attraction to roots, movement toward optimal depth in soil, and as a collimating stimulus.

Key Words—*Meloidogyne incognita*, nematode, carbon dioxide, chemotaxis, video camera, microcomputer, tracking, soil.

INTRODUCTION

An important question in soil ecology is how animals orient in this complex environment. A specific question of particular importance to agriculture is how

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plant-parasitic nematodes locate their hosts. Carbon dioxide is a known attractant for several phytoparasitic nematodes (Prot, 1980). While CO_2 is the most abundant root exudate, its relative importance as an attractant under natural conditions is debatable. Prot (1980) concluded that CO_2 may be one of the attractants for nematodes toward host roots, but that its influence probably does not extend further than 1 or 2 cm from the roots.

A major problem in determining the influence of CO_2 in situ has been the lack of quantitative data for minimum concentrations and gradients required to elicit attraction and for the strength of the response. Using a computer–video tracking system, we have measured these parameters for the root-knot nematode *Meloidogyne incognita*.

METHODS AND MATERIALS

The computer tracking system was similar to that previously described (Dusenbery, 1985a,b), except that an IBM XT computer with an Imaging Technology camera interface was used. Several other modifications were made to accommodate the plant-parasitic nematodes which are smaller, slower (Goode and Dusenbery, 1985), and more sensitive to thermal gradients (J.A. Diez, M.J. Pline, and D.B. Dusenbery, unpublished data) than the free-living nematodes studied previously. The tracking interval was increased from 1 to 5 sec. The illumination source was switched from an incandescent bulb to a 14-W U-shaped fluorescent bulb to reduce the heat produced. To reduce thermal gradients, air was recirculated through a heat exchanger coupled to a water bath and over the chamber which contained the nematodes. A Computar® enlarging lens (f1.9, 55 mm) provided a fast lens with a flat field at close focus. Increased magnification produced a field of 20 × 17 mm. Since the video image is digitized into an array of 512 × 480 pixels, the pixel spacing was 39 μm along the flow and 35 μm across the flow.

Meloidogyne incognita were grown on tomato plants (Goode and Dusenbery, 1985). Several hundred 1- to 2-day-old juveniles (J2) from egg masses acclimated to 23°C for several days were placed on a thin sheet (30 × 50 mm) of 2% agar poured on a glass plate (75 × 150 × 5.5 mm). This plate was inverted onto a similar glass plate to which rubber tubes had been cemented to form an open-ended chamber (35 × 130 × 5 mm) with an input tube at one end (Figure 1).

Hydrated air was a carrier for the CO_2 stimulus. To compare the sensitivity of the nematodes to CO_2 under different baseline CO_2 concentrations, we used a carrier of either low hydrocarbon compressed air containing 0.003% CO_2 or compressed air containing 1.04% CO_2. The compressed air was blown through a flowmeter and activated charcoal trap into a gas washing bottle submerged in a thermostatically controlled water bath. The hydrated air then flowed into the

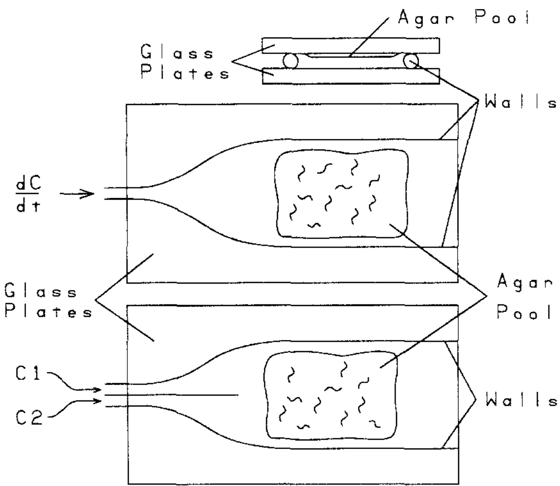

FIG. 1. Diagram of the two flow chambers used. The top figure is a cross-section of either chamber. The middle figure is the chamber used to generate a temporal, spatially uniform stimulus. The lower figure is of the chamber used to generate a steady spatial gradient. "C1" and "C2" stand for two different concentrations of CO_2 in air flowing through the chambers; "*dC/dt*" indicates a temporal change in concentration.

chamber and over the agar sheet. Air flow within the chamber was laminar. Various concentrations of CO_2 (0.25–100%) were premixed with hydrated air in a syringe pump and injected at various flow rates (0.01–0.04 ml/sec) into the constantly flowing (0.25, 0.5, or 1.0 ml/sec) hydrated air for 2 min. Mixing occurred in 35 cm of small-diameter Teflon tubing before entering the chamber. This procedure increased the concentration delivered to the nematodes by 0.0025% to 16% CO_2. Sufficient time (3–6 min) was allowed between exposures for behavior to return to a stable baseline for at least 1 min. Concentrations of CO_2 were confirmed by gas chromatography. Each concentration was tested with 4–10 replicates.

To measure the response of the nematodes to gradients of CO_2, the chamber was modified by cementing two input tubes at one end of the chamber and separating them by a thin vertical partition of plastic extending 3 cm into the chamber (Figure 1). The stream of hydrated air (containing 0.003% or 1.04% CO_2) was split before entering the chamber, and CO_2 of various concentrations was injected by syringe pump into one of the two air flows. A switching valve controlled which side of the chamber received added CO_2. When two gases with different CO_2 concentrations were used with this chamber, diffusion occurred between them as they flowed through the chamber. This established a concentration gradient across the direction of flow. Concentrations of CO_2 in the gradients were determined by gas chromatography. This procedure was ef-

fective in creating near-linear gradients of CO_2 across the width of the chamber in the area of the agar sheet.

In the first series of experiments, involving temporal step changes in CO_2 concentration, the position of each of 150–300 nematodes was determined every 5 sec and the absolute value of locomotion in two orthogonal directions was determined and summed. Reversals (a change in direction of locomotion of >90°; Dusenbery, 1985a) were also determined every 5 sec and summed. In the second series of experiments, involving CO_2 gradients, the amount and direction of locomotion in each of two orthogonal directions (i.e., parallel and perpendicular to the direction of flow) was determined every 5 sec and separately summed to indicate the net movement of the average position of 200–300 nematodes along the flow and along the gradient of CO_2 created in the chamber. The absolute amount of locomotion as described above was also measured.

RESULTS

Low CO_2 Baseline. Figure 2 illustrates the response of *M. incognita* to sudden small increases in CO_2 concentration under conditions of low (0.003%) CO_2 baseline concentration. Figures 2–5 show responses for only a sample of the concentrations tested. The nematodes reacted quickly to changes in concentration $\geq 0.01\%$ CO_2 by increasing their rate of locomotion and decreasing their frequency of reversals. At a carrier flow rate of 1.0 ml/sec, the CO_2–air mixture reached the nematodes in 10 sec, and their initial response began about 5–10 sec later. Peak response occurred within 1 min. Some adaptation was usually observed during the second minute of the stimulus with a slight decrease in locomotion and increase in reversals. At concentrations $\leq 0.04\%$ CO_2, movements returned to prestimulus levels usually within 60 sec after the CO_2 was turned off.

Figure 3 presents similar data for large changes in concentration. At concentrations $\geq 6\%$ CO_2, the response was depressed and acquired a biphasic character. The initial changes in movement lasted less than a minute before decreasing. After the stimulus was removed, there was a second response, similar to the first. In all cases, the rate of locomotion and reversal frequency changed in opposite directions.

High CO_2 Baseline. Figure 4 illustrates the response of *M. incognita* to sudden small increases in CO_2 concentration under the condition of high CO_2 baseline concentration (1.04%). As with low CO_2 baseline concentration, the nematodes responded quickly to CO_2, but the pattern of response was different. For concentration changes $\leq 0.03\%$ CO_2, no response was detected. For a change of 0.06% CO_2, the rate of locomotion increased and reversal frequency

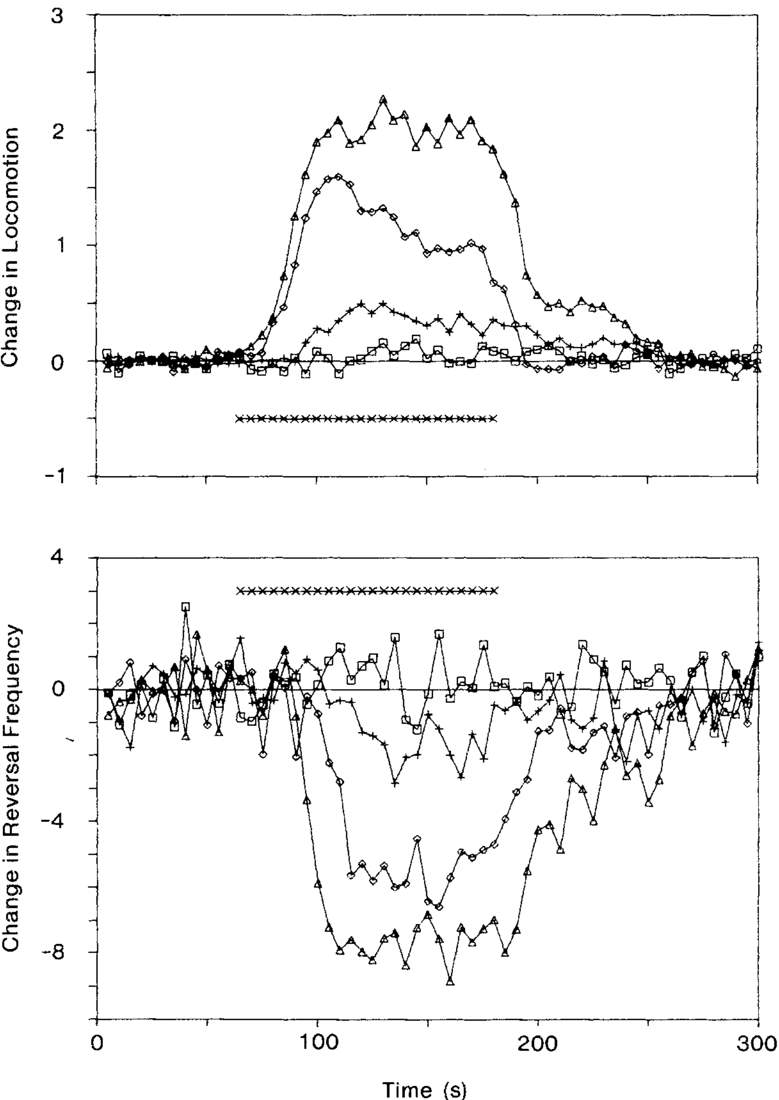

FIG. 2. Time course of changes in movements in response to small, temporal step changes in CO_2 concentration with low (0.003%) CO_2 baseline concentration. Locomotion, presented as the change from the prestimulation value, is the average distance in pixels the nematodes have moved between successive determinations of their position (trackings). Reversal frequency, presented as the change from the prestimulus value, is the average number of reversals detected per 100 nematodes per tracking. During the times indicated (X), CO_2 was added to the air flow to increase concentration by (□) 0.0075, (+) 0.01, (◇) 0.02, or (△) 0.04% volume CO_2/volume gas.

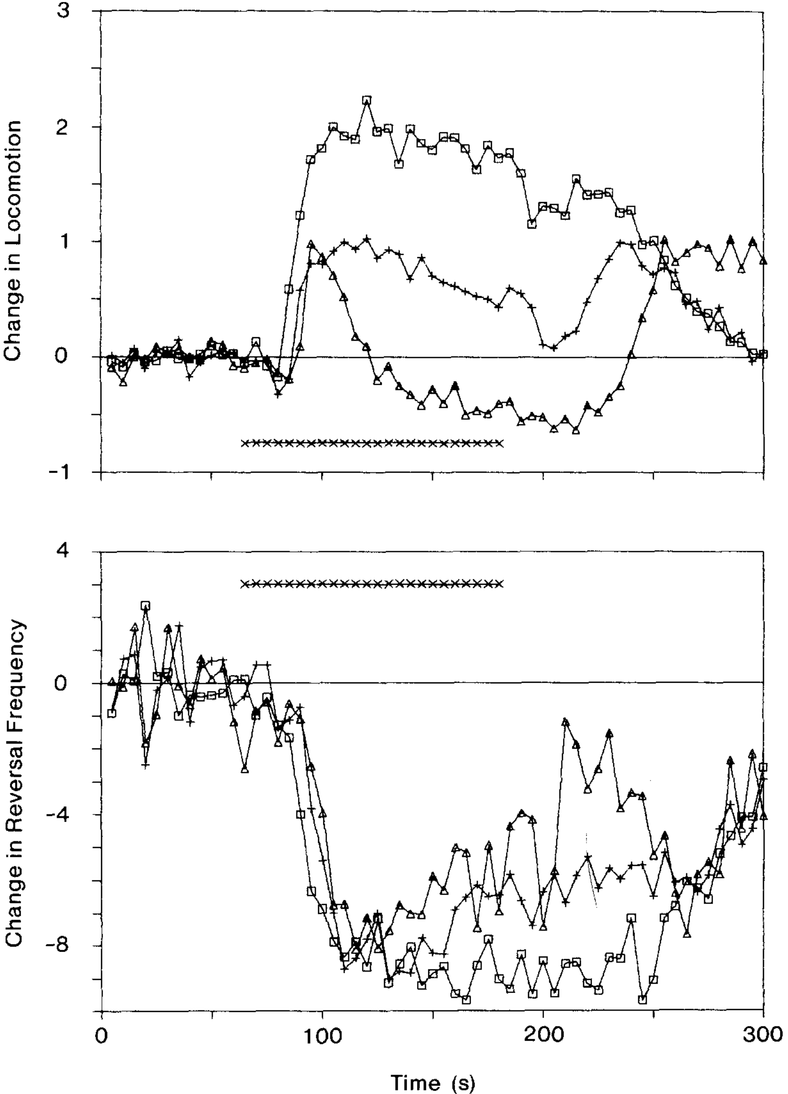

FIG. 3. Time course of changes in movements in response to large, temporal step changes in CO_2 concentration. Same as Figure 2, except the concentration increases are larger: (□) 4% CO_2, (+) 8% CO_2, or (△) 16% CO_2.

decreased. These movements returned to prestimulus levels in about 30 sec. When the stimulus was turned off, there was an abrupt decrease in the rate of locomotion and increase in reversal frequency. This pattern of response was discernible but weak at 0.04% CO_2. Not until the stimulus concentration reached

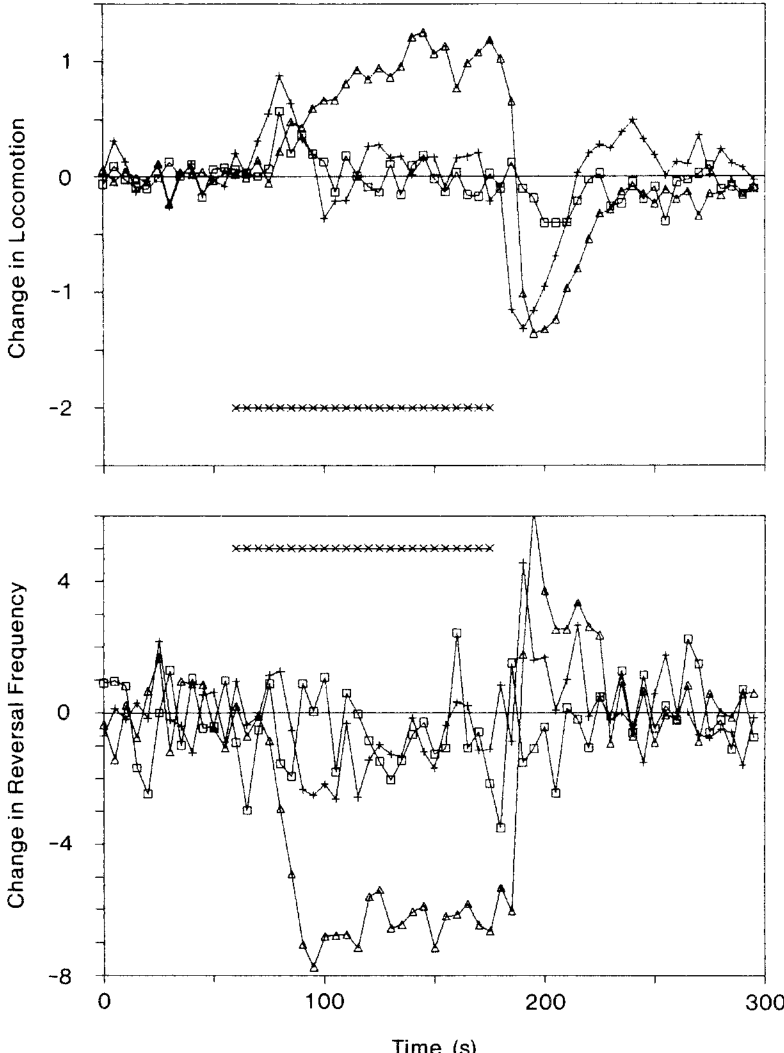

FIG. 4. Time course of changes in movements in response to small, temporal step changes in CO_2 concentration with high (1.04%) CO_2 baseline concentration (see Figure 2). During the times indicated (X), CO_2 was added to the air flow to increase concentration (volume CO_2/volume gas) by (□) 0.04% CO_2, (+) 0.06% CO_2, or (△) 2% CO_2.

2.0% CO_2 did the rate of locomotion remain elevated and the frequency of reversals remain depressed throughout the stimulus application. The characteristic response to the removal of the stimulus occcurred at all concentrations $\geq$0.06%.

Figure 5 presents data for large changes in concentration with high CO_2 baseline air. Results were similar to Figure 3 in showing the depression of response within 1 min of initial stimulus application.

To more precisely define the effect of concentration on the strength of the response, the average change in locomotion (or reversal frequency) per tracking for the second minute of the 2-min stimulus application was calculated for 75 experiments using low CO_2 baseline air and 75 experiments using high CO_2 baseline air (Figure 6). Change was measured with respect to a prestimulus value calculated for each experiment from the average locomotion (or reversal frequency) per 5 sec tracking for 1 min immediately preceding application of the stimulus.

With low CO_2 baseline air, prestimulus values ranged from 1.4 to 1.9 pixels displacement per nematode, and from 11 to 14 reversals per 100 nematodes. The smallest stimulus to which the nematodes responded was a concentration change of 0.01% CO_2. The strength of the response changed little between 0.04% and 4% CO_2. In this range, the rate of locomotion doubled and the reversal frequency was half its prestimulus value. The response was significantly depressed over 6% CO_2. The rate of locomotion actually fell below prestimulus levels with the application of 12% and 16% CO_2.

High CO_2 baseline air elicited prestimulus movements of 2.2–3.8 pixels displacement per nematode, and 7–12 reversals per 100 nematodes. Because the nematodes acclimated so quickly to small step changes in CO_2 concentration with high CO_2 baseline air, Figure 6 does not illustrate the response of the nematodes to threshold concentrations as clearly as with low CO_2 baseline air. While Figure 4 clearly indicates that the nematodes reacted both to the application and removal of a 0.06% CO_2 stimulus, there is a barely detectable rise in locomotion in Figure 6. As with low CO_2 baseline air, CO_2 concentrations $\geq 6\%$ caused general depression of movement, and the rate of locomotion fell below prestimulus levels with the application of 12% and 16% CO_2.

In other experiments, nematodes were subjected to 20% CO_2 for 3 min followed by 100% CO_2 for 3 min. The rate of locomotion was depressed by 70% with 20% CO_2 and by 93% with 100% CO_2. Following the removal of 100% CO_2, the rate of locomotion slowly increased to prestimulus levels.

Gradients. To determine clearly whether these responses lead to effective migration of the nematodes in a gradient of CO_2, a concentration gradient was established across the direction of flow in the gradient flow chamber (Figure 1). Often, the nematodes showed a significant net migration with the same gas flowing through both sides. This was presumably due to shallow thermal gradients to which these animals are extremely sensitive (J.A. Diez, M.J. Pline, and D.B. Dusenbery, unpublished data). Thermal gradients were minimized, and a background rate of migration was established. Stimulus was added to one side of the flow and then switched to the other side. If the net migration changed

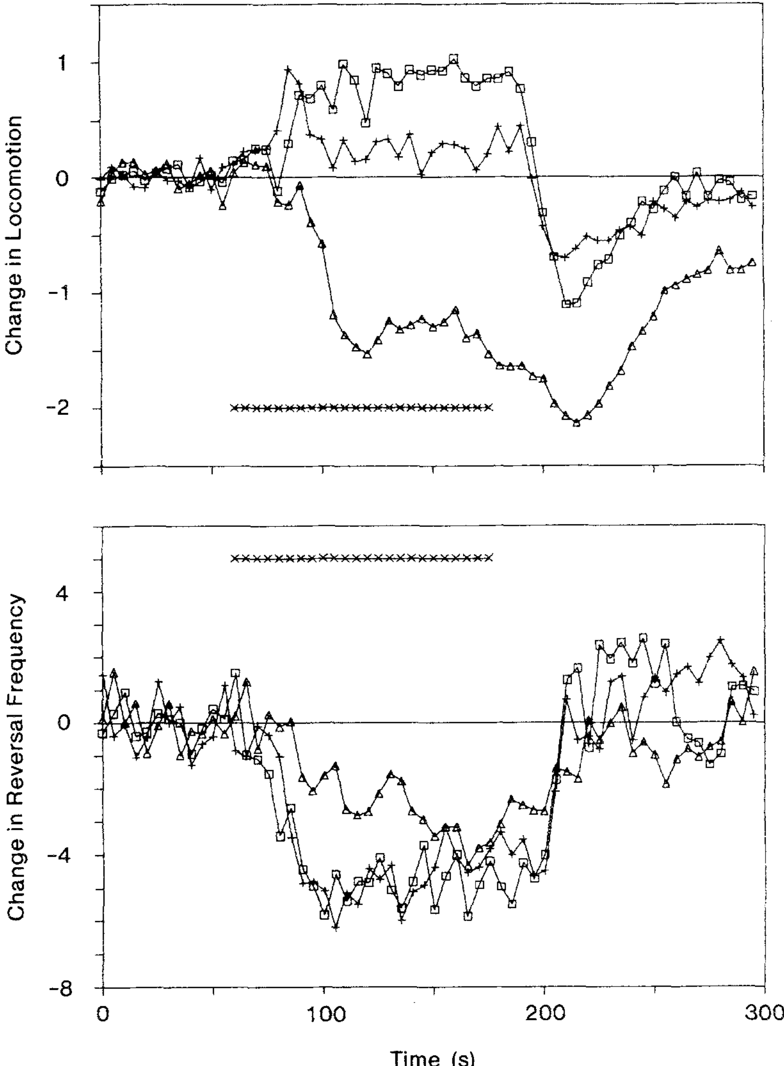

FIG. 5. Time course of changes in movements in response to large, temporal step changes in CO_2 concentration. Same as Figure 4, except the concentration increases are larger: (□) 4% CO_2, (+) 8% CO_2, or (△) 16% CO_2.

consistently either toward or away from the side with the stimulus, an effective migration to the chemical stimulus was established. Figure 7 shows that the nematodes migrated toward the CO_2. Migration away from CO_2 was not found with any tested concentration.

Table 1 summarizes results from 40 experiments that measured the re-

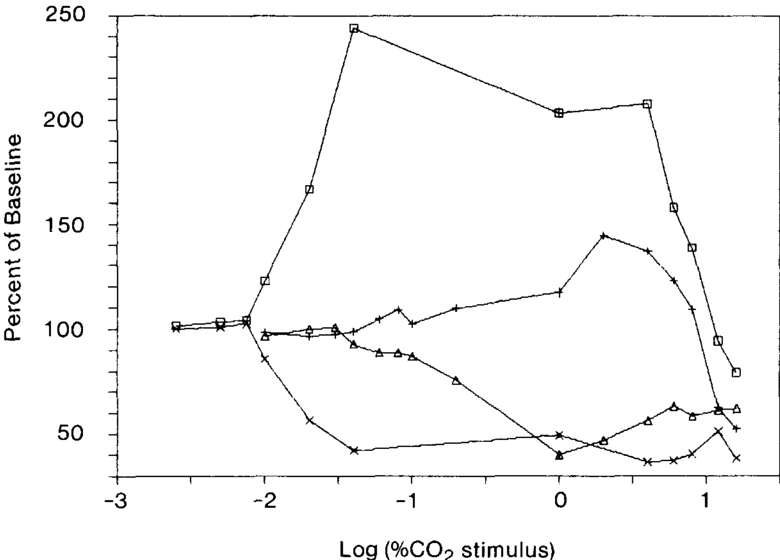

FIG. 6. Relative changes in locomotion and reversals as a function of concentration. Each data point represents the average locomotion or reversal frequency during the application of a stimulus concentration as a percent of the average prestimulus value. The stimulus is the change in concentration from the baseline value. Locomotion (□) and reversal frequency (×) with low CO_2 (0.003%) baseline concentration. Locomotion (+) and reversal frequency (△) with high CO_2 (1.04%) baseline concentration.

sponse of the nematodes to gradients of CO_2 containing low concentrations ranging from 0.02% to 0.24% CO_2. Relative gradient steepness was calculated as the percent change in CO_2 concentration per centimeter at the center of the field of camera view. The assumption that gradients were linear was supported by periodic measurements of CO_2 concentration at regular intervals along typical CO_2 gradients. The reported response is for locomotion perpendicular to the flow of air. The response for each experiment was the average rate of locomotion per nematode toward higher CO_2 concentrations (up the gradient) for the last 4 min of a 6-min gradient duration minus the average rate of locomotion per nematode for the last 4 min of a 6-min period either immediately preceding or immediately following the gradient application.

For each range of gradient strengths reported in Table 1, the response was the average of *N* experiments converted to cm/hr. Initial analysis of responses, grouped by relative gradient steepness, revealed little correlation between response strength and relative gradient steepness. When responses were grouped by absolute gradient steepness (and mean CO_2 concentration within the gradients), the rank sum test revealed that the nematodes detected and responded

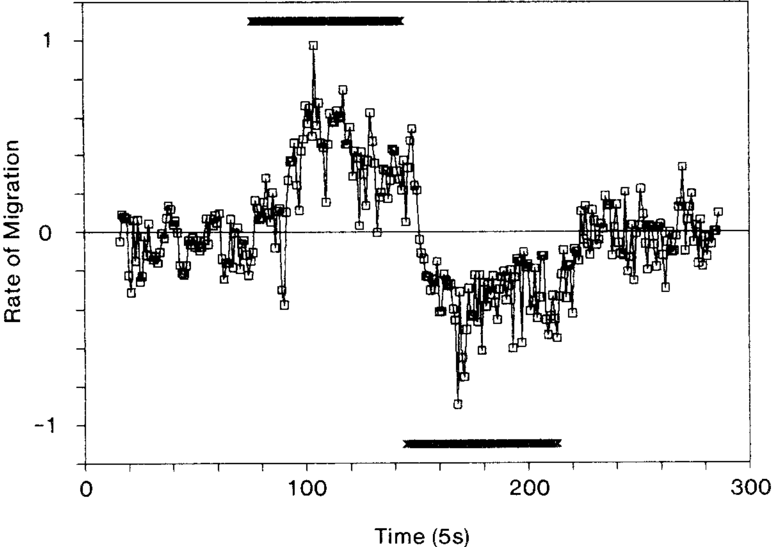

FIG. 7. Record of rate of migration in a steady gradient. The rate of migration across the flow is presented in units of pixels per nematode per tracking. Positive values indicate net movement to the left and negative to the right. The bar at the top indicates times during which 0.16% CO_2 was added to the left-hand side of the flow (containing 0.003% CO_2), creating a concentration gradient of 30% change/cm. The bar at the bottom indicates addition to the right side, creating a concentration gradient of 38% change/cm. At other times, no CO_2 was added.

TABLE 1. EFFECT OF GRADIENT STEEPNESS ON RATE OF MIGRATION AT LOW CONCENTRATIONS

Gradient type	Concentrations Mean (% CO_2)	Gradients: Relative (% change/cm)	Gradients: Absolute (% CO_2/cm)	Response (cm/hr): Mean	SD	*N*	P^a
Control	0.003	0	0	0.08	0.16	12	
Measured	0.026–0.028	16–12	0.004–0.006	0.07	0.06	4	>0.2
Measured	0.033–0.041	37–50	0.014–0.016	0.12	0.28	8	>0.5
Measured	0.055–0.082	30–38	0.020–0.024	0.66	0.36	8	<0.001
Measured	0.149–0.184	27–38	0.040–0.061	0.72	0.33	8	<0.0001

[a] *P* is the probability that rate of migration measurements came from the same population as the control measurements as determined by the rank sum test.

TABLE 2. EFFECT OF GRADIENT STEEPNESS ON RATE OF MIGRATION AT HIGH CONCENTRATIONS

Gradient type	Concentrations Mean (% CO_2)	Gradients Relative (% change/cm)	Gradients Absolute (% CO_2/cm)	Response (cm/hr) Mean	Response (cm/hr) SD	Response (cm/hr) N	P[a]
Control	1.04	0	0	0.04	0.16	12	
Estimated	1.05–1.07	0.5–1.0	0.006–0.01	0.26	0.25	14	<0.02
Measured	1.15–1.52	1.8–9.3	0.02–0.14	0.38	0.32	10	<0.005
Measured	1.69–2.02	12–20	0.22–0.41	0.24	0.37	16	<0.04

[a] P is the probability that rate of migration measurements came from the same population as the control measurements as determined by the rank sum test.

to absolute gradients of $\geq 0.024\%$ CO_2/cm at a mean CO_2 concentration of $\geq 0.082\%$. Their response to gradients with lower concentrations of CO_2 was not significantly different from the control experiments, even though relative gradient steepness was comparable or higher.

Table 2 summarizes results from 52 experiments using gradients of CO_2 with moderate concentrations ranging from 1.04% to 2.37% CO_2. All calculations were performed as in Table 1. Very shallow gradients were not accurately measurable using gas chromatography and were estimated conservatively by extrapolating from the relationship of injected CO_2 concentrations to measurable, steeper gradients. An analysis of grouped responses by the rank sum test indicated that the nematodes detected and responded to relative gradients of $\geq 1.0\%$ change/cm, which corresponded to absolute gradients of $\geq 0.01\%$ CO_2/cm.

DISCUSSION

Sensitivity. This research determined minimal concentrations and gradients for behavioral responses of root-knot nematodes to carbon dioxide. Experiments using sudden jumps in concentration and measuring individual rates of movement indicated that the minimal concentration for a response was about 0.01 volume percent (Figures 2 and 6). Experiments measuring response to gradients demonstrated that the minimal concentration was about 0.05% CO_2 (Table 1). These values are well below the concentrations usually found in the soil atmosphere (Boynton, 1941; Nye, 1981). Thus, the minimal sensitivity does not normally limit the response in soil. Rather, the response is likely limited by the minimal detectable change in concentration.

This aspect of the response was tested by exposing the nematodes to a

baseline concentration of 1.04% and increasing the concentration by small amounts. Measurement of individual movements in response to sudden changes indicated a threshold change of about 0.05% CO_2 (Figure 4) or a relative change of 5%. Experiments measuring the response to gradients demonstrated a threshold less than 0.01% CO_2/cm (Table 2). These values are significantly lower than the limit of 0.1% CO_2/cm established by Klingler (1963) for directed responses of *Ditylenchus dipsaci*. In terms of the relative steepness of the gradient, the limit for *M. incognita* is less than 1% change/cm (Table 2). The corresponding value calculated from Klingler's graph (1963, Figure 9) is about 50% change/cm. Thus, these experiments demonstrate a much higher degree of sensitivity to carbon dioxide than has previously been demonstrated for any nematode.

The juveniles continued to be attracted to carbon dioxide at higher concentrations. The only limit was a decrease in all movements at concentrations above 10% CO_2. This is probably due to the anesthetic effects of carbon dioxide known in other nematodes (Davis et al., 1986).

Mechanism. Klingler (1963) observed that *D. dipsaci* were capable of direct orientation (klinotaxis) to gradients. Our observed decreases in rate of change of direction with increases in CO_2 concentration (Figures 2–6) suggest that indirect orientation via klinokinesis may also occur. Similar observations were made with the response of *Caenorhabditis elegans* to CO_2 (Dusenbery, 1985b) and, using the tether technique, with the response of both *M. incognita* (Goode and Dusenbery, 1985) and *C. elegans* (Dusenbery, 1980) to NaCl. The major difference is that in most previous cases the response adapted in less than a minute. This type of response was observed at the high baseline concentration (Figure 4), but not at the low concentration (Figure 2). Adaptation makes indirect orientation much more efficient. Thus, the sensory–response system may function optimally at higher concentrations. However, higher rates of migration were observed at lower concentrations.

Both direct and indirect mechanisms may be used by nematodes in responding to other stimuli, with indirect orientation occurring at low concentrations and direct orientation taking place near the source at higher concentrations (Dusenberry, 1980). If this is true for *M. incognita* and *D. dipsaci*, the sensitivity observed by Klingler may differ from that observed here simply because different aspects of the response were measured.

The data allow estimation of the degree of orientation achieved by the nematodes. We simply divide the net distance moved per nematode (= the displacement of average position of all nematodes) in a given time interval by the total distance moved per nematode. The net distance moved is the square root of the sum of the squares of net movement in the x and y directions, after first compensating for the difference in pixel lengths. Because total distance moved was measured by the computer program as the sum of the absolute dis-

tance traveled in the x and y directions, it must first be multiplied by a factor of 0.79 (the average ratio of the true distance moved to the sum of the two orthogonal distances moved, assuming movement in random directions). If all nematodes were moving in the same direction for the entire time interval, the result would be 100%. If all were moving in random directions, the result would be 0%.

In 91 experiments where no CO_2 gradient existed, the mean degree of orientation was 7.2% ± 5.2% SD. In 56 experiments where gradient intensities were at or above threshold, the mean was 9.9% ± 5.7% SD. While this suggests a 38% increase in directed locomotion in the presence of CO_2 gradients, the degree of variation in results, the lack of complete independence of measurements, and the unknown effects of ubiquitous thermal gradients precludes stating that CO_2 gradients alone caused the increased orientation. We conclude that the nematodes can achieve a degree of orientation of about 10% in a gradient of stimuli. This can be interpreted as meaning that in a typical situation about 10% of the nematodes are moving in a particular direction, while the rest move at random.

Rate of Migration. An important question in understanding host location by plant-parasitic nematodes concerns the rate at which they can move along a stimulus gradient. An estimate can be made by using the computer tracking system to measure the movement of the average position of a group of nematodes in the x and y directions, compensating for the difference in pixel lengths, and taking the square root of the sum of the squares of the two values. Under baseline conditions when no CO_2 gradients existed, the average rate of migration was about 0.4 cm/hr. Under conditions when CO_2 gradients were above threshold, the average rate of migration was about 0.7 cm/hr.

Function. A variety of other plant-parasitic nematodes have been reported to be attracted to carbon dioxide (Prot, 1980). This has naturally led to the suggestion that this response allows them to find plant roots by moving up the concentration gradient created by rhizosphere production of carbon dioxide. However, some have suggested that it could only act within 1 or 2 cm of the root (Prot, 1980). Since we have determined the sensitivity with more precision, it is worth investigating this question quantitatively.

Nye (1981, Appendix) provides a theoretical basis for determining concentrations of carbon dioxide in the rhizosphere. Using his symbols and typical values,

$$Cg_r = Cg_a - aF \ln (r/a)/D$$

where Cg is the concentration of CO_2 in the gas phase; $a = 0.037$ cm, the radius of a typical root; $F = 1.59 \times 10^{-6}$ ml/cm^2/sec, rate of CO_2 production; $D = 0.065$ cm^2/sec the diffusion constant of CO_2 in soil.

The gradient is obtained

$$dCg_r/dr = aF/Dr$$

The maximum value, at the root surface, is 0.0025% CO_2/cm. This is much smaller than the 0.02% CO_2/cm threshold found at low baseline concentrations (Table 1). But the experiments at higher concentrations (Table 2) are more relevant, and in this case we could not establish gradients that were lower than 0.006% CO_2/cm. Thus, although attraction to roots requires an extraordinary sensitivity to shallow gradients, it remains a possibility (Dusenbery, 1987).

Another potential function of the response is to lead the nematode to the optimal depth in the soil profile. Boynton (1941) provides many measurements of carbon dioxide concentration at various levels in apple orchards. Analysis of data from his Table 9 for approximately 500 samples taken at 1-, 2-, and 3-ft depths shows that mean concentrations at the three depths were 2.2, 3.6, and 5.0% CO_2, and 90% of the differences between adjacent levels were in the range 0.4–5% CO_2. This range corresponds to gradients of 0.01–0.16% CO_2/cm. Theoretical analysis of CO_2 profiles in soil (Wesseling, 1962) suggests a range of 0.003–0.2% CO_2/cm in the upper 40 cm. Since nematodes showed a clear response to gradients less than 0.01% CO_2/cm (Table 2), sufficiently strong gradients should exist under most conditions. Since carbon dioxide generally increases with depth, the nematodes should move downward, away from the surface.

A third possibility is that this response serves simply to maintain a straight path. All organisms have limits on how accurately they can move along a straight line without reference to an external stimulus. In the absence of any cues as to the location of a target, the best strategy for an organism may be to move in a straight path in order to sample as large an environment as possible in a given time. Circling is inefficient and invariably happens when an animal has no directional references. Stimuli that serve such a function might well be termed "collimating stimuli." It is possible that carbon dioxide is such a stimulus. If so, carbon dioxide gradients do not lead the nematode to plant roots or even to a particular level in the soil but simply provide a directional reference. To fulfill this function, the only requirement is that the gradients be above threshold and extend over distances that are large compared to the size of the nematode. The rapid diffusion of carbon dioxide, which makes gradients around roots shallow, ensures that what gradients do exist extend over relatively large distances.

The quantitative effect of a collimating stimulus can be analyzed using a random walk with step size L at a frequency f for a time period t. Let $L = 0.01$ cm, roughly the size of soil particles and accessible spaces. A reasonable value for f is 0.1 Hz. Let $t = 86{,}400$ sec or a day. The root-mean-square distance moved, x, in three dimensions is

$$x = L(ft/3)^{1/2} = 0.54 \text{ cm}$$

On the other hand, if the walk is all in one direction

$$x = Lft = 86 \text{ cm}$$

In other words, the nematode travels 160 times further in a straight path. Our observations indicate that the nematodes typically achieve only about 10% orientation. This would suggest 10 cm/day as a more realistic estimate, which is still superior to completely random searching.

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ROLE OF BENZOXAZINONES IN ALLELOPATHY BY RYE (*Secale cereale L.*)[1]

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Abstract—Two phytotoxic compounds [2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one (DIBOA) and 2(3*H*)-benzoxazolinone (BOA)] were previously isolated and identified in 35-day-old greenhouse-grown rye shoot tissue. Both compounds were also detected by TLC in greenhouse-grown root and field-grown shoot tissue. The concentration of DIBOA varied in the tissues, with the greatest quantity detected in greenhouse-grown shoots. DIBOA and BOA were compared with β-phenyllactic acid (PLA) and β-hydroxybutyric acid (HBA) for activity on seed germination and seedling growth and were consistently more toxic than either compound. Dicot species tested, including lettuce (*Lactuca sativa* L.), tomato (*Lycopersicon esculentum* Mill.), and redroot pigweed (*Amaranthus retroflexus* L.), were 30% more sensitive than the monocots tested. Of the two benzoxazinone compounds, DIBOA was most toxic to seedling growth. DIBOA and BOA reduced chlorophyll production in *Chlamydomonas rheinhardtii* Dangeard, by 50% at 7.5×10^{-5} M and 1.0×10^{-3} M, respectively. Both DIBOA and BOA inhibited emergence of barnyardgrass (*Echinochloa crusgalli* L. Beauv.), cress (*Lepidium sativum* L.), and lettuce when applied to soil, indicating their potential for allelopathic activity.

Key Words—Hydroxamic acids, benzoxaquinone DIBOA, BOA, allelochemicals, allelopathy.

INTRODUCTION

Allelopathy, where one plant chemically interferes with the germination, growth, or development of another plant, has been implicated in many aspects

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of higher plant interactions (Muller, 1966; Whittaker and Feeny, 1971; Rice, 1974; Swain, 1977; Lovett, 1982). Difficulties in separating allelopathy from competition and detection of small quantities of biologically active compounds have hindered past research. As a result, there are few instances which clearly implicate a particular chemical in the observed interference between two plants. Even where chemicals have been identified, their significance and role in higher plant interactions under field conditions remain unclear (Harborne, 1977).

Winter rye (*Secale cereale* L.) is an annual grass that has proven useful as a rotational species in a variety of cropping systems (Overland, 1966; Benoit et al., 1962) and has been found to contribute organic matter, reduce soil erosion, and enhance water penetration and retention (Benoit et al., 1962; Blevins et al., 1971). An increased use of reduced-tillage systems employing cover crops such as winter rye will undoubtedly be important to agriculture in the future. Residues at the soil surface are inherent to reduced tillage systems. This residue may interact with weeds and/or crops to inhibit growth of plants. The interference is believed to arise from chemicals released into the environment via both root exudates and plant residues.

Although there are numerous reports of toxicity by rye and its residues (Cubbon, 1925; Hill, 1926; Osvald, 1953; Patrick and Koch, 1958; Patrick, 1971; Kimber, 1973; Chou and Patrick, 1976; Putnam and DeFrank, 1982; Barnes and Putnam, 1983; Shilling et al., 1985), none have conclusively explained the interference. Fuerst and Putnam (1983) proposed a set of criteria to be met for proof of allelopathic interference. Through a series of experiments leading up to and including the present work, we have attempted to meet these criteria when evaluating the allelopathic activity in rye residues.

Initially, the symptoms of interference must be identified in the natural state with experiments designed to eliminate competitive and indirect forms of interference (Fuerst and Putnam, 1983). In no-till field studies designed to separate physical from chemical mulch influences, ''Wheeler'' rye residues significantly reduced weed density and biomass over both no-residue and poplar (*Populus*) excelsior (PE) controls (Putnam et al., 1983). Greenhouse studies which simulated the no-till system confirmed field experiments (Barnes and Putnam, 1983; 1985). Indicator species emerging through the rye residue in greenhouse bioassays were chlorotic and stunted, although nutrient supplies were superoptimal.

Bioassays testing the effects of greenhouse-grown residues in soil on early stages of seed germination and seedling growth produced the greatest toxicity when seed was placed in close proximity to the residue (Barnes and Putnam, 1985). The soil diminished the activity, although growth reduction still occurred when residues were placed 15 mm away. Radicle elongation was a more sensitive measure of toxicity than germination, per se. Shoot tissue was about twice as active as root tissue on the species tested with 0.8 g/150 g soil placed

5 mm away resulting in 50% inhibition of root length. Lettuce (*Lactuca sativa* L. "Ithaca") was particularly sensitive as rye residues caused the apical root meristem to become discolored and root growth was subsequently inhibited. Phytotoxicity from shoot tissue or extracts persisted in the absence of soil microorganisms, suggesting they were more toxic than decomposition products.

The second step in proof of allelopathy is chemical isolation, identification, and assay (Fuerst and Putnam, 1983). Aqueous extracts of greenhouse-grown rye shoot tissue were separated via solvent partitioning, and all fractions were bioassayed for relative activity on cress (*Lepidium sativum* L. "Curly") germination and seedling growth. The greatest toxicity occurred from the diethyl ether fraction, and two compounds inhibitory to cress seedling growth were identified from this fraction: 2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one (DIBOA) and its breakdown product, 3(3*H*)-benzoxazolinone (BOA) (Barnes et al., 1986).

The purpose of this research was to further characterize the activity and assess the role of DIBOA and BOA in allelopathy by rye. This is directed toward final steps in proof where it is necessary to simulate the interference by supplying the toxin as it might be supplied in nature and to quantify the release, movement, and uptake of the toxins produced (Fuerst and Putnam, 1983).

METHODS AND MATERIALS

Comparison of Organic Extracts from Field- and Greenhouse-Grown Rye. Field grown "Wheeler" rye shoots for extraction were obtained from a planting made on October 2, 1983. On May 11, 1984, rye (preboot stage) was mowed down. Four days later, shoot tissue was collected, dried at 50°C, ground through a 40-mesh screen in a Wiley mill, and stored at −20°C until extracted.

Thirty-five-day-old greenhouse-grown rye shoots were cut, dried, ground, and stored similarly. Root tissue of 35-day-old greenhouse-grown rye was rinsed free of most soil and processed as shoot material. From this point all tissues were treated similarly.

For qualitative comparisons, equivalent weights of tissues were extracted for 24 hr with equal volumes of H_2O. Aqueous fractions were precipitated with Me_2CO and sequentially partitioned against a series of solvents of increasing polarity (hexane, Et_2O, CH_2Cl_2, EtOAc). All organic fractions were dried over $MgSO_4$ (anhydrous), concentrated, and weighed for percent recovery. Organic fractions were also qualitatively compared via thin-layer chromatography (TLC) on silica plates (Merck SF-254, 250 μm) in two solvent systems (EtOAc and CH_2Cl_2–MeOH (95:5)]. Each extract (100 μg) was spotted, developed, and detected by UV-visible, vanillin–H_2SO_4, $CeSO_4$–H_2SO_4, and $FeCl_3$–HCl.

Preparation of Extracts for Quantification. Dried rye tissue (1.0 g) and

H_2O (12 ml) were added to test tubes, vortexed for 1 min, and sonicated for 3 hr (32°C). The residue was transferred from test tubes by rinsing nine times sequentially with H_2O (2 ml) and suction filtered through Whatman No. 2 filter paper until dry. The residue was discarded, and the pH of the filtrate was adjusted to 2.0 with concentrated HCl. The filtrate was then heated for 1 min at 65°C, cooled in an ice bath, and filtered (Millipore, 0.45 μm). A 5-ml aliquot of this sample was extracted with two (4 ml) volumes of EtOAc. EtOAc was removed with a syringe, transferred to a second set of tubes, and dried by blowing a stream of nitrogen gas over the sample maintained at 50°C. Samples were redissolved in 3 ml of MeOH and filtered (Millipore, 0.45 μm).

Quantification of Hydroxamic Acids. DIBOA was quantified by the $FeCl_3$ method (Corcuera et al., 1978; Hamilton, 1964; Long et al., 1974; Sullivan et al., 1974; Argandona et al., 1980; Zuniga et al., 1983). Hydroxamic acids form with $FeCl_3$ reagent (50 g of $FeCl_3 \cdot 6H_2O$; 500 ml 95% EtOH; 5 ml of 1.5 M HCl) a blue complex whose absorbance is measured at 590 nm. Fifty microliters of $FeCl_3$ solution was added to each extract and concentrations were determined by comparing absorbance of the extract with a standard curve made with DIBOA. The purest DIBOA crystals from previous separations were used. All samples were replicated three times. The validity of the method has been discussed (Woodward et al., 1979).

Purification of DIBOA for Bioassays. DIBOA used for all assays was purified via preparative TLC. Any organic extracts containing DIBOA from previous solvent extractions were applied to prewashed silica plates (Whatman PLK5F 1000 μm) and ascendingly developed in $CHCl_3$–MeOH (9 : 1). Portions of the plates were sprayed with $FeCl_3$–HCl which specifically reacts blue with DIBOA. The corresponding zone on the unsprayed portion of the plate was scraped, ground with a mortar and pestle, and eluted with Me_2CO. The Me_2CO–silica slurry was suction filtered through fritted glass funnels, concentrated, and checked for purity by TLC (Whatman SF-254). The resultant pink-colored solution contained DIBOA and crystallized, but it still had a few contaminants. Therefore, the preparative TLC procedure was repeated on the resultant sample an additional three times (until pure by TLC). The purified DIBOA was then filtered (0.45 μm) to ensure removal of silica which would influence rate of application in bioassays.

Seed Germination and Seedling Growth Bioassays. DIBOA and BOA were bioassayed for activity on seed germination and seedling growth. Initially, BOA extracted from rye was compared with BOA obtained from Aldrich Chemical Company, for activity on cress. Equivalent weights of chemicals were applied in MeOH to Whatman No. 1 filter paper in Petri dishes (60 × 15 mm). Pure MeOH, similarly applied to filter paper, was used as a control. After solvent evaporation, 1.5 ml H_2O and 10 cress seeds were added to each dish. Root and shoot lengths were measured after 72 hr dark incubation in a high-humidity growth chamber (27°C).

Shilling et al. (1985) have previously identified and implicated β-phenyllactic acid (PLA) and β-hydroxybutyric acid (HBA) in rye residue toxicity. These chemicals are commercially available (Sigma Chemical Company) and were compared to DIBOA and BOA for their relative activity and selectivity on seed germination and seedling growth of several weeds [large crabgrass (*Digitaria sanguinalis* L. Scop.), barnyardgrass (*Echinochloa crusgalli* L. Beauv.), proso millet (*Panicum milaceum* L.) and redroot pigweed (*Amaranthus retroflexus* L.) with 20 seeds tested per replication] and crops [lettuce (*Lactuca sativa* L. "Ithaca") and tomato (*Lycopersicon esculentum* Mill. "Lafayette") with 10 seeds tested per replication]. The experimental design was a randomized complete block with four replications. Germination and average root and shoot length data were obtained after 72 hr.

For bioassay on seed germination and seedling growth, pure chemicals were applied in MeOH or H_2O (depending on solubility) to filter paper in Petri dishes. The final volume of H_2O in all dishes was 1.5 ml. Seeds were added and Petri dishes were dark incubated prior to evaluation. The experimental design was a randomized complete block with four replications.

Algae Growth Bioassay. DIBOA and BOA were also tested for activity on the alga, *Chlamydomonas rheinhardtii* Dangeard. The bioassay was developed by Hess (1980) and modified by Ries and Wert (personal communication). The stock culture was grown for one week in aerated nutrient solution in a growth chamber (30°C/18°C; 103 $\mu E/m^2/sec$; 16 hr day length). Prior to bioassay, the culture was subjected to 1 min of CO_2 gas (0.57 hl/min/2 l algal solution). Aliquots (10 ml) of algal solution were pipetted into test tubes (16 $\times$ 100 mm), and chemicals were added in 200 μl of Me_2CO. The control tubes received 200 μl of pure Me_2CO. All tubes were placed in the growth chamber for the bioassay.

After 28 hr incubation, samples were centrifuged for 15 min to pellet all cellular material, after which the supernatant was removed by aspiration. The pellet was resuspended in 5 ml of 80% Me_2CO in H_2O to solubilize the chlorophyll. After a second centrifugation (5 min), the supernatant was decanted and absorbance at 652 nm was determined. Zero-time, or initial, growth was determined on four (10-ml) aliquots of algae taken prior to addition of chemicals.

The experimental design was a randomized complete block with four replications. The effect of DIBOA and BOA on growth of algae over a 28-hr period was determined by subtracting the zero-time absorbance from the absorbance of the treated sample and expressed relative to the Me_2CO control.

Soil Activity of Hydroxamic Acids. Uptake of rye allelochemicals by susceptible plant species may be from direct contact with tissue or through the soil. To test the potential for uptake from soil, DIBOA from rye and BOA (Aldrich Co.) were applied to the soil immediately after planting indicator species [cress, barnyardgrass, and lettuce (10 seeds each)]. Pots (43 $\times$ 43 mm) were filled

with soil [Spinks loamy sand (Psammentric hapludalf, sandy, mixed, mesic)], and indicators were planted 3 mm deep. To reduce disturbance to the seed and soil interface, pots were subirrigated prior to spraying. Chemicals were dissolved in 4 ml of 50% Me_2CO in H_2O and uniformly applied to soil with an air-forced TLC spray reagent applicator at rates of 25, 50, and 100 kg/hectare. Controls received 4 ml of 50% Me_2CO in H_2O. Pots were placed in the greenhouse (28°C/18°C; 15-hr day length) under fluorescent lights (168 μE/m^2/sec) and subirrigated during bioassay. Emergence was recorded at 4 and 14 days. The experimental design was a randomized complete block with four replications.

RESULTS AND DISCUSSION

Comparison of Organic Extracts from Field- and Greenhouse-Grown Rye. Since DIBOA and BOA were implicated as the most active allelochemicals in 35-day-old greenhouse-grown rye (Barnes et al., 1986), it was necessary to confirm their presence in field-grown tissue harvested at similar stages of growth. DIBOA was found in the Et_2O, CH_2Cl_2, and EtOAc fractions, while BOA was only detected in the Et_2O fraction (Table 1). In both greenhouse- and field-grown herbage, the greatest quantity of DIBOA partitioned into the Et_2O fraction, and all fractions were generally very similar in appearance. DIBOA and

TABLE 1. DETERMINATION OF DIBOA AND BOA BY TLC IN ORGANIC EXTRACTS FROM AQUEOUS EXTRACTIONS OF RYE ROOT AND SHOOT TISSUE

Tissue	Organic Extract	DIBOA[a]	BOA[b]
Shoots	Hexane		
Field	Et_2O	+[c]	+
	CH_2Cl_2	+	
	EtOAc	+	
Greenhouse	Hexane		
	Et_2O	+[c]	+
	CH_2Cl_2	+	
	EtOAc	+	
Roots	Hexane		
Greenhouse	Et_2O	+	+
	CH_2Cl_2	+	
	EtOAc	+[c]	

[a] Based on $FeCl_3$ detection.
[b] Based on $CeSO_4$ detection.
[c] Fraction contains greatest quantity of DIBOA relative to others from that tissue.

TABLE 2. RECOVERY OF ORGANIC FRACTIONS OBTAINED FROM SEQUENTIAL PARTITIONING OF ROOT AND SHOOT EXTRACTS[a]

	Initial aqueous extract (%, w/w)		
Fraction	Field rye shoots	Greenhouse rye shoots	Greenhouse rye roots
Hexane	0.37	0.13	0.11
Et_2O	0.55	0.96	0.56
CH_2Cl_2	0.17	0.22	0.04
EtOAc	0.18	0.59	0.19
Total	1.27	1.90	0.90

[a] Dried tissue, (50 g), was extracted with 1 liter H_2O (24 hr; 4°C). Aqueous extracts were sequentially partitioned against solvents (250-ml volumes; 6–8 times), and concentrated on a rotary evaporator. Entire sample was transferred to preweighed glass tubes for recovery.

BOA were also detected in the same extracts of root tissue, although the EtOAc fraction contained the greatest quantity of DIBOA, and the entire range of compounds differed considerably.

Recovery of fractions by solvent partitioning also varied among tissues extracted (Table 2). Although Et_2O extracted more compounds from all tissues relative to other solvents, greenhouse-grown shoot tissue contained the greatest quantity of Et_2O-extractable compounds. Field-grown shoot tissue contained more hexane-soluble compounds than greenhouse-grown rye roots or shoots. Taking all fractions together, greenhouse-grown shoot tissue contained the greatest percentage of solvent extractable compounds.

Quantification of Hydroxamic acids. The content of hydroxamic acids has been shown to vary with the species (Zuniga et al., 1983), age of the plant, and organ assayed (Argandona et al., 1981). Most of the previous work on quantification of hydroxamic acids in rye has involved fresh tissue from young seedlings grown in the greenhouse (Argandona et al., 1980; Zuniga et al., 1983), which may differ from the content in residues of older, field-grown plants. As DIBOA is the predominant hydroxamic acid detected in rye (Hofman and Hofmanova, 1969; Zuniga et al., 1983) and the only compound which reacted with $FeCl_3$ in TLC separations, its concentration in dried tissues was determined colorimetrically.

Thirty-five-day-old greenhouse shoots contained up to 28-fold more hydroxamic acids than the other tissues (Table 3). These data represent only one harvest each of greenhouse and field plants and do not allow for assessment of variability due to age, environment, etc. The apparent difference in hydroxamic acid content between the two shoot tissues may account for the differential re-

TABLE 3. CONCENTRATION OF HYDROXAMIC ACIDS IN GREENHOUSE-GROWN RYE ROOTS AND SHOOTS AND FIELD GROWN RYE SHOOTS[a]

Tissue	Source	Concentration (μg hydroxamic acids/g dry wt)[b]	SD
Roots	Greenhouse	171.2	13.2
Shoots	Greenhouse	2865.4	13.3
Shoots	Field	104.0	13.3

[a]Determined colorimetrically (590 nm).
[b]Concentration was determined from mean of three samples.

covery of solvent-extractable compounds. Argandona et al. (1980) found the content of hydroxamic acids in rye to decrease with increasing age, which may further explain the large concentration difference between field- and greenhouse-grown shoots. The difference in concentration between roots and shoots of greenhouse rye is probably responsible for the differential activity demonstrated by the tissues in greenhouse and Petri dish bioassays of residues (Barnes and Putnam, 1985).

Activity on Seed Germination and Seedling Growth. The commercial availability of BOA provided an opportunity for further evaluation of the biological activity. The Aldrich product and BOA extracted from rye were initially compared for activity on cress root and shoot growth (Figure 1). BOA isolated from rye and Aldrich BOA similarily reduced cress seedling growth. Aldrich BOA is of higher purity and readily available and was therefore substituted for rye BOA in all remaining bioassays.

As results of toxicity by allelochemicals can vary with the bioassay and indicator species tested, chemicals must be tested in similar bioassays for comparison of activity. Shilling et al. (1985) have previously implicated PLA and HBA in allelopathic activity of field-grown rye residues. The relative activity of these compounds was compared with DIBOA and BOA in our seed germination and seedling growth bioassays (Figures 2 and 3). Overall, DIBOA and BOA were consistently more inhibitory than PLA and HBA to germination and seedling growth of all weeds and crops tested. Monocotyledenous and dicotyledenous plants varied in their response and sensitivity to the four compounds tested.

Of the four chemicals, DIBOA was most active against the monocot species (Figure 2A–C). While no chemical inhibited barnyardgrass germination, DIBOA significantly reduced germination of large crabgrass and proso millet. Root growth of grass species was more sensitive than shoot growth, with greatest activity by DIBOA. BOA reduced growth of large crabgrass and proso millet

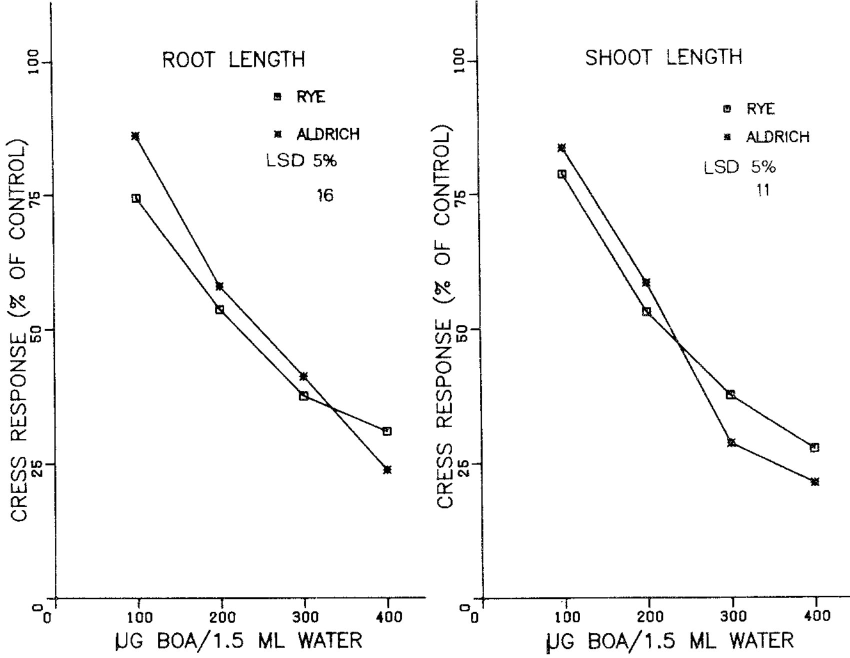

FIG. 1. Comparison of activity of BOA from Aldrich Co. and BOA extracted from rye on cress seedling growth.

at high concentrations but had little effect on barnyardgrass. The only significant activity by PLA was a reduction of large crabgrass root and shoot length at the highest concentration. Response of smooth crabgrass [*Digitaria ischaemum* (Schreb.) Muhl.] was similar to large crabgrass (data not presented). HBA had no effect at any concentration on any growth parameter tested. In general, germination was less sensitive than seedling growth to chemicals tested.

In contrast, both germination and seedling growth of dicot species tested were significantly inhibited by the benzoxazinone compounds tested (Figure 3A–C). BOA was inhibitory to germination of all three dicot species, while DIBOA showed significant reductions only at the higher concentrations. Both PLA and HBA had little effect on germination of the test species. Root and shoot growth of dicots were similarily affected by the chemicals tested. DIBOA and BOA were most toxic to seedling growth. BOA, in particular, caused lettuce root meristems to turn black. The necrosis of the apical root meristem closely resembled symptoms evident on lettuce germinating in the soil/residue Petri dish bioassays. PLA resulted in some significant reduction of dicot seedling growth, but it was still less active than DIBOA and BOA. Once again, HBA had no effect on any growth parameter of dicots tested.

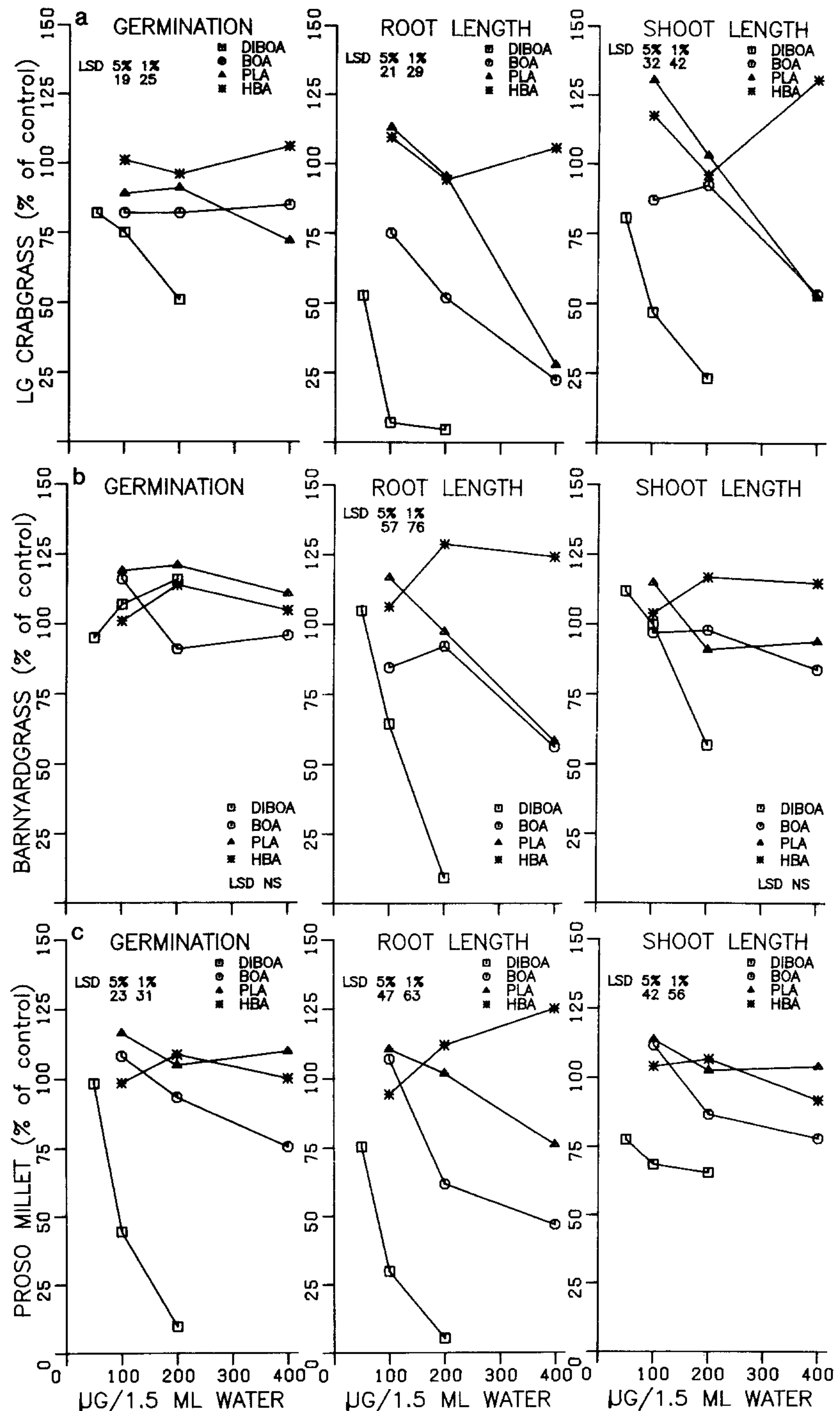

FIG. 2. Response of (A) large crabgrass, (B) barnyardgrass, and (C) proso millet to various concentrations of DIBOA, BOA, PLA, and HBA in Petri dishes.

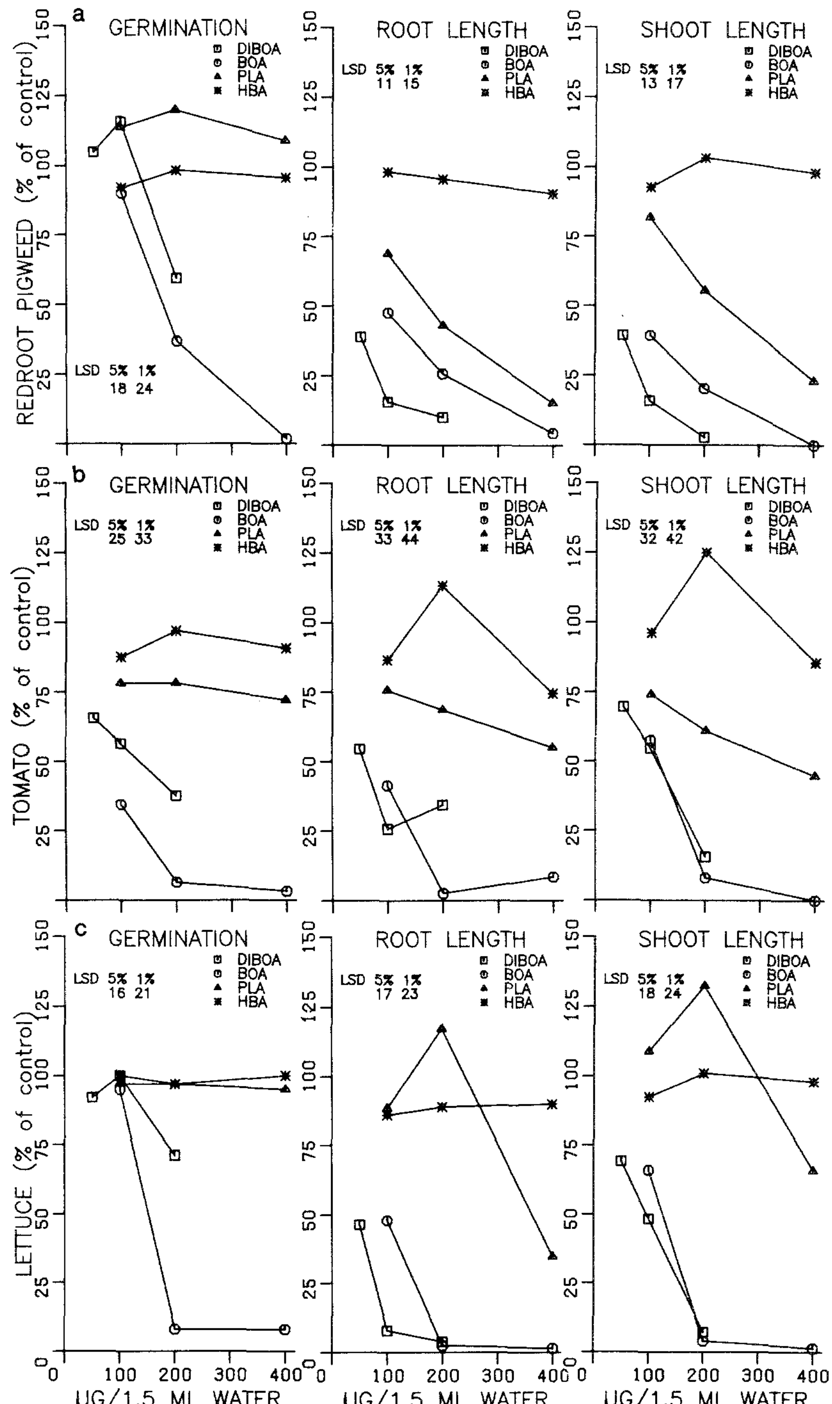

FIG. 3. Response of (A) redroot pigweed, (B) tomato, and (C) lettuce to various concentrations of DIBOA, BOA, PLA, and HBA in Petri dishes.

On average, the dicots were 30% more sensitive than the monocots to all rates of all chemicals tested. DIBOA and BOA were anywhere from 2 to 30 times more active than PLA and HBA. Of the two benzoxazinone compounds, BOA was more toxic to dicot germination, while DIBOA had greater activity on germination of monocots. Both chemicals similarily reduced dicot seedling growth, while only DIBOA had any significant effect on seedling growth of monocots.

Activity on Growth of Algae. Symptoms of DIBOA and BOA injury on cress when bioassayed in the light included a characteristic chlorosis starting at the leaf margin and progressing toward the midvein. As the concentration of DIBOA increased, the color of the cotyledons changed from a dark green to pale yellow and white. The injury resembled that of several commercial photosynthetic-inhibitor herbicides. Therefore, DIBOA and BOA were bioassayed on *Chlamydomonas*. At the highest concentrations tested, both compounds killed the algae (Figure 4). Although all cells appeared dead, the results from BOA

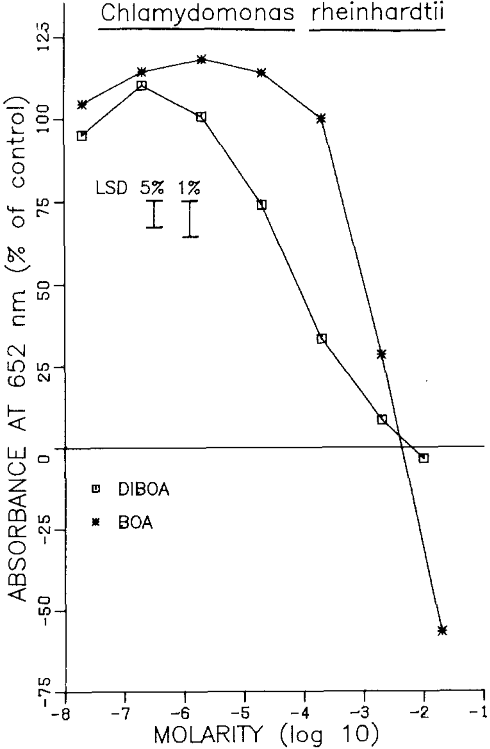

FIG. 4. Response of *Clamydomonas rheinhardtii* Dangeard to various concentrations of DIBOA and BOA based on absorbance at 652 nm.

at the highest concentrations may be somewhat misleading as it interfered with absorbance at 652 nm. Fifty-percent inhibition of growth occurred at ca 7.5×10^{-5} M and 1.0×10^{-3} M for DIBOA and BOA, respectively. At low concentrations, both DIBOA and BOA stimulated algae growth relative to control over a 28-hr period.

Queirolo et al. (1981) found both 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and its glucoside, extracted from maize, inhibited both cyclic and noncyclic photophosphorylation in spinach (*Spinacea oleracea* L.) chloroplasts. Inhibition of coupled electron transport was attained at concentrations of 1 and 4 mM, respectively. Several years ago, BASF Co. manufactured and evaluated a benzoxazinone related herbicide, Benzazin (BASF 1700H), with photosynthetic activity. Moreland and Hill (1963) proposed that the H—N—C=O fragment found in many polycyclic urea herbicides was related to activity. This fragment is present in BOA and reduced forms of DIBOA. Chlorosis was a symptom of injury by rye residues on several indicators (Barnes and Putnam, 1983) and may be related to the effects of DIBOA and BOA on photophosphorylation and electron transport.

Activity of DIBOA and BOA in Soil. Uptake of allelochemicals from rye residue may be by direct contact with tissue fragments or through the soil (Barnes and Putnam, 1985). Therefore, the pure compounds DIBOA and BOA were applied to soil for evaluation of activity on emergence of three indicator species. All rates of DIBOA and the higher rates of BOA completely inhibited emergence of both cress and lettuce four days after application (Table 4). Barnyardgrass emergence was less sensitive to the chemicals, although high rates of both compounds significantly reduced emergence relative to controls. Barnyardgrass seedlings which did emerge appeared to be stunted and chlorotic.

Two weeks after chemical application, emergence of lettuce and cress was

TABLE 4. EMERGENCE OF BARNYARDGRASS (BYGR), CRESS, AND LETTUCE 4 DAYS AFTER SPRAY APPLICATION OF DIBOA AND BOA TO SOIL

Chemical	Rate (kg/hectare)	% of control		
		BYGR	Cress	Lettuce
DIBOA	25	88	0	0
	50	20	0	0
	100	5	0	0
BOA	25	112	21	72
	50	63	0	0
	100	15	0	0
LSD 5%		31	19	20

TABLE 5. EMERGENCE OF BARNYARDGRASS (BYGR), CRESS, AND LETTUCE 14 DAYS AFTER SPRAY APPLICATION OF DIBOA AND BOA TO SOIL

Chemical	Rate (kg/hectare)	% of control		
		BYGR	Cress	Lettuce
DIBOA	25	65	11	10
	50	19	0	0
	100	7	0	0
BOA	25	81	51	72
	50	61	9	16
	100	32	0	0
LSD 5%		29	19	24

still inhibited completely by the highest rates of DIBOA and BOA (Table 5). Although still reduced relative to control, some cress and lettuce germinated and emerged in the lower rates of DIBOA after 14 days. In general, emergence of barnyardgrass at 14 days was similar to that at four days.

The concentrations of benzoxazinones applied to soil in this study are considerably higher than the potential calculated for production in field situations if the data from fall-planted, spring-harvested rye (Table 3) are representative. In field studies where shoot residue biomass was 4.9 Metric Tons (MT)/hectare (rye killed at pre boot stage), the potential hydroxamic acid concentration was ca 0.5 kg/hectare. This figure does not take into account the relative contribution from root tissue. Calculations of hydroxamic acids in residues of 35-day-old rye were based on 4.7 MT/hectare [biomass of 40-day-old spring planted rye (Barnes, 1981)] and indicate a potential for ca. 13.5 kg/hectare from shoots and ca. 0.8 kg/hectare from roots. None of the field calculations accurately assess the possible contribution of BOA to the total activity. When the pure compounds were bioassayed in separate studies at rates lower than 10 kg/hectare, there was no inhibition and occasional stimulation by the chemicals (data not included).

The stimulatory effects of hydroxamic acids at lower concentrations may result from their ability to chelate iron (Waid, 1975; Lewin, 1984). Tipton and Buell (1970) have determined the stability constants for ferric iron complexes with two hydroxamic acids in maize [DIMBOA ($\log K = 21.3$); DIBOA ($\log K = 19.4$)] and found them to be much greater than that of citric acid ($\log K = 11.9$) which is reported to function in microbial iron metabolism (Lewin, 1984) and absorption and transport in higher plants (Sillen and Martell, 1964). Tipton and Buell concluded that at the concentrations (10^{-5} M to 10^{-3} M) present in young maize plants, a higher proportion of ferric iron must be bound as complexes of the hydroxamic acids.

Argandona et al. (1980) have similarily determined the concentration of hydroxamic acids in leaf extracts of different aged greenhouse-grown rye. In order to transform their concentrations for 34-day-old rye to kilograms hydroxamic acid per hectare, the following assumptions have been made: (1) 10 kg fresh weight is equivalent to 1 kg dry weight; (2) the predominant hydroxamic acid in rye is DIBOA with a weight of 181 g/mol; and (3) a similarily aged spring-grown rye produces ca. 4.7 MT dry tissue/hectare in the field. According to the concentrations presented by Argandona et al. (1980), 34-day-old rye contains 1.9 mmol hydroxamic acids/kg fresh weight or a potential of 16.2 kg hydroxamic acids/hectare. This calculation is somewhat greater than our data, but it further supports the potential for high levels in rye.

Although no data are available on the concentration of benzoxazinones in field-grown, spring-planted, rye, it is logical to assume that the trend of decreasing hydroxamic concentrations with increasing age, determined by Argandona et al. (1980), holds true. Two major differences between spring- and fall-planted rye are age and the exposure to cold. Both of these factors may influence the quantity and quality of chemicals produced by rye, as distinct developmental changes including emergence, shooting, heading, and flowering occur in winter rye as it matures. Temperature has the greatest influence on the duration of each stage in a given locality—light, moisture, and nutrients being other important factors (Nuttonson, 1958). As both spring-planted and greenhouse-grown ryes lack the significant exposure to cold, concentrations in similarily aged greenhouse tissue may provide a rough approximation of the field tissue. The potential levels of 13.5 and 16.2 kg hydroxamic acids/hectare calculated from concentrations determined by the authors and Argandona et al. (1980), respectively, could be considered at least 10 times greater than most rates of commercial herbicides used today. They are, however, about 10–100 times less active than most synthetic herbicides.

While our major field obsevations for proof of allelopathy have been based on phytotoxicity by residues of fall-planted, spring-killed rye, there is evidence for toxicity by spring-planted rye (Barnes, 1981). Residues of 40-day-old spring-planted rye reduced total weed density by 69% and total weed biomass by 32% when compared to a PE control mulch. As age may be an important variable and could be managed by timing the kill, the potential exists for maximizing benzoxazinone concentrations in the field. Additional studies which quantify and compare concentrations in both fall- and spring-planted rye killed at different ages would provide data useful in this regard. Ideally, the actual release of benzoxazinones into soil, and uptake by plants, perhaps using radiolabeled material, should also be documented for conclusive proof of their role in allelopathy.

While phytotoxicity by residues in nonsterile soil was significant after incubation for 72 hr (Barnes and Putnam, 1985), benzoxazinones may be easily degraded by microorganisms. Undoubtedly, several compounds, in addition to

the solvent extractable benzoxazinones, contribute toward toxicity by rye residues. This is evidenced by the fact that almost 50% of the initial aqueous extract activity was associated with the protein precipitate and final aqueous fractions (Barnes et al., 1986). The identification of benzoxazinones, and their subsequent implication in allelopathy by rye, has been based on steps directed toward isolation of the most active chemicals. Although the ether extract accounted for only 12% of the potential toxicity, it was 11–25 times more active than the protein and final aqueous fractions.

Some allelochemicals may leach from green rye or in the early stages after it is killed. Additional toxicity by residues may also result from phytoactive degradation products. Chou and Patrick (1976) found significant toxicity with residues of rye which had decomposed for up to 30 days. BOA is the major chemical breakdown product of DIBOA and, in our studies, shows greater activity against germination of all dicot species tested. Although the $FeCl_3$ method precludes quantitation of BOA and subsequent determination of its significance in rye residue toxicity, levels should presumably increase as DIBOA is broken down. Monitoring the breakdown and resultant toxicity in the soil over time should provide evidence for the persistance of both chemicals.

The importance of allelochemicals produced in response to injury or infection is indicated by the benzoxazolinone series of compounds which occur naturally in many grain plants (Beck and Reese, 1976). Previously, their role and significance in resistance to insects and disease organisms have been investigated more thoroughly than their role in allelopathy.

With regard to their plant activity, the greenhouse bioassays provided evidence for the toxicity of the benzoxazolinone series of compounds on whole plants in simulated no-till systems. The similar injury symptoms noted where residues had previously been applied and, further, where pure compounds are applied, strongly support the premise of allelopathic interference by rye with benzoxazinones playing a prominent role.

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IDENTIFICATION OF SEX PHEROMONE OF CALENDULA PLUME MOTH, *Platyptilia williamsii*

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Abstract—The sex pheromone of the calendula plume moth, *Platyptilia williamsii* was identified as (Z)-11-hexadecenal (Z11-16:Ald). Extracts of female sex pheromone glands contained several compounds when analyzed by capillary and packed-column GLC. However, airborne collections of volatiles from glands contained only one of these compounds, having the same retention time as Z11-16:Ald. GC-MS and microozonolysis analyses of the natural product were consistent with those of synthetic Z11-16:Ald. In a flight tunnel, males oriented upwind and touched sources of Z11-16:Ald and gland extract with equal frequency. Field tests of synthetic Z11-16:Ald already have shown it to be a potent sex attractant for males of this species. This study further supports the hypothesis that *P. williamsii* and a sympatric species, *Platyptilia carduidactyla*, are not reproductively isolated by chemical differences in the composition of the sex pheromone, but rather by temporal differences in sexual activities.

Key Words—*Platyptilia williamsii*, Lepidoptera, Pterophoridae, calendula plume moth, insect pheromones, (Z)-11-hexadecenal, reproductive isolation, insect behavior.

INTRODUCTION

The calendula plume moth, *Platyptilia williamsii* Grinnell, is part of a composite-feeding subgroup of the genus *Platyptilia* (Lange, 1950). It can be an occasional pest of greenhouse flowers (Lange, 1942), has been reported to feed (rarely) on artichokes (Lange, 1950), and is most common in composite wildflowers of coastal California. This species is very closely related to, and sym-

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patric with, the artichoke plume moth, *Platyptilia carduidactyla* (Riley) (Lange, 1942). Under natural conditions there is little cross-attraction between the two species, but manipulation of the photoperiodic regimes that synchronizes sexual activities leads to cross-attraction and even interspecific mating (Haynes and Birch, 1986). Furthermore, (Z)-11-hexadecenal (Z11-16:Ald), the identified sex pheromone of *P. carduidactyla* (Klun et al., 1981) was effective in attracting males of both species to traps (Haynes and Birch, 1986). However, the sex pheromone of *P. williamsii* has remained unknown. The present study provides evidence that Z11-16:Ald is the sex pheromone of *Platyptilia williamsii*, and there is no behavioral or chemical evidence of additional components. These findings support the important role of temporally based rather than chemically based reproductive isolation between *P. williamsii* and *P. carduidactyla*.

METHODS AND MATERIALS

Infested seaside daisies, *Erigeron glaucus*, were collected near Bodega Bay, California. Larvae and pupae were dissected out of the plant, and larvae were transferred to potted *Calendula officinalis*, on which they were allowed to complete development. Pupae were kept individually in 3.9 × 2.2-cm-diam. plastic vials with nylon screen tops. On emergence, adult males and females were separated and housed in separate environmental chambers (16:8 hr light-dark, 21°C-15.5°C). Adults had access to 8% sugar water on a cotton wick.

Calling behavior of 3- to 5-day-old females was observed every half hour [following the procedures of Haynes and Birch (1984a)] for the duration of the scotophase and until calling had ceased for 3 hr in the photophase in order to determine the optimal time to extract pheromone from the females.

Extracts of pheromone glands were made between the sixth hour of the scotophase and the beginning of the photophase. The ovipositor and the associated bilobed sex pheromone gland were forcibly extruded and removed with a pair of fine-tipped forceps. Several types of gland extracts were prepared, including (1) individual glands dipped for 2 sec in 5 μl of glass-distilled CS_2, (2) pooled extracts of ca. 20 female-equivalents in which the glands were dipped for 2 sec in 200 μl CS_2, and (3) pooled extracts of ca. 20 female equivalents each in which the glands were left in 200 μl of CS_2 or hexane overnight.

Airborne collections of volatiles from forcibly extruded pheromone glands were carried out following the procedure outlined by Baker et al. (1981). A single female was inserted abdomen first into the large-diameter end of a glass tube that tapered to an internal diameter of 1.5 mm. At the small distal end of the tube, a 0.5-mm hole allowed the ovipositor and sex pheromone gland to emerge when pressure was applied to the head of the moth with a pipe cleaner. When the bilobed sex pheromone gland was fully extruded, the glass tube was inserted through a Teflon-coated rubber septum into the all-glass collector de-

scribed by Haynes et al. (1983). After the 10-min collection period, an internal standard (either 1 ng of (*Z*)-11-octadecenyl acetate or 1 ng of (*Z*)-5-undecenyl acetate) was added to the glass wool at the distal end of the collector before it was rinsed out with ca. 100 μl of CS_2. The volume of CS_2 was reduced to ca. 1 μl for GLC analysis.

Gland extracts were analyzed by GLC using a Varian model 3700 GLC equipped with a flame ionization detector and a capillary and packed column. A 30-m DB-5 capillary column was run using the following conditions: temperature program 100°C for 2 min, 30°C/min to 190°C; He flow rate through column, 4 ml/min; N_2 flow, 26 ml/min; H_2 flow, 23 ml/min; and air flow, 300 ml/min. A 4-m column packed with 3.6g 10% Silar 10C on 100–120 mesh Chromosorb W was used under the following conditions: isothermal at 170°C; N_2 flow, 45 ml/min; H_2 flow, 22 ml/min; air flow, 330 ml/min.

To determine the functional group of the pheromone, 18 female equivalents extracted in 200 μl CS_2 (dipped for 2 sec) were blown down to apparent dryness under N_2 in a 1-ml reaction vial. Red-Al® (50 μl, Aldrich Chemical Co.) was added to the vial, and immediately 2 N NaOH (100 μl) was added. The resulting solution was extracted two times with 0.25 ml hexane, and the hexane fraction was then washed twice with 0.5 ml water. The hexane fraction was blown down to apparent dryness, and CS_2 (10 μl) was added. Five microliters were injected onto the Silar 10C column for analysis; the remainder was blown down to apparent dryness, and acetyl chloride (50 μl, Aldrich) was added to the vial. After 30 min at 24°C, this solution was blown to apparent dryness, CS_2 (5 μl) was added, and then injected onto the Silar 10C column.

GC-MS analysis was conducted using a 12-m DB-1 capillary column, a Hewlett-Packard 5880A GC, and a Hewlett-Packard 5970 Mass Selective Detector using a temperature program with initial temperature 65°C for 6 min, 8°C/min to 200°C. An extract containing 15 female equivalents extracted in hexane overnight was blown down to apparent dryness under a stream of N_2, CS_2 (10 μl) was added, and the resulting solution reduced to ca. 2 μl before injection into the GC-MS.

For determination of the double-bond position, microozonolysis (Beroza and Bierl, 1967) was carried out on only the GC fraction that contained the one compound detected in airborne collections. Twenty female equivalents of glands extracted in CS_2 overnight were cleaned up first by collecting this GC fraction as it eluted from the Silar 10C and then from the DB-5 column, using a glass capillary tube (12.7 cm × 1.3 mm ID) packed with ca. 2 mg of glass wool. This resulted in ca. 20 ng of the compound previously detected in airborne collections with no detectable contaminants. For 1 sec, ozone was bubbled into 50 μl of pure CS_2 in a 4 cm × 3-mm-ID test tube in an acetone bath of Dry Ice. The cleaned-up natural product was then injected into the CS_2–O_3 solution, and immediately 30 ng of triphenylphoshine was added, cleaving the ozonide

at the position of the original double bond. The volume of CS_2 immediately was reduced to ca. 1 μl under a N_2 stream and analyzed by capillary GLC. Ozonolysis reactions also were run on the following synthetic standards: (Z)-7-, (Z)-9-, (Z)-11-hexadecenal, (Z)-13-octadecenal, and (Z, E)-2,13-octadecadienol.

Behavioral tests were carried out in the wind tunnel described by Vetter and Baker (1983), which was modified from that of Miller and Roelofs (1978). Males were transferred from their original individual holding cages to individual screen cages (3.5 × 3.3 cm diam.), during the photophase preceding the flight tunnel test. During the peak hour of calling activity 7–9 h after the scotophase began, males were released individually. Their response was tested to 10 ng of Z11–16:Ald, 10 ng of the peak of interest from CS_2 extracts of female glands, and CS_2 alone. The light level during the test was 0.3 lux, which allowed observation of the following behavioral categories: wing fanning, flight, upwind flight in the pheromone plume, and contact with the pheromone source. The wind speed in the tunnel was 0.5 m/sec and the temperature was 20°C.

RESULTS AND DISCUSSION

Observations of female *P. williamsii* indicated that under the specified photoperiod and temperature regime, the moths everted their sex pheromone glands during the last $1\frac{1}{2}$ hr of the scotophase and for up to 2 hr into the photophase. Airborne collections and extracts of pheromone glands were only conducted during this $3\frac{1}{2}$-hr period. Because there is a well-defined periodicity in responsiveness of male *P. williamsii* that is synchronous with calling behavior of females (Haynes and Birch, 1986), flight tunnel tests were conducted over the same time period as pheromone extractions, but always under scotophase conditions.

Analysis of individual gland extracts dipped for 2 sec in CS_2 on the DB-5 capillary column revealed a compound with the same retention time as Z11–16:Ald with several additional peaks having longer retention times (Figure 1A). Airborne collections of volatiles from extruded glands were free of these later peaks (Figure 1B). Similarly, individual gland extracts and pooled extracts analyzed on the Silar 10C packed column contained up to four detectable peaks (Figure 2A). The largest of these agreed in retention time with Z11–16:Ald. In airborne collections, the only detectable peak had a retention time identical to that of synthetic Z11–16:Ald. The Silar 10C column effectively separates *cis* and *trans* isomers of monounsaturated 16-carbon aldehydes, and many of the positional isomers.

Reduction of a pooled gland extract with Red-Al yielded a peak with the same retention time as synthetic (Z)-11-hexadecenol on the Silar 10C column. In addition, the peak corresponding to Z11–16:Ald was no longer detected.

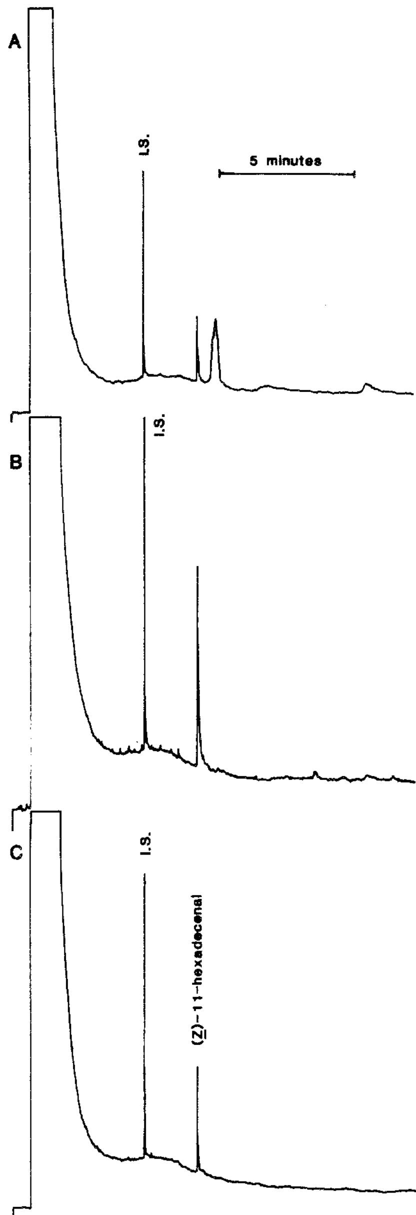

FIG. 1. (A) GLC analysis on DB-5 capillary column of sex pheromone gland extracted for 2 sec in CS_2 with internal standard, (Z)-5-undecenyl acetate. (B) Airborne collection of volatiles emitted from sex pheromone gland with internal standard. (C) Synthetic standards: 1 ng (Z)-5-undecenyl acetate and 0.5 ng Z11-16:Ald.

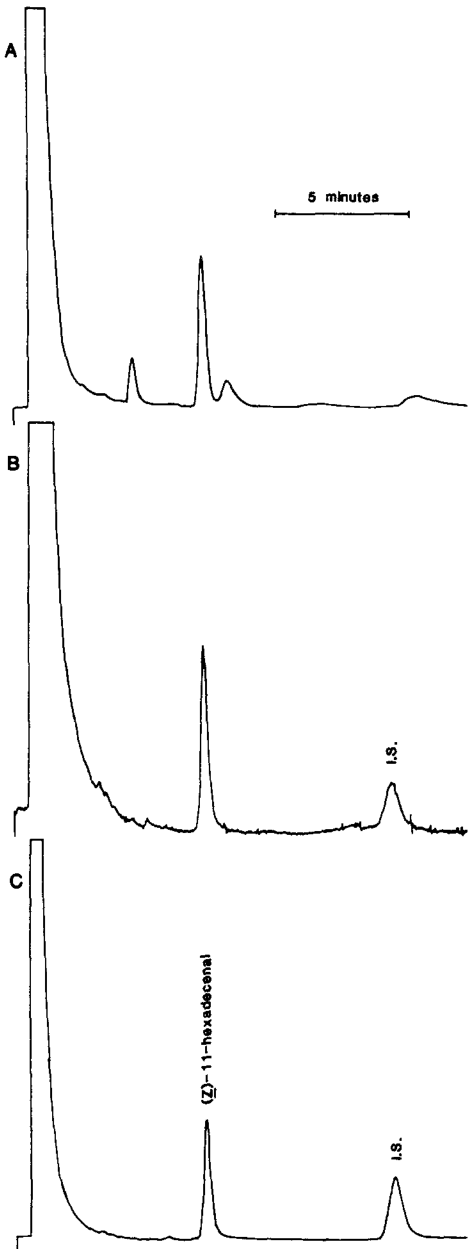

FIG. 2. (A) GLC analysis on Silar 10C packed column of four female equivalents from glands extracted for 2 sec in CS_2 with no internal standard. (B) Airborne collections of volatiles emitted from sex pheromone gland with internal standard, (Z)-11-octadecenyl acetate. (C) Synthetic standards: 10 ng Z11–16: Ald and 10 ng (Z)-11-octadecenyl acetate.

Acetylation of the reduced product yielded a compound with the retention time of (Z)-11-hexadecenyl acetate and loss of the putative alcohol product. These data provided additional evidence that the peak of interest was an aldehyde. Identical derivitization of synthetic Z11–16:Ald yielded the products with the same retention times as those from natural extract.

GC-MS analysis of 15 female equivalents containing ca. 40 ng of the putative pheromone (retention time 21.07 min) gave a good match to the spectrum obtained from 20 ng of synthetic Z11–16:Ald (base peak at *m/e* 55 and $M^+ - 18$ at *m/e* 220). The M^+ peak expected at *m/e* 238 was not detected in synthetic standards of 20 ng or less but was detected in standards of 100 ng (ca. 1% of base peak), and in the 40-ng sample of natural extract (ca. 1% of base peak).

The GC-purified natural extract sample was analyzed on DB-5 and found to contain no detectable peaks other than the putative pheromone. This purified sample was then ozonized and examined on the DB-5 column for reaction products. A peak that agreed in retention time with an ozonolysis product (undecanedial, retention time 5.1 min) of synthetic Z11–16:Ald was detected. A compound with the same retention time was detected in the ozonolysis products of (Z, *E*)-2,13-octadecadienol, but not the dial products of (Z)-7-hexadecenal (R_t 2.41 min), (Z)-9-hexadecenal (R_t 3.96), and (Z)-13-octadecenal (R_t 6.35 min). The separation of the ozonolysis products was sufficient to allow the position of the double bond to be determined unambiguously to be between the 11 and 12 carbons in the 16-carbon aldehyde.

Behavioral data from the flight tunnel clearly supported the role of Z11–16:Ald as the sex pheromone of *P. williamsii*. There was no significant difference between the behavior elicited by 10 ng of synthetic Z11–16:Ald (>98% free of other sex pheromone-like volatile impurities) and a gland extract containing 10 ng of the putative pheromone plus other potential minor components obtained by the method of extraction (glands in CS_2 overnight) (Table 1). In tests of solvent controls, there was some background level of wing fanning and flight (although significantly less than that stimulated by natural extract or the synthetic compound, but upwind flight and source contact were observed only in response to extract or synthetic Z11–16:Ald.

Field tests also supported the identification of Z11–16:Ald as the sex pheromone of this species (Haynes and Birch, 1986). Traps baited with 10 8-mil black hollow fibers loaded with Z11–16:Ald (provided courtesy of the Controlled Release Division of Albany International) captured more, although not significantly more, males than traps baited with two virgin females [20.4 ± 31.8 and 17.3 ± 28.6 (SD) males per trap per week], while blank traps caught no males. Higher release rates of Z11–16:Ald resulted in the capture of significantly fewer males (Haynes and Birch, 1986), suggesting an upper limit for upwind flight to the sex attractant.

Based on these chemical, behavioral, and trapping data, there is sufficient evidence to classify Z11–16:Ald as the sex pheromone of *P. williamsii*. No

TABLE 1. CONDITIONAL PROBABILITIES OF BEHAVIORAL TRANSITIONS OF MALE *Platyptilia williamsii*'S RESPONSE TO EXTRACTS OF SEX PHEROMONE GLANDS, SYNTHETIC (Z)-11-HEXADECENAL, AND SOLVENT CONTROL IN FLIGHT TUNNEL ASSAY[a]

Treatments	N	Stationary to wing fanning	Stationary to flight	Flight to upwind flight	Upwind flight to source contact	Overall (Stationary to source contact)
Treatments	N	Stationary to wing fanning	Stationary to flight	Flight to upwind flight	Upwind flight to source contact	Overall Stationary to source contact
Gland extract[b]	30	0.93a	0.97a	0.55a	1.00a	0.53a
Z11-16:Ald[c]	30	0.87a	1.00a	0.57a	0.88a	0.50a
Solvent control[d]	20	0.40b	0.50b	0b	—	0b

[a] Proportions in the same column are significantly different if they do not share a letter in common according to Ryan's (1960) multiple comparison test for proportions.
[b] Gland extract contains 10 ng of the putative pheromone component in 10 μl CS_2.
[c] Ten nanograms of synthetic (Z)-11-hexadecenal in 10 μl CS_2.
[d] Ten microliters of CS_2.

other pheromone components are implicated in any of these studies. Haynes and Birch (1986) demonstrated that calling behavior by females of *P. williamsii* and *P. carduidactyla* is temporally separate, with female *P. carduidactyla* emitting pheromone during the first half of the night and *P. williamsii* calling during the second half of the night. In addition, males only seek conspecific females during these times. Male *P. williamsii* are attracted to sex pheromone from calling female *P. carduidactyla* when the females' photoperiod is manipulated in a way that synchronizes the sexual activities of the two species (Haynes and Birch, 1986). This is strong evidence that the composition of the sex pheromone cannot be responsible for reproductive isolation between the two species. No minor pheromonal components of *P. carduidactyla* other than Z11-16 : Ald have been detected by gas chromatography (Klun et al., 1981; Haynes et al., 1983) nor are they suspected from field tests (Klun et al., 1981) or flight tunnel observations of behavior (Haynes and Birch, 1984b). Temporal differences between the sexual activities of the two species, rather than chemical differences in the pheromone blend, are sufficient to ensure that cross-attraction does not normally occur. Apparently this type of reproductive isolation is rare, since most sympatric species use blend differences with or without temporal differences (Cardé and Baker, 1984).

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LACK OF CHEMICAL FACTORS IN HOST-PLANT RESISTANCE OF ALFALFA TOWARDS ALFALFA WEEVIL:
Ovicidal Activity of Some Coumarin Derivatives

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Abstract—A resistant line of alfalfa was studied in an attempt to identify possible chemical factors responsible for alfalfa weevil (*Hypera brunneipennis*) resistance. Bioassays were developed to measure ovicidal, larval development, pupation, and adult development rates. Serial alfalfa extracts of a weevil-resistant line showed no effect in the bioassays when compared to those of a weevil-susceptible line. It was concluded that resistance substances are not present but that resistant lines are simply more tolerant to weevil attack. Alfalfa constitutents and synthetic analogs were also screened in the egg hatch bioassay. Among these were a series of 3-acyl-4-hydroxycoumarins. 3-Acyl-4-hydroxycoumarins with short-chain acyl groups showed good ovicidal rates, but activity decreased with increasing length of the acyl group.

Key Words—*Medicago sativa*, alfalfa, *Hypera brunneipennis*, *H. postica*, Coleoptera, Curculionidae, alfalfa weevil, host-plant resistance, bioassay, feeding deterrency, ovicide, antigrowth, coumarins.

INTRODUCTION

The Egyptian alfalfa weevil, *Hypera brunneipennis* (Boheman), was initially introduced into the American southwest in 1939 and is a destructive pest on commercial alfalfa in California (App and Manglitz, 1972). Morphologically it is indistinguishable from and differs from the closely related species, *H. postica* (Gyllenhal), largely in its overwintering behavior and its degree of parasitization, respectively. Much effort has been expended in a search for weevil-resistant alfalfa lines and the enhancement of weevil-resistant traits has been a

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central theme in a number of alfalfa breeding programs (Sorensen et al., 1972). To date, however, pronounced resistance to the weevil in commercial alfalfa lines has been elusive.

The life cycle of the alfalfa weevil has a number of different stages which may be sensitive to potential resistant substances in the plant. After about three weeks as an adult, the female weevil begins laying clusters of eggs in the hollow alfalfa stems. The choice of stems for egg laying by the female is highly dependent on their diameter (Norwood et al., 1967a; VanDenburgh et al., 1966). The females lay about two egg clusters per week with 1–30 eggs per cluster for the remainder of their reproductive life. The eggs hatch after an incubation period of one to two weeks. The resulting newly hatched larvae feed on the growing plant tip and are fully grown in three to four weeks. Pupation lasts one to two weeks. The duration of these different development phases is, of course, highly dependent on humidity, temperature, and photoperiod.

It is not known which stage(s) of the insect's life cycle is effected by possible plant substances in resistant alfalfa lines. Among the possible modes of alfalfa resistance having a chemical basis is the presence of: (1) ovicidal materials causing a decrease of the egg hatch rate; (2) feeding deterrent or antigrowth materials causing a decrease in larval development rate; (3) substances which would extend the duration of pupation; (4) materials causing a decrease in pupation rates, and (5) substances causing a decrease in the adult development rate.

This paper describes efforts to develop bioassay procedures designed to measure inhibition of various phases of weevil development. The bioassays were used to search for possible antibiotic activity in alfalfa extracts and constituents during experiments attempting to identify chemical factors contributing to weevil resistance in alfalfa. A number of synthetic materials related to alfalfa constituents have also been screened in the ovicidal bioassay for activity against weevil eggs.

METHOD AND MATERIALS

Maintenance of Weevil Colony. Weevils, biotype Colusa, were maintained on caged alfalfa bouquets of Caliverdi under an 8-hr light and 16-hr dark photoperiod. This light regimen was necessary to prevent the insects from going into diapause (Schroder and Steinhauer, 1976). The stems were immersed in water and replaced every few days as needed. Eggs were collected from excised stems of the discarded alfalfa when needed. Plant material for all operations was raised in a greenhouse. The commercial cultivar Weevlchek (Buker et al., 1983) was used consistently as the resistant line and Caliverdi as the weevil-susceptible line.

Extracts and Test Compounds. The aerial portion of 30-day-old alfalfa plants was cut and air dried, ground, and exhaustively extracted serially in a Soxhlet extractor using hexane, acetone, methanol, and water in sequence. Solvent was removed from the extracts using a rotary evaporator. The thick tarry residue was used directly in the bioassays described below.

The 3-acyl-4-hydroxycoumarins were prepared according to published procedures from 4-hydroxycoumarin and alkanoic acid (Klosa, 1956).

Bioassay of Larval Development. The Berger diet containing 0.5 g alfalfa/cup was made up as previously described (Chan et al., 1978). Extracts to be tested were added until they constituted 5% of the final diet. The diet slices were placed in 10 polystyrene cups and each cup inoculated with two 48-hr-old larvae. The cups had mesh lids to prevent buildup of condensed moisture. Diets and cups were changed every three to four days to prevent accumulation of fungal contamination. After 11 days on the diet, the surviving larvae were counted and weighed.

Egg Hatch Bioassay. Filter paper was impregnated with an aliquot of solution containing 0.05 g of test material in acetone. The concentration of the solution and area of spot were arranged so that the final concentration of test substance was 0.01 mg/cm^2 of filter paper. The acetone was allowed to evaporate, and the filter paper was dampened with 3 ml of sterile water, placed in a Petri dish with 20 weevil eggs on the paper, and incubated in an environmental cabinet at 25°C and 80% relative humidity. The number of eggs hatched was counted after seven days and compared with the appropriate controls under the same conditions. All test compounds which were ovicidal at 0.01 mg/cm^2 were retested at 0.001 mg/cm^2. The data for active substances in the tables is at this concentration.

Plant Damage Ratings. The ratings for plant resistance were carried out on caged alfalfa bouquets. In each of these cages were placed two bouquets of alfalfa in water: one resistant, Weevlchek; and one susceptible, Caliverdi. Each bouquet contained 30 alfalfa stems. Fifty adult weevils were placed in each cage and the cages maintained at ambient temperature with an 8-hr light and 16-hr dark photoperiod. After five days, the bouquets were removed and evaluated for damage using an arbitrary 1–10 rating scale and judged by six independent observers. There was no significant difference in damage between the two different lines.

Determination of Ovipositional Rates. Two separate caged bouquets of Caliverdi alfalfa and two similar bouquets of Weevlchek alfalfa were inoculated with 50 adult weevils to a cage. Each bouquet consisted of 10 stems in water, and the cages were maintained under the conditions described for the damage rating experiment. After four days, the stems were sliced open and the number of egg clusters and eggs per cluster counted.

DISCUSSION

Bioassays were developed to measure the effect of plant extracts and chemical constituents on larval development, pupation rates, fecundity, and ovicidal activity.

Several artificial diets for the alfalfa weevil have been previously proposed (Hsiao and Hsiao, 1974; Hsiao, 1969; Nash and Tombes, 1966). In our experience these diets proved to be only marginally acceptable, especially with neonate larvae. As a result the standard Berger diet frequently used on *Heliothis* was employed (Chan et al., 1978). As Nash and Tombes (1966) reported, the diet still leaves much to be desired since survival rate and weight gain on the synthetic diet is so poor relative to insects raised on alfalfa. The addition of various alfalfa extracts to the standard Berger diet did not improve it. To be successful with the Berger diet, it was necessary to start with second-instar larvae to achieve an acceptable weight gain. The use of larvae which had spent their first 48 hr on alfalfa greatly increased the survival rates on the diet. The weights of the 48-hr-old introduced larvae ranged from 1.15 to 1.3 mg. After 20 days on the diet their weights ranged from 10.6 to 14.2 mg. Insects living on susceptible alfalfa plants over the same period ranged in weight from 15.9 to 18.9 mg. Pupation rates after 14 days on the diet were 60–75%. There was no difference in insect weights on the controls and those diets containing acetone extracts of susceptible or resistant alfalfa, the marc remaining from the extracts or whole alfalfa leaf powder.

By far the most convenient bioassay was the measurement ovicidal activity. Excised alfalfa weevil eggs gave a 95–100% hatch rate in seven days when incubated on damp filter papers pretreated with alfalfa extracts or other test materials. There was no difference in hatch rate between the controls and those treated with extracts of susceptible or resistant alfalfa lines.

To determine if plant resistance involved the egg-laying phase of weevil reproduction (Norwood et al., 1967b), the average number of eggs per cluster and average number of egg clusters per stem were measured in resistant and susceptible lines. Again there was no difference between the susceptible and resistant lines. The susceptible Caliverdi line averaged 1.7 clusters/stem and 7 eggs/cluster while the resistant line, Weevlchek, averaged 1.9 clusters/stem and 6 eggs/cluster.

Since no antibiotic difference could be detected between resistant and susceptible alfalfa lines when surveyed by the bioassay procedures described above, whole, intact plant material was studied in an attempt to demonstrate the presence of resistance in Weevlchek. This was done in a choice test using neonate larvae on intact, but detached alfalfa stems. There was no preference in plant material consumed, no difference in the amounts eaten, and no difference in the weights of emergent larvae from the two alfalfa lines. The mean weight of 10 newly emerged larvae from either line was 0.25 mg.

As a result, it is concluded that weevil resistance in the resistant line, Weevlchek, and probably all commercial resistant alfalfa lines, is not based on antibiosis but is due to greater plant tolerance to insect damage. Some degree of tolerance in Weevlchek has been previously suggested (Sorensen et al., 1972).

The ovicidal bioassay was also extended to screen a number of alfalfa constituents and related substances for activity. The ovicidal activity of coumarin, particularly on *Drosophila* species, has been well documented (Nakajima and Kawazu, 1980; Kagan et al., 1983). The intense activity of coumarin verified on alfalfa weevil eggs in the ovicidal bioassay prompted the examination of a number of related coumarin derivatives (Table 1). Coumarin and 7-methoxycoumarin showed good inhibition of egg hatch. Replacement of the methyl ether group by other alkyl or ester groups resulted in loss of activity. The addition of further substituents to the 7-methoxycoumarin system also resulted in loss of activity. Transfer of the methoxy group to other positions resulted in decreased activity (6-methoxy) or total loss of activity (4- or 8-methoxy). The dimethylpyranocoumarin, seselin, previously reported (Tanaka et al., 1985) as ovicidal against spider mite eggs, was inactive in the weevil bioassay.

Among the coumarin derivatives tested for their effect on egg hatch rates were a series of 3-acyl-4-hydroxycoumarins which show a wide range of biological activity against bacteria and insects. The results are displayed in Table 2. In common with aphid feeding deterrency (Dreyer et al., 1987) and bactericidal sporostatic activity (Lewis and Jurd, 1972) the egg hatch rate was totally suppressed by those 3-acyl-4-hydroxycoumarins with short alkyl groups. The presence of all the structural features found in 3-acyl-4-hydroxycoumarins apparently is required for ovicidal activity since separation of the structural features in 4-hydroxycoumarin and 3-acetylcoumarin resulted in loss of activity in each case (Table 1).

The inhibition of egg hatch of the 3-acyl-4-hydroxycoumarins declined with increasing length of the 3-acyl group. Under the conditions of the experiment, those members of the series with eight or more carbons in the acyl group were inactive. This order of activity parallels bacterial sporostatic action (Lewis and Jurd, 1972) for these same compounds. Again members with small acyl groups showed the highest sporostatic activity which decreased as the size of the acyl group increased. A similar order of activity was found for pea aphid feeding deterrents except that the change in activity occurred with the C_4 butyryl analog (Dreyer et al., 1987). To what degree this falloff in activity is due to decreasing water solubility is not clear. Antibacterial activity of the 3-acyl-4-hydroxycoumarins increased with increasing size of the 3-acyl group and reached a maximum with the decanyl derivative (Toda et al., 1958; Ukita et al., 1951). Insecticidal activity of 3-acyl-4-hydroxycoumarins has also been previously noted (Laeuger et al., 1944). Of two related tricarbonylmethanes, one, dehydroacetic acid, was ovicidal, while the second, the lichen constituent, usnic acid, was inactive. Coumestrol, saponin, and dicoumarol, constituents associ-

TABLE 1. OVICIDAL EFFECT OF COUMARIN AND DERIVATIVES ON WEEVIL EGG HATCH

Substituent	Egg hatch rate (%) after 7 days
Coumarin	0
7-Hydroxy	100
7-Methoxy	0
7-Geranyloxy	100
7-Benzyloxy	100
7-Benzoyloxy	100
4-Hydroxy	100
4-Methoxy	100
4-Benzoyloxy	100
6-Methoxy	45
8-Methoxy	100
5,7-Dimethoxy	100
7-Hydroxy-6-methoxy	100
7-Methoxy-6-hydroxy	100
6,7-Dimethoxy	100
6,7-Dihydroxy	100
6-Glucopyranosyloxy-7-hydroxy (esculin)	100
4-Methyl-7-methoxy	100
4-Methyl-7-benzyloxy	100
4-Hydroxy-7-methoxy	100
4,7-Dimethoxy	100
3-Acetylcoumarin	100
3-Benzoylcoumarin	100
Other related compounds and alfalfa constituents	
Seselin	100
5-Methoxy-3′,4′-dihydro-4′-oxoseselin	100
3′,4′-Dihydro-4-oxoallo-xanthoxyletin	100
Precocene I	0
3-Acetyl-2-methyl-7,8-benzochromone	0
Xanthotoxin	100
Coumestrol	100
Dicoumarol	100
Dehydroacetic acid	0
Usnic acid	100
Quercetin	100
Morin	100
Saponin	100

TABLE 2. OVICIDAL EFFECT OF 3-ACYL-4-HYDROXYCOUMARINS ON WEEVIL EGGS

R =	Egg hatch (%) after 7 days
CH_3	0
n-C_2H_5	0
n-C_3H_7	0
iso-C_3H_7	0
n-C_4H_9	0
n-C_5H_{11}	0
n-C_6H_{13}	10
n-C_7H_{15}	100
n-C_8H_{17}	100
n-C_9H_{19}	100
n-$C_{11}H_{23}$	100
n-$C_{13}H_{27}$	100

ated with alfalfa, were inactive, as were several representative flavonoids. It has been previously shown that there was no effect of high saponin alfalfa lines on their associated insects (Pedersen et al., 1976; Parker and Pedersen, 1975).

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FEEDING DETERRENCY OF SOME 4-HYDROXYCOUMARINS AND RELATED COMPOUNDS: Relationship to Host-Plant Resistance of Alfalfa Towards Pea Aphid (*Acyrthosiphon pisum*)

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Abstract—A series of 3-acyl-4-hydroxycoumarins, structurally related to dicoumarol, as well as several alfalfa constituents including coumestrol were tested for their feeding deterrency towards the pea aphid. Feeding deterrency of the 3-acyl-4-hydroxycoumarins decreased as the size of the 3-acyl group increased.

Key Words—*Medicago sativa*, alfalfa, *Acyrthosiphon pisum*, pea aphid, Homoptera, Aphididae, host-plant resistance, phytoalexin, 4-hydroxycoumarins, coumestrol, symbiotes, feeding deterrency.

INTRODUCTION

Aphids are a major, worldwide pest on crop plants. In many cases, plant breeders have been fairly successful in developing aphid-resistant varieties. However, because of the adaptability of aphids to form new, virulent biotypes, resistance in cultivars is frequently overcome in relatively short time periods.

The mechanism of resistance in many crop plants towards aphids is frequently ascribed to the presence of secondary plant constituents which have toxic or feeding-deterrent effects (Dreyer and Jones, 1981). The evidence supporting this view comes mostly from bioassays which employ artificial diets to which plant extracts or purified compounds have been added. From the bioassay data, a dose–response curve can be constructed from which an ED_{50} value can be obtained. This ED_{50} is the concentration of material that deters 50% of the

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aphids from feeding and serves as a convenient comparative value to describe feeding deterrency between a variety of plant compounds.

A disadvantage to testing plant extracts or compounds in the above manner is that most aphids probe either intercellularly or bypass vacuoles containing toxic substances. Since aphids feed in the phloem, if such bioactive plant substances are not located either intercellularly or in the phloem, they play a minimal role in host-plant resistance.

Role of Pectin in Host-Plant Resistance. More recently, compelling evidence has been advanced which extends and supports an earlier idea (Adams and McAllan, 1958) that host-plant resistance towards phloem-feeding aphids, which probe intercellularly, is related to the ease with which the aphid's salivary pectinase catalyzes the depolymerization of intercellular pectin (Dreyer and Campbell, 1984; Campbell and Dreyer, 1985). Pectin is a polysaccharide chiefly found in the middle lamella and primary cell wall which cements plant cells together (Ishii, 1984). Pectinase-catalyzed depolymerization of intercellular matrix pectin mediates probing by the aphid stylets in attaining the phloem.

In the case of sorghum, it has been shown by electronically monitoring probing behavior that stylets of an aphid pest, the greenbug [*Schizaphis graminum* (Rondani)], reach the phloem in a susceptible line more than twice as fast as in an aphid-resistant line (Campbell et al., 1982). This rate of access to the phloem is associated with the rate of depolymerization of the isolated plant pectin catalyzed by the aphid's pectinase system (Dreyer and Campbell, 1984; Campbell and Dreyer, 1985). It is further argued that aphids evolved an intercellular mode of probing to avoid many potentially deleterious secondary plant constituents which are normally compartmentalized inside specialized storage cells or vacuoles (Matile, 1984).

Host-plant-resistance studies on sorghum have been extended to the pea aphid on alfalfa and the results support the idea that pectin, again, plays a major role in mediating aphid probing and controlling access to the phloem (Dreyer et al., 1986). The salient points are: (1) The pea aphid is a phloem feeder and probes intercellularly. (2) It takes more than twice as long for the pea aphid to reach the phloem of an aphid-resistant line as on a susceptible alfalfa line. (3) Once in the phloem, the pea aphid feeds equally well on resistant as on susceptible alfalfa lines. (4) Pectin fills the intercellular spaces in plants and acts as an intercellular cement that binds cells together. (5) The rate of enzymatic catalyzed depolymerization of the pectin isolated from different alfalfa lines correlates with plant resistance to aphids. (6) Aphid resistance in alfalfa can be enhanced in susceptible lines by the application of plant growth bioregulators, for example CCC, which have been shown to influence pectin composition.

These results argue for a major role of intercellular pectin in host-plant resistance to aphids and that pectin functions by mediating aphid stylet penetration towards the phloem. The structural features of pectin, a complex poly-

saccharide (Jarvis, 1984), which govern the depolymerization rate by enzymes are, to date, only partially understood.

The aphid pectinase relationship with plant pectin has many parallels with the mechanism of bacterial or fungal pathogen attack on plants (Bateman, 1976). Indeed, the aphid's salivary pectinase is very likely a product of the extensive bacterial endosymbiotes (Campbell and Dreyer, 1985) that are ubiquitously found in sap-sucking insects (Homoptera) (Koch, 1960). It appears likely that the differences in aphid biotypes (Diehl and Bush, 1984) are due to differences in the bacterial endosymbiotes (Houk and Griffiths, 1980) rather than inherent biochemical differences in the insect (Campbell and Dreyer, 1985). Circumstantial evidence supporting this view includes the induction of the aphid's ability to feed on otherwise unacceptable hosts as well as the frequent appearance of new, more virulent biotypes.

Natural Products and Host-Plant Resistance to Aphids. In spite of a central role proposed for pectin in governing host-plant resistance to aphids, there are some plants where a persuasive argument can be made for a role of secondary plant constituents in aphid resistance, especially if such constituents are transportable in the phloem. In certain lupins aphid resistance correlates with the concentration of quinolizidine alkaloids in the plant (Wink et al., 1982; Wegorek and Krzymanska, 1970). It has been shown that these alkaloids are transported in the phloem (Wink and Witte, 1984). These results argue that the role of certain secondary plant constituents in host-plant resistance to aphids cannot be dismissed out of hand.

METHODS AND MATERIALS

The 3-acyl-4-hydroxycoumarins were prepared according to published procedures (Klosa, 1956) from 4-hydroxycoumarin and the *n*-alkanoic acid. The bioassay, using biotype Chualar of the pea aphid, was carried out using the Akey diet (Akey and Beck 1971). The bioassay procedure has been described in detail (Dreyer et al., 1981).

RESULTS AND DISCUSSION

This paper describes the feeding deterrency of a series of related 3-acyl-4-hydroxycoumarins towards the pea aphid. These compounds are structurally related to dicoumarol, a known alfalfa constituent, and other anticoagulants which, in general, retard the growth of gram-positive bacteria (Dadak and Hodak, 1966). The pea aphid, *Acyrthosiphon pisum* (Harris), is a significant pest on alfalfa and was selected as the test system because alfalfa is a known phytoalexin producer with relatively well-studied chemistry.

TABLE 1. FEEDING DETERRENCY OF SOME 3-ACYL-4-HYDROXYCOUMARINS AND RELATED COMPOUNDS TOWARDS PEA APHID

Compound	ED_{50} (ppm)
R =	
n-CH_3	370 ± 15
n-C_2H_5	370 ± 25
n-C_3H_7	>1000
iso-C_3H_7	>1000
n-C_4H_9	no effect
n-C_5H_{11}	no effect
n-C_6H_{13}	not tested
n-C_7H_{15}	not tested
n-C_8H_{17}	no effect
n-C_9H_{19}	no effect
Dehydroacetic acid	>1000 ppm
Dicoumarol	no effect at 2500 ppm
Commercial saponin	no effect at 2500 ppm
Coumestrol	no effect at 1000 ppm

Although none of the hydroxycoumarins were strongly active, compounds of this series with a small alkyl residue in the 3-acyl group showed the greatest feeding deterrency towards the pea aphid (Table 1). The activity declined so that there was little or no effect when the acyl residue was three carbons or larger. This order of activity for these compounds paralleled bacterial sporostatic action (Lewis and Jurd, 1972). In this case, members with small acyl groups showed the highest sporostatic activity with decreasing activity as the size of the acyl group increased. This series of 3-acyl-4-hydroxycoumarins are known to be active antibacterial agents against gram-positive but not against gram-negative bacteria (Toda et al., 1958; Ukita et al., 1951). The order of compounds showing feeding-deterrent activity against the pea aphid was the reverse of bactericidal activity previously described against gram-positive bacteria. Structure–activity studies (Toda et al., 1958; Ukita et al., 1951) showed increasing antibacterial activity towards gram-positive bacteria with increasing size of the acyl group. Activity reached a maximum with the 3-decanoyl derivative. Insecticidal activity of 3-acyl-4-hydroxycoumarins has also been previously noted (Laeuger et al., 1944).

Two different modes of action of secondary plant constituents on aphids might be distinguished. One is a mechanism by which compounds act as bactericides on the endosymbiotes and the other when these substances act directly as feeding deterrents or toxicants on the aphid itself. Compounds having a deleterious effect on the symbiotes might have a profound effect on the aphid's

probing ability if the pectinase arose from the symbiotes. In the present case, it has not been determined if the activity of the hydroxycoumarins has a direct effect on the aphid or the endosymbiotes.

Degradation of plant pectin catalyzed by the aphid pectinase system could give rise to oligosaccharides which can act as elicitors in initiating phytoalexin biosynthesis (Darvill and Albersheim, 1984). Such oligosaccharides have been identified in the honeydew of aphids, indicating their potential for translocation (Campbell, 1985).

It has been reported that pea aphid or spotted alfalfa aphid infestations on alfalfa results in very high increases in coumestrol levels (Loper, 1960). Similar coumestrol increases result from fungal infestations on alfalfa (Loper et al., 1967). Coumestrol, dicoumarol and saponin, all alfalfa constituents, were bioassayed for their feeding deterrency and showed no effect up to 1000 ppm. Coumestrol has been reported to have poor antifungal activity (Perrin and Cruickshank, 1969; Van Etten, 1967) and modest antibacterial properties (Lyon and Ward, 1975) so that, even though its formation may be triggered by aphid probing, it apparently has little effect on the insect. It should be recognized that phytoalexins are not always effective in protecting plants from pathogens. The selective inhibition or modification of aphid symbiotes may not only be a possible insect control method but may also yield information about the nature and role of symbiotes in insect host-plant relationships.

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ATTRACTION OF SPHINGID MOTHS (Lepidoptera: Sphingidae) TO 10,12-HEXADECADIENYL ALDEHYDES AND ACETATES: Evidence of Pheromone Components[1]

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Abstract—A field survey of 10,12-hexadecadienyl alcohols, acetates, and aldehydes showed attraction to a wide range of sphingid moths. Data are presented showing the attraction of *Smerinthus jamaicensis*, *Smerinthus cerisyi*, *Pachysphinx modesta*, *Hemaris diffinis*, and *Proserpinus flavofasciata* to these compounds. Mass spectral, EAG, and EAD data show evidence for the presence of these dienes in female extracts of *S. cerisyi*, *Hyles gallii*, and *Sphinx drupiferarum*.

Key Words—Attractant, pheromone, 10,12-hexadecadienal, 10,12-hexadecadienyl acetate, *Smerinthus jamaicensis*, *Smerinthus cerisyi*, *Paonias excaecatus*, *Paonias myops*, *Pachysphinx modesta*, *Hemaris diffinis*, *Proserpinus flavofasciata*, *Hyles gallii*, *Hyles euphorbiae*, *Sphinx vashti*, *Sphinx drupiferarum*, Lepidoptera, Sphingidae.

INTRODUCTION

Bombykol, *E*10,*Z*12-16:OH[2], was the first pheromone component ever isolated and identified (Butenandt et al., 1959) from the extraction of 500,000 female scent glands of *Bombyx mori* L. Improved isolation and identification techniques have led to the discovery of hundreds of additional pheromones and

[1]NRCC No. 25857.
[2]In this paper the chemical nomenclature is abbreviated as follows: *Z* and *E* indicate the *cis* and *trans* configurations of the double bond, the number following *Z* or *E* indicates the position of that double bond, the last number indicates the carbon chain length, the terminal functional groups are :Ac = acetate; :OH = alcohol; :Ald = aldehyde.

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attractants but relatively few involved 10,12-hexadecadienyl alcohols, aldehydes, or acetates (hexadecadienyls). Bombykol was also identified in the female gland extracts of *Bombyx mandarina* by Kuwahara and Yen (1979).

Kasang et al. (1978a) have shown bombykal (*E*10,*Z*12–16:Ald) is a second component of the *B. mori* pheromone and *E*10,*E*12–16:OH is present in its female scent gland (Kasang et al., 1978b). Starratt et al. (1979) identified bombykal as a pheromone component of a sphingid *Manduca sexta* (L.), and Hall et al. (1980) identified *E*10,*E*12–16:Ald as a pheromone component of the noctuid, *Earis insulana* (Boisduval).

Hyles euphorbiae (L.), an Old World sphingid, has been introduced into North America, and most recently to Saskatchewan, as a biological control of leafy spurge (*Euphorbia esula* L.) (Harris, 1984). We are presently investigating the pheromone of *H. euphorbiae* as a potential population monitor, and initial studies have indicated that hexadecadienyl aldehydes are present in female abdominal tip extracts, (Underhill, unpublished). This evidence, as well as the proven pheromone activity of 10,12-hexadecadienyl compounds in several lepidopteran families and previous success in using conjugated dienyl compounds as attractants for a wide range of Lepidoptera (Reed and Chisholm, 1985), led us to synthesize and field test the 10,12-hexadecadienyls. We report here field trapping, electroantennograph (EAG), and coupled gas chromatograph–electroantennographic detector (GC-EAD) data that suggest several members of Sphingidae family use 10,12-hexadecadienyl compounds as pheromone components. We also present mass spectral data that show the presence of one of these compounds in female abdominal tip extracts.

METHODS AND MATERIALS

The chemicals used in this study were synthesized and purified in this laboratory. The *EE*, *EZ*, and *ZE* alcohols were synthesized using methods described by Chisholm et al. (1981). The *E*10,*E*12–16:OH and *E*10,*Z*12–16:OH were recovered from a Wittig condensation reaction of the ylide of butyltriphenylphosphonium bromide with (*E*)-12-tetrahydropyranyloxy-2-dodecenal. *E*10,*E*12–16:OH and *Z*10,*E*12–16:OH were recovered from the Wittig condensation reaction of the ylide of 10-acetoxydecyltriphenylphosphonium bromide with (*E*)-2-hexenal.

Synthesis of the *ZZ* isomer, and purification and conversion of all alcohols to aldehydes and acetates was similar to that described by Chisholm et al. (1985). *Z*10,*Z*12–16:OH was synthesized by the catalytic coupling of 10-undecynol with 1-bromopentyne and the subsequent dicyclohexylborane reduction of the diyne to the diene.

Structures were verified by 90-MHz NMR, IR, and mass spectrometry. Isomeric purity was greater than 99% and was checked on a Hewlett Packard

5890A gas chromatograph with flame ionization detector (FID) using a 0.32-mm-ID × 30-m DB-5 fused silica capillary column (J. & W. Scientific, Rancho Cardova, California).

Chemical ionization mass spectral analyses of female tip extracts were done with a Finnigan 3300 GC-MS using a 0.32-mm × 60-m DB-5 fused silica column with hydrogen as carrier gas and methane as reagent gas.

Injections were made at an oven temperature of 40°C, heated to 120°C and temperature programmed 4°/min to 280°C. A multiple-ion detection (MID) method was used to scan for characteristic ions of the 10,12-hexadecadienyls which included ions with m/z 169, 219, 221, 235, 237, 239, 253, 265, 279, and 321.

From May 2 to September 10, 1985, field trapping was carried out 100 km northeast of Saskatoon, Saskatchewan, Canada, in a forest area containing spruce, pine, birch, aspen, and poplar trees and a number of herbaceous shrub species. The sticky wing traps (Phero Tech Inc., Vancouver, B.C.) contained chemical lures impregnated into rubber septa (Arthur H. Thomas Co. Philadelphia, Pennsylvania, No. 1780 J07) and protected from oxidation with 100 μl of a solution of 10% buylated hydroxytoluene in acetone. The traps were hung on tree branches 1–2 m from the ground and spaced at least 10 m apart. Inspections of traps and recording of captures were done weekly, and lures were replaced on June 4 and July 12.

The survey consisted of 96 nonreplicated traps containing each of the 10,12-hexadecadienyls as a single component at 500 μg dose and two component lures formulated by adding an additional 50 μg of a compound which differed from the primary component by a single change in functional group or isomeric geometry; also, 500-μg doses of single-component 10-hexadecenyl and 12-hexadecenyl compounds were surveyed.

Light trapping of moths was done using 8-W portable UV light traps (Bioquip Products, Santa Monica, California) in the area of the survey and in an area 10 km south of Saskatoon.

EAG responses of moths to synthetic chemicals were recorded following the method of Chisholm et al. (1975) using light-trapped males of unknown age which were maintained in the laboratory at 5°C and used within 36 hr of capture. EAG data presented are from single male moths which gave reproducible responses. Although the intensity of the absolute responses may have differed from those of other males of the same species, the relative responses were very consistent. EAD responses (Arn et al., 1975) of male antennae to synthetic standards and female extracts were done with light-trapped or hand-caught males and females. The majority of light-trapped females had mated, and extracts showed no EAD activity. Female abdominal tips were excised and extracted with methylene chloride for 0.5 hr. Extracts were concentrated and separated chromatographically using a modified gas chromatograph (Hewlett Packard

5710A) with a 0.32-mm × 30-m DB-5 fused silica capillary column. The effluent was split in a 7:3 ratio to an FID and EAD. Injections of ~1 female equivalent (FE) of extracts or 2- to 5-ng quantities of standards were injected splitless at 40°C for 30 sec and the oven heated to 90°C and temperature programmed 4°/min to 230°C. Good separations of the geometrical isomers of the 10,12-hexadecadienyls were achieved with typical separations being greater than 10 sec and resolutions of better than 1.0.

Replicated experiments were set out when possible to verify captures in the unreplicated survey.

Summed trap captures from randomized, three-times replicated, field tests were transformed $\sqrt{X + 0.5}$, where X is the number of male moths captured in the trap, and then submitted to an analysis of variance, and significantly different means separated by Duncan's multiple-range test.

RESULTS AND DISCUSSION

The 1985 survey attracted five sphingid species: *Smerinthus jamaicensis* (Drury), *Smerinthus cerisyi* Kirby, *Pachysphinx modesta* (Harris), *Hemaris diffinis* (Boisduval), and *Proserpinus flavofasciata* (Walker) (Tables 1 and 2) as well as at least eight other lepidopteran species (unpublished data). Although the survey traps were not replicated, male captures were replicated over time, and attractive lure components were clearly evident for a number of sphingids. The best example is that of *S. cerisyi*, where all baits containing Z10,*E*12-16:Ald at the 500-μg dose (Table 2) except where Z10,*E*12-16:Ac or *E*10,*E*12-Z10,*E*12-16:Ac (500 μg) + Z10,*E*12-16:Ald (50 μg) caught more than six times as many moths as any other trap. In a replicated test (Table 3), this two-component mixture at 550 μg was a significantly better attractant than the mixture at 110 μg or the acetate alone at 500 μg. Z10,*E*12-16:Ald alone or in combination with any other hexadecadienyl compound as well as the corresponding hexadecenyl compounds failed to attract *S. cerisyi*. EAGs done on male *S. cerisyi* (Figure 1) showed strong responses to its attractant components.

Smerinthus jamaicensis males were caught in traps containing Z10,*E*12-16:Ald at the 500-μg dose (Table 2) except where Z10,*E*12-16:Ac or *E*10,*E*12-16:Ald was a second component. EAG data (Figure 1) for *S. jamaicensis* showed a strong response to its attractant, Z10,*E*12-16:Ald, as well as relatively large responses to *E*10,*E*12-16:Ald, and to Z10,*E*12-16:Ac, which suggests they may have inhibited trap capture in the survey. Corresponding hexadecenyl compounds were not attractive in the field, and EAG analysis showed only weak responses.

Pachysphinx modesta males were captured along with *S. cerisyi* in traps containing Z10,*E*12-16:Ac at 500 μg except in traps where Z10,*E*12-16:Ald was a second component (Tables 2 and 3). EAG data for *P. modesta* (Figure

TABLE 1. SUMMARY OF SPHINGIDS ATTRACTED TO FIELD TRAPS DURING 1985

Sphingidae		Attractant (μg)	Total number trapped	Trapping period
Subfamily: Sphinginae				
Tribe: Smerinthini	*Smerinthus jamaicensis*	Z10,*E*12-16:Ald (500)	37	May 23–July 23
	Smerinthus cerisyi	Z10,*E*12-16:Ac (500) +Z10,*E*12-16:Ald (50)	258	May 17–July 29
	Pachysphinx modesta	Z10,*E*12-16:Ac (500)	42	June 10–Sept. 10
Subfamily: Macroglassinae				
Tribe: Dilophontini	*Hemaris diffinis*	Z10,*E*12-16:Ald (500)	12	June 10–July 2
Tribe: Macroglossini	*Proserpinus flavofasciata*	*E*10,*E*12-16:Ald (500)	5	June 4

TABLE 2. CAPTURES OF MALE MOTHS IN SURVEY TRAPS (NONREPLICATED, MAY 10 to SEPTEMBER 10, 1985)

Lure composition (μg)	*P. modesta*	*S. jamaicensis*	*S. cerisyi*	*H. diffinis*
*E*10,*E*12-16:Ac (500)	0	0	0	0
*E*10,*Z*12-16:Ac (500)	1	0	0	0
*E*10,*Z*12-16:Ac (500) + *Z*10,*E*12-16:Ac (50)	1	0	0	0
*Z*10,*E*12-16:Ac (500)	2	0	10	0
*Z*10,*E*12-16:Ac (500) + *E*10,*E*12-16:Ac (50)	12	0	8	0
*Z*10,*E*12-16:Ac (500) + *E*10,*Z*12-16:Ac (50)	10	0	8	0
*Z*10,*E*12-16:Ac (500) + *Z*10,*Z*12-16:Ac (50)	3	0	1	0
*Z*10,*E*12-16:Ac (500) + *Z*10,*E*12-16:Ald (50)	0	0	62	2
*Z*10,*E*12-16:Ac (500) + *Z*10,*E*12-16:OH (50)	4	0	8	0
*Z*10,*Z*12-16:Ac (500)	0	0	0	0
*Z*10,*E*12-16:Ald (500)	0	6	0	0
*Z*10,*E*12-16:Ald (500) + *Z*10,*E*12-16:Ac (50)	0	0	0	2
*Z*10,*E*12-16:Ald (500) + *E*10,*E*12-16:Ald (50)	0	0	0	0
*Z*10,*E*12-16:Ald (500) + *E*10,*Z*12-16:Ald (50)	0	4	0	0
*Z*10,*E*12-16:Ald (500) + *Z*10,*Z*12-16:Ald (50)	0	12	0	2
*Z*10,*E*12-16:Ald (500) + *Z*10,*E*12-16:OH (50)	0	3	0	0
*Z*10,*E*12-16:OH (500)	0	0	0	1
*Z*10,*E*12-16:OH (500) + *Z*10,*E*12-16:Ald (50)	0	0	0	2

TABLE 3. CAPTURES OF *S. cerisyi* AND *P. modesta* IN BAITED TRAPS CONTAINING *Z*10,*E*12-HEXADECADIENYL ACETATE AND ALDEHYDE[a]

Lure composition (μg)	Mean No. males captured/trap (±SD) *S. cerisyi*	*P. modesta*
*Z*10,*E*12–16:Ac (500)	5.0 (±1.7)*b*	3.3 (±1.1)*c*
*Z*10,*E*12–16:Ac (100) + *Z*10,*E*12–16:Ald (10)	5.7 (±5.0)*b*	0*d*
*Z*10,*E*12–16:Ac (500) + *Z*10,*E*12–16:Ald (50)	23.7 (±8.5)*a*	0*d*

[a]Three replications May 23–July 15, 1985. Means followed by the same letter are not different ($P = 0.05$).

1) shows strong responses for its attractant *Z*10,*E*12–16:Ac and also *E*10,*Z*12–16:Ac and *Z*10,*E*12–16:Ald. The presence of this aldehyde in *S. cerisyi* female extracts (see below) may indicate that, as well as being an attractant component of *S. cerisyi*, it serves to inhibit the attraction of *P. modesta* and thereby aids in species isolation.

From field observations it was noticed that the day flyer *Hemaris diffinis* was readily attracted to lures containing *Z*10,*E*12–16:Ald but few of these agile flyers landed in traps. Specimens for EAG analysis were literally "hand caught" when males were attracted to contaminated hands after handling lures containing 10,12-hexadecadienals. The EAG profile shows strong responses to all the hexadecadienals (Figure 2). Again, the corresponding hexadecenyl aldehydes were not attractive in the field nor were they EAG active.

Proserpinus flavofasciata was similar to *H. diffinis* in that very few of this agile, bee mimic were captured (five males) but all were taken in traps containing *Z*10,*E*12–16:Ald as a major component. No suitable specimens were obtained for EAG or EAD analysis.

A few specimens of each of the two *Paonias* species were light trapped, and EAG profiles showed strong responses for only the 10,12-hexadecadienyl acetates (Figure 2). One *Paonias exceactus* was captured in a trap containing *E*10,*Z*12–16:Ac (500 μg) + *E*10,*Z*12–16:Ald (50 μg).

Data were obtained supporting the occurrence of 10,12-hexadecadienyl acetates and aldehydes in female abdominal tip extracts of *S. cerisyi*, *Sphinx drupiferarum* (J.E. Smith), and *Hyles gallii* (Rottenburg).

Female abdominal tip extracts were obtained from light-trapped *S. cerisyi*, and GC-EAD analysis with conspecific male antennae to 1 FE showed responses corresponding to retention times of *Z*10,*E*12–16:Ac (140 μV signal) and *Z*10,*E*12–16:Ald (50 μV signal) (Figure 3). This evidence, along with trapping and EAG data, suggests strongly that these are actual pheromone components.

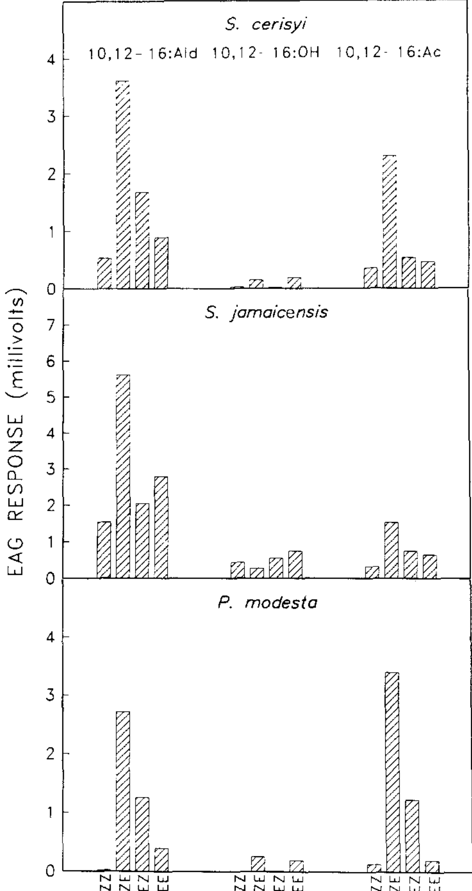

FIG. 1. EAG responses of males to synthetic 10,12-hexadecadienyls at 1-μg doses for *S. cerisyi* and *S. jamaicensis* and 10-μg doses for *P. modesta*.

Light-trapped *S. drupiferarum* females were collected and abdominal tip extracts were prepared and analyzed by GC-EAD using the antennae from light-trapped *S. vashti*. Analysis showed responses corresponding to the retention times of *Z*10,*E*12-16:Ald (80 μV signal), *E*10,*Z*12-16:Ald (430 μV signal), and *E*10,*E*12-16:Ald (80 μV signal) (Figure 3). GC-MS analysis using the MID method for *S. drupiferarum* tip extracts showed a peak at the same retention time of synthetic *E*10,*Z*12-16:Ald with characteristic ions: $(M + 29)^+$, *m/z* 265; $(M + 1)^+$, *m/z* 237; $(M - 1)^+$, *m/z* 235; and $[(M + 1) - 18]^+$, *m/z* 219 in the same relative ratios as synthetic *E*10,*Z*12-16:Ald.

Both MS and GC data showed the *E*10,*Z*12-16:Ald present in amounts greater than 30 ng/female. The *ZE* and *EE* may be present in picogram quan-

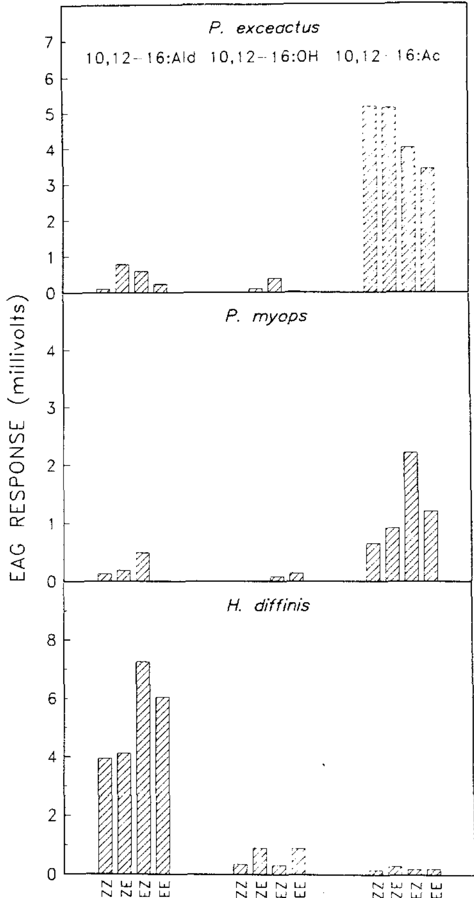

FIG. 2. EAG responses of males to synthetic 10,12-hexadecadienyls at 10-μg doses.

tities, but they were not verified by MS analysis. No *S. drupiferarum* were captured in baited traps, but the EAD shows the presence of at least one and possibly three 10,12-hexadecadienals in the extract and a multicomponent mixture may be necessary for attraction. *Hyles gallii* was not found in the trapping survey area (light trap or survey traps), but males and females were obtained from the light trap south of Saskatoon (no survey traps). Female abdominal tip extracts showed EAD responses corresponding to the retention time and presence of *E*10,*E*12–16 : Ald (130 μV signal) when using a male *H. gallii* antenna. *Hyles euphorbiae* male antennae (obtained from a lab culture) responded to female *H. gallii* extracts, indicating the presence of *E*10,*Z*12–16 : Ald (50 μV signal). GC-EAD analysis of *H. gallii* to standard solutions of hexadecadienyls showed strong responses to the *EE* and *EZ* aldehydes, with *EE* being the strongest.

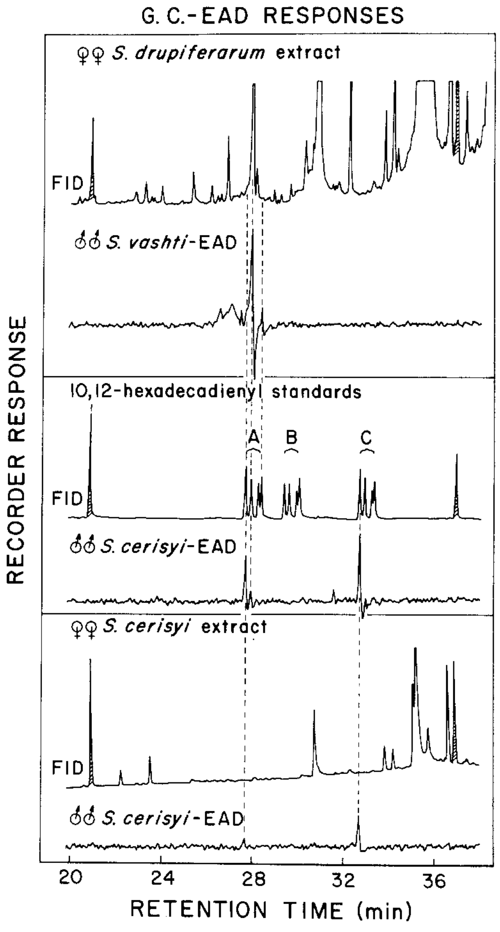

FIG. 3. Representative EAD and associated FID responses of male sphingids to female extracts (FID 0.7 FE : EAD 0.3 FE) and synthetic standards (FID 2.8 ng : EAD 1.2 ng). The elution order of the 10,12-hexadecadienyl isomers was *ZE*, *EZ*, *ZZ*, and *EE* for the aldehydes (A), alcohols (B), and acetates (C).

On several occasions the presence of only scales in the sticky traps indicated a lack of moth retention, particularly for the larger moth species. When the large *P. modesta* was captured in the sticky traps, the traps became saturated when they contained only three or four moths, and results may have been more conclusive if a retentive, nonsaturating trap had been used.

A comparison was made of captures of *S. cerisyi* and *S. jamaicensis* in sticky wing traps and large (9-liter) double inverted cone-orifice traps with varying orifice diameters (Steck and Bailey, 1978). An opening of 2 cm was necessary to allow the *Smerinthus* species to enter but these were found to be no

better than the sticky wing traps. None of the other sphingids attracted to wing traps could be trapped using the inverted cone-orifice trap.

A different problem arose with the more agile flyers such as *Hemaris diffinis*, *Proserpinus flavofasciata*, and possibly the *Sphinx* and *Hyles* species. These hovering, nectar feeders very rarely landed in the sticky traps even though some were obviously attracted to the lures.

In North America there are two subfamilies of Sphingidae, containing five tribes. Our data show attraction to and/or the presence of 10,12-hexadecadienyl acetates or aldehydes in species representing four tribes. A chemotaxonomic approach using hexadecadienyl compounds could be used for any other sphingid species whose pheromones need to be identified.

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TECHNIQUE FOR VISUALIZATION OF EPIDERMAL GLANDULAR STRUCTURES IN PLANTS

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Abstract—The distribution of microscopic glandular structures (hairs, capsules, nodules) on a leaf or other plant part can be recorded visually by pressing the plant part against an indicator surface (filter paper, thin-layer chromatography plate) soaked in Tollens' reagent. The glandular sites are revealed instantly on the test surface as dark spots.

Key Words—Chemical ecology, plant defenses, glandular trichomes, Tollens' reagent.

Leaves and stalks of plants are commonly beset with microscopic glandular structures, such as secretory hairs, capsules, or nodules, widely believed to be defensive (Levin, 1973; Stipanovic, 1983). Because of their intrinsic botanical interest, and because they are often the source of unusual chemicals, such glands have been the subject of considerable research (Levin, 1973; Stipanovic, 1983; Rodriguez et al., 1984). Visualization of the glands may prove problematic, particularly in the field, since it may be possible only by use of magnifying devices. Some structures, particularly nodular glands, which may be embedded in the leaf tissue itself, may not be apparent. We here describe a simple technique whereby the epidermal glandular specializations of a plant can be quickly demonstrated and recorded. The technique involves obtaining a printout of the glands by pressing leaves or other vegetative portions of the plant against a chemically pretreated indicator surface.

Filter paper or, better, commercial thin-layer silica-gel chromatography plates, can serve as indicator surfaces. Pieces of these materials, cut to appropriate size, are soaked just prior to use in freshly prepared Tollens' reagent (Tollens, 1882a,b; Feigl, 1966). This is made by adding droplets of aqueous

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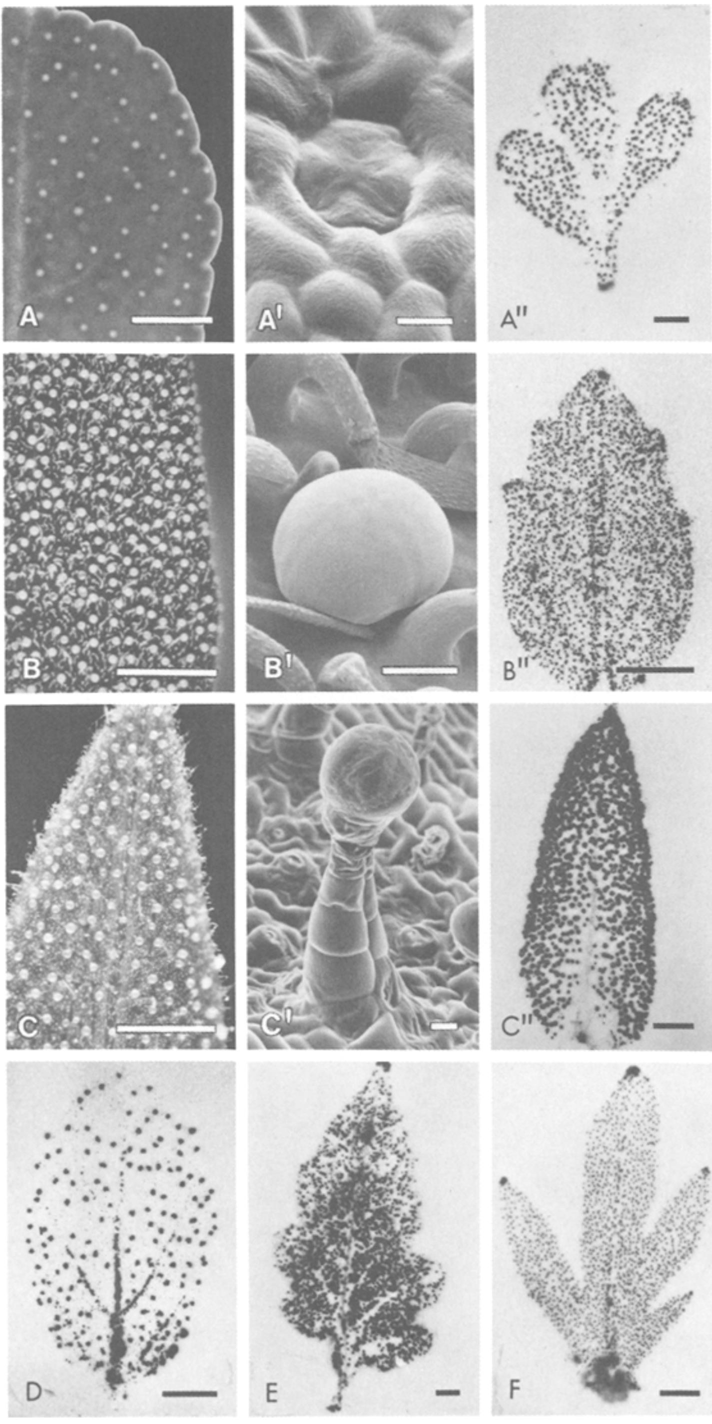
potassium hydroxide (10%) to aqueous silver nitrate (10%), and dissolving the resulting silver oxide precipitate by addition of aqueous ammonia (10%). The soaked pieces are blotted off, and then pressed tightly (to ensure rupture of glandular capsules and nodules) for some seconds against the plant surface to be tested. Glandular sites are immediately revealed as a pattern of dark spots (metallic silver) on the indicator surface. Tollens' reagent deteriorates with time (caution: explosive potential!), but fresh preparation from the stable precursors poses no problem, even under field conditions, for which purpose the technique is particularly useful, since it can afford a rapid means for screening plant surfaces for incidence, as well as varying density, of glandular defenses. Microscopic closeups of representative leaf glands, as well as typical printouts of glandular patterns, are shown in Figure 1. Note how the technique reveals even nodular glands (Figure 1A–A″), which may not be apparent with a hand lens (Figure 1A was taken with a microscope and dark-field transmitted light).

Our initial choice of Tollens' reagent was prompted by an interest in cat thyme (Figure 1 B–B″), whose glandular capsules contain the terpenoid aldehyde dolichodial (Pagnoni et al., 1976). We soon realized, however, that this traditional aldehyde reagent gave distinct glandular printouts with a diverse group of plants, including some not known to produce aldehydic exocrine secretions. At least two factors could account for this unexpectedly broad reactivity. First, it is likely that some aldehydes are present, if only at low levels, in almost every epidermal plant secretion. And second, many other functionalities can reduce ammonia-complexed silver ion, as implied by Tollens himself, and documented extensively by subsequent workers (Tollens, 1882a,b; Feigl, 1966).

FIG. 1. Glandular leaf structures: morphology and chemical printout patterns. (A–A″) *Ruta graveolens* (family Rutaceae); (A) portion of leaf (dark-field, transmitted light) showing pinpoint pattern of translucent glandular nodules; (A′) scanning electronmicrograph (SEM) of single nodule; (A″) printout pattern of leaf. (B–B″) *Teucrium marum* (cat thyme, family Labiatae); (B) leaf portion (horizontal incident light) showing stalkless glandular capsules; (B′) SEM of capsule; (B″) printout pattern of leaf. (C–C″) *Heterotheca psammophila* (camphorweed, family Compositae); (C) leaf portion (horizontal incident light) showing glandular hairs; (C′) SEM of glandular hair with apical secretory globule; (C″) printout pattern of leaf. (D, E, F) Leaf printout patterns of (D) marjoram (*Origanum majorana*, family Labiatae), (E) tomato (*Lycopersicum esculentum*, family Solanaceae), and (F) absinthe (*Artemisia absinthium*, family Compositae). The chemicals produced by these glandular structures include a diversity of terpenoids, acetogenins, and phenylpropanes, among others (Karrer, 1958; Pagnoni et al., 1976; Rodriguez et al., 1984; Stipanovic, 1983); *Heterotheca* produces a mixture of terpenoids, including camphor and borneol (Silverstein, West, Webster, Hummel, and Eisner, unpublished results). Reference bars: A, B, C = 1 mm; A′, B′, C′ = 20 μm; A″, B″, C″, D–F = 2 mm.


A
A'
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B
B'
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C
C'
C''
D
E
F

Basified silver ion solution was once purported to be useful for diagnosing "die mysteriöse mit den namen Leben bezeichnete Erscheinung." (Loew and Bokorny, 1881) While we cannot claim the reagent to test for life itself, it does seem to provide a simple means for studying one small chemical aspect of the life of plants.

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INTERACTION OF NUCLEAR POLYHEDROSIS VIRUS WITH CATECHOLS:
Potential Incompatibility for Host-Plant Resistance against Noctuid Larvae

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Abstract—Two major orthodihydroxy phenolics of *Lycopersicon esculentum*, rutin and chlorogenic acid, have previously been identified as potential sources of host-plant resistance against the tomato fruitworm *Heliothis zea*. We report here the possible incompatibility of these chemically based resistance factors with viral control of *H. zea*. We have found that both rutin and chlorogenic acid significantly inhibited the infectivity of nuclear polyhedrosis viruses. Chlorogenic acid, when added to tissue culture medium containing TN-368 ovarian cells, inhibited the infectivity of a multiply embedded virus (AcMNPV) by over 86%. Rutin or chlorogenic acid, when fed to *H. zea*, inhibited the infectivity of a singly embedded nuclear polyhedrosis virus (HzSNPV), with the greatest degree of inhibition occurring at low doses of viral inoculum. Additionally, the ingestion of these phytochemicals significantly prolonged the survival time of virally infected *H. zea* larvae. These results suggest that the effectiveness of nuclear polyhedrosis viruses in controlling *H. zea* populations may be adversely affected by varieties of *L. esculentum* with significant levels (eg. 3.5 μmol/g wet weight) of rutin or chlorogenic acid.

Key Words—*Heliothis zea, Spodoptera exigua, Noctuidae, Lycoperiscon esculentum*, nuclear polyhedrosis virus, chlorogenic acid, rutin, orthodihydroxy phenolics, flavonoids, host-plant resistance, microbial control agents, biological control, antibiosis, tomato, pathogenesis.

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INTRODUCTION

It is normally assumed that the use of host-plant resistance (HPR) against insect pests is compatible with the concurrent use of biological control (BC) agents (Adkisson and Dyck, 1980; Bergman and Tingey, 1979; Kogan, 1975), presumably because of the lesser degree of evidence to the contrary (Pimentel and Wheeler, 1973; Starks et al., 1972). This assumption may not be valid in certain instances where HPR is based upon chemical antibiosis. For example, it was suggested that the unwitting use of tomatine in HPR programs against noctuid larvae may be incompatible with the survival of endoparasitoids, as evidenced by the poisoning of the endoparasitic ichneumonid *Hyposoter exiguae* by tomatine (Campbell and Duffey, 1979; Campbell and Duffey, 1981). This potential incompatibility may apply as well to other types of BC agents such as bacteria, fungi, and viruses.

The literature on the compatibility of HPR with microbial control agents is exceptionally meager (Jones, 1983). It has been theorized that the nutritional state of the insect, as well as the type of plant products ingested, can predispose the insect to disease (Price et al., 1980; Schultz, 1983; Vago, 1963; Steinhaus, 1956). The factual evidence to support this contention is usually correlational; however, in several instances the potential of specific natural products to modify the course of disease has been documented. In one case, the leaf resin of a leguminous plant, *Hymenaea courbaril*, was shown to induce mortality in virally infected *Spodoptera exigua* (Stubblebine and Langenheim, 1977). In another instance, the nonprotein amino acid, *L*-canavanine, which occurs in many leguminous plants, was shown to enhance the pathogenicity of *Bacillus thuringiensis* in the hornworm *Manduca sexta* (Felton and Dahlman, 1984). Also, plant-derived caffeic acid and other simple phenolics when ingested by the silkworm, *Bombyx mori*, were shown to have antibacterial activity against the pathogenic *Streptococcus faecalis* (Koike et al., 1979; Iizuka et al., 1974). Additionally, in the gut of *B. mori*, enzymatic degradation of chlorophyll-a leads to a chlorophyll–protein complex which confers antiviral activity against a nuclear polyhedrosis virus (Hayashiya, 1978). In contrast to this, a number of studies have implicated the presence of unidentified antimicrobial substances in the foliage as being responsible for or having the potential to reduce the efficacy of the pathogens (Hare and Andreadis, 1983; Ramoska and Todd, 1985; Kushner and Harvey, 1962; Maksymiuk, 1970; Merdan et al., 1975; Morris, 1972; Smirnoff, 1967).

The tomato plant, *Lycopersicon esculentum*, has a number of natural products that have been implicated in resistance to insects, such as tomatine (Campbell and Duffey, 1981; Juvik and Stevens, 1982; Sinden et al., 1978), proteinase inhibitors (Ryan and Green, 1974; Broadway, *et al.* 1986), and orthodihydroxy phenolics including rutin and chlorogenic acid (Isman and Duffey, 1982a,b). Some of these natural products from *Lycopersicon* are also thought

to contribute to the plant's ability to resist pathogens (Carrasco et al., 1978; Chadha and Brown, 1974; Cleveland and Black, 1983; Matta et al., 1969; Mendez and Brown, 1971; Roddick, 1974; Schlosser, 1977). The ability of natural products to serve as bases of resistance against both insects and pathogens suggest that their use might be highly compatible with integrated pest management. However, since it is possible for plant natural products to inhibit the activity of pathogens against insects, as discussed above, it is also possible that these natural products may be incompatible with the concurrent use of microbial control agents.

In light of the ability of caffeic acid to act antibacterially against the pathogenic bacterium *Streptococcus faecalis* in the gut of *B. mori* (Koike et al., 1979; Iizuka et al., 1974), the possibility of the incompatibility of orthodihydroxy phenolics (rutin and chlorogenic acid) with microbial control agents exists in the tomato systems where these phenolics might be used as a basis of resitance (Isman and Duffey, 1982a,b) against the tomato fruitworm. The ability of some phenolics (rutin and quercetin, as examples) to inhibit infectivity and/or inhibit replication of certain RNA and DNA viruses has been documented (Verma, 1973; Harborne et al., 1975; Beladi et al., 1977). These phenolics have the ability to oxidatively couple with viral protein coats and viral nucleic acids (Pierpoint et al., 1977; Davies et al., 1978; Mink et al., 1966) and modify their properties; therefore, it is reasonable to assume that such oxidative coupling to insect nuclear polyhedrosis viruses (NPVS) may influence the degree of infectivity.

In this present study, we wished to determine the effect of rutin and chlorogenic acid on the infectivity of nuclear polyhedrosis viruses. The antiviral effects of these phytochemicals were assayed under both in vitro and in vivo conditions to assess the potential compatibility of host-plant resistance based on these phenolics with viral control of the tomato fruitworm.

METHODS AND MATERIALS

Cell Line and Culture Media. For the in vitro virus assay, the *Trichoplusia ni* (TN-368) cell line was used and grown under conditions similar to those described by Hink (1970). The tissue culture medium (TCM) contained 90 ml Grace's insect tissue culture medium, 8 ml of fetal bovine serum, 8 ml whole egg ultrafiltrate, 0.5 g bovine albumin, 0.3 g yeastolate, 0.3 g lactalbumin hydrolysate, and 100 μg/ml each of the antibiotics streptomycin sulfate and penicillin.

Virus. The virus used in the in vitro assay was a multiply embedded nuclear polyhedrosis virus (MNPV) originally isolated from *Autographa californica* (Speyer) by Vail et al. (1971) and designated as AcMNPV-13. The AcMNPV-13 polyhedral inclusion bodies (PIB) were produced in *Spodoptera*

exigua (Hübner) larvae. The virus used in the in vivo assay was a singly embedded virus (SNPV) originally isolated from *H. zea* larvae and designated as HzSNPV. The virus was obtained as the commercial formulation Elcar from Sandoz-Wander (Wasco, California).

Insects. Eggs of *Heliothis zea* were obtained from a laboratory colony of Shell Development Company, Modesto, California. Larval *H. zea* were reared in an environmental chamber at 27°C with a 16:8 light–dark photoperiod in 25-well plastic rearing trays obtained from Bio-Serv Inc. (Frenchtown, New Jersey). Larvae were fed semidefined artificial diet (No. 976L3) purchased from Bio-Serv Inc. with streptomycin sulfate (100 ppm) and 5.5% aureomycin (1000 ppm) added as antibiotics. Chlorogenic acid and rutin were purchased from Sigma Chemical Co. (St. Louis, Missouri) and were mixed in the diet at a concentration of 3.5 μmol/g diet on a wet weight basis. This concentration has been found to reduce larval weight gain by approximately 50% compared to larvae fed control diet in a standardized bioassay (Isman and Duffey, 1982b).

In Vitro Assay of Viral Infectivity with MNPV. The in vitro virus assay used was an endpoint titration in Falcon 96-well microtiter plates. The TN-368 cells were used 24 hr after transfer while in the log phase of growth and were diluted to give a concentration of 4.5×10^4 cells/ml TCM. Each well was seeded with 0.2 ml of cell suspension. Nonoccluded virus was diluted by 10-fold or twofold with TCM and was added to the wells at 0.1 ml inoculum/well. Control wells containing TN-368 cells received 0.1 ml of TCM in lieu of the viral inoculum. For each dilution of inoculum, 24 wells or replicates were treated with the virus.

To test the effect of orthodihydroxy phenolics on viral infectivity, rutin or chlorogenic acid was added to the TCM (without cells added) immediately prior to seeding the plates with the TN-368 cells. The control treatment did not contain added phenolics. Then the rutin or chlorogenic acid–TCM mixture was heated to 36°C for 1 hr with stirring, after which the solution was immediately filtered through a sterile 0.22-μm Falcon 150-ml filtration unit. The final concentrations of the two phenolics used in the endpoint titrations were 0.11 and 2.2 μmol/ml TCM for rutin and chlorogenic acid, respectively (as determined by spectroscopy; Pesez and Bartos, 1974). The resulting concentration of rutin was less than that of chlorogenic acid because of the limited solubility of rutin in aqueous medium. The addition of these phytochemicals did not significantly alter the pH of the TCM. Approximately 50% of the cells became melanized by the 5th day in treatments using chlorogenic acid. Additionally, chlorogenic acid when added to the TCM at much higher concentrations than were used in these assays (10×) did inhibit cell growth by approximately 30%. The effect of these phenolics on viral infectivity was determined by endpoint titration as described above for untreated TCM.

The seeded plates were incubated at 27°C for five days, and then each well was checked for the presence of infected cells under an inverted light micro-

scope. The presence of a single infected cell in a well, as determined by the presence of polyhedra, was considered to be a positive response to infection. The median tissue infective dose ($TCID_{50}$) and 95% confidence limits were calculated by the method of Reed and Muench (1938).

In Vivo Assay of Viral Infectivity with SNPV. Larval *H. zea* were reared from neonates in 25-well plastic trays on control diet or diet supplemented with rutin or chlorogenic acid. Approximately 75 μl of the respective artificial diet was added to each well of a Falcon 96-well microtiter plate and a specified dose of HzSNPV in 1 μl of H_2O was added to the diet surface. Controls in each treatment received 1 μl of H_2O in lieu of virus. Newly molted third-instar *H. zea* (ca. 6 hr postecdysis) were then individually placed in the wells of the microtiter plates and parafilm was used to cover the wells to prevent larvae from escape. After 36 hr, larvae that had consumed all of the diet were transferred to 18-ml clear plastic cups containing a surfeit of the respective diet. At least four doses of virus and a control were used in each diet treatment with a minimum of 50 larvae per dose. Mortality was monitored on a daily basis and LD_{50} values for each diet treatment were computed from 10-day mortality figures using a probit analysis procedure developed by the Statistical Analysis System (SAS).

RESULTS

Results from the in vitro assay indicated that chlorogenic acid significantly reduced AcMNPV infectivity in both replicates (Table 1). In the first replicate, the infectivity of the virus was reduced by 70.7% compared to the control, while in the second replicate, an 86.4% reduction in infectivity was observed. In both replicates, the effect of rutin on infectivity (7.1% and 25.0% reduction)

TABLE 1. EFFECT OF ORTHODIHYDROXY PHENOLICS ON IN VITRO AcMNPV INFECTIVITY

Media	$TCID_{50}$ (x 10^7)[a]	*N*	Inhibition %
Replicate 1			
Control	1.4	120	
Chlorogenic acid (2.2 μmol/ml)[b]	0.41[b]	120	70.7
Rutin (0.11 μmol/ml)	1.3	120	7.1
Replicate 2			
Control	2.8	144	
Chlorogenic acid (2.2 μmol/ml)[b]	0.38[b]	144	86.4
Rutin (0.11 μmol/ml)	2.1	144	25.0

[a] $TCID_{50}$/ml TCM.
[b] Significantly less than the control at $P < 0.05$.

TABLE 2. EFFECT OF ORTHODIHYDROXY PHENOLICS ON IN VIVO HzSNPV INFECTIVITY

Diet	LD (95% fiducial limits)[a]	Regression equation	N
Control		$Y = 3.18 + 1.16X$	275
LD_{10}	2.92 (1.27-5.03)		
LD_{50}	37.36 (26.56-54.93)		
LD_{90}	478.86 (254.04-1292.0)		
Chlorogenic acid			
(3.5 μmol/g)		$Y = 1.72 + 1.94X$	290
LD_{10}[b]	10.67 (6.77-14.85)		
LD_{50}	48.76 (38.21-62.67)		
LD_{90}	222.81 (156.79-363.99)		
Rutin			262
(3.5 μmol/g)		$Y = 1.96 + 1.61X$	
LD_{10}[b]	12.26 (6.87-18.55)		
LD_{50}[b]	76.51 (56.99-102.49)		
LD_{90}	477.25 (317.46-843.27)		

[a] LD_{50} = PIBs/ insect.
[b] Significantly different from control at $P < 0.05$.

was not significant as determined by an overlap of the 95% confidence limits of the $TCID_{50}$ between rutin and control treatments. The observed differences in antiviral activity between rutin and chlorogenic acid may be due to the differences in their concentrations used in this assay.

Results from the in vivo assay indicate that at viral concentrations below the LD_{50}, larvae fed either rutin or chlorogenic acid were much less sensitive to the virus than larvae fed control diet (Table 2). For example, at a dose of 12 polyhedral inclusion bodies (PIB)/insect, 34% mortality was observed in larvae fed control diet while larvae on rutin or chlorogenic acid diets showed significantly lower ($P < 0.05$ by chi-square analysis) mortalities of 8% and 15.5%, respectively. The LD_{10}s for larvae on rutin- or chlorogenic acid-supplemented diets were respectively 4.21× and 3.66× greater than the control (Table 2). At concentrations above the LD_{50}, the response to rutin was not as significant. For example at the LD_{50}, rutin significantly inhibited HzSNPV infectivity by over 51%, but at the LD_{90} the effect of the virus was statistically similar to the control infected larvae. Although larvae ingesting chlorogenic acid showed decreased sensitivity to HzSNPV at the LD_{50}, the response was not considered significant because of overlapping confidence limits for the LD_{50}s. The LD_{90} for chlorogenic acid-treated larvae was also not significantly different from the controls.

In addition to affecting the relative susceptibility of larval *H. zea* to HzSNPV, ingestion of rutin and chlorogenic acid significantly prolonged the survival time of virally infected larvae (Table 3). For instance, at a dose of 200

TABLE 3. EFFECT OF ORTHODIHYDROXY PHENOLICS ON SURVIVAL TIME OF LARVAE INFECTED WITH HzSNPV

Treatment	Mean survival time (days)[a]
Control	6.50
Chlorogenic acid (3.5 μmol/g)	7.14[b]
Rutin (3.5 μmol/g)	7.84[b]

[a] Survival time until death following ingestion of virus.
[b] Significantly different from the control at $P < 0.01$ by Tukey's studentized range test.

PIB/insect, the survival time of infected larvae fed control diet was 6.50 days but was extended to 7.14 and 7.84 days, respectively, in larvae that fed on chlorogenic acid- or rutin-supplemented diet. A significant prolongation of the survival time of infected larvae was observed for each concentration of virus which produced a lethal infection. The survival time of infected larvae was defined as the survival time until death following ingestion of virus in lethally infected larvae. Significant differences between mean survival times were determined by Tukey's studentized range test.

DISCUSSION

We show that both rutin and chlorogenic acid in vivo inhibit the infectivity of a nuclear polyhedrosis virus in larval *H. zea* (Table 2). The effect of both phytochemicals was greatest at low concentrations of viral inoculum. Under field conditions, the occlusion bodies of NPV are inactivated rapidly in ultraviolet light on tomato foliage (Young and Yearian, 1974) and, therefore, larvae would be expected to consume small amounts of inoculum. We predict the effect of ingestion of rutin or chlorogenic acid on viral infectivity would be greatest under natural conditions. Because both phenolics may be involved in host-plant resistance to *H. zea* (Isman and Duffey, 1982a,b), these data suggest a potential incompatibility of host-plant resistance with viral control of *H. zea* in tomatoes under normal field conditions. The concentrations of rutin and chlorogenic acid used in the bioassays (3.5 μmol/g wet weight) are representative of the concentrations that occur in the foliage of many *L. esculentum* varieties (Isman and Duffey, 1982b). For example, the foliar concentration of rutin and chlorogenic acid in the commercial fresh market variety, Castlemart, averaged approximately 3.7 and 3.6 μmol/g wet weight, respectively, during the course of a growing season in 1984 (unpublished data). As a result, the efficacy of HzSNPV as a mortality factor in *Heliothis* populations in tomato ecosystems

may be reduced by the use of varieties of *Lycopersicon* with high rutin and/or chlorogenic acid content.

Additionally, we have preliminary evidence suggesting that these phenolics may adversely affect other parameters of the disease process which may be important in modifying the occurrence and progression of viral epizootics. A short survival time of infected insects and a high rate of production of infective stages are considered important factors in determining the epizootic potential of a pathogen (Anderson and May, 1981). For instance, the survival time of infected larvae was significantly prolonged in larvae feeding upon diets containing either chlorogenic acid or rutin. Also, fewer occlusion bodies containing the infectious virions would be liberated in the environment in larvae consuming orthodihydroxy phenolic-containing diets because of the reduction in the relative numbers and size of infected larvae. This present study points to the necessity of developing a mechanistic understanding of the multitrophic effects of plant natural products before attempting to manipulate their concentrations in planta.

The mechanism(s) by which these phenolics modify the infectivity of nuclear polyhedrosis viruses has not been determined. Chlorogenic acid and rutin are able to bind to the protein coat of certain viruses which may render the virus incapable of initiating infection (Mink, 1965; Pierpoint et al., 1977). Thus, the observed mitigation of infectivity may be due to a reduction in the ability of the virus to attach to host receptor sites and/or due to changes in the solubility of the viral matrix protein in the midgut of the host. Moreover, these phenolics may directly inactivate the virions. Following uncoating of the virus within the midgut epithelial cells and the subsequent release of viral DNA, the viral nucleic acid may be susceptible to modification by these phenolics (Mink, 1965).

Because both chlorogenic acid and rutin are ubiquitous in occurrence among plant species (Harborne et al., 1975; Harborne, 1973) and are thus an adventitious dietary component of many herbivorous insects, the results from this study may have broad implications to the study of insect–plant interactions. It is reasonable to assume that the ingestion of these phytochemicals by lepidopterous insects may offer some protection from viral infections and may even affect the occurrence of viral epizootics in larval populations. This study emphasizes the necessity of considering pathogenicity and host-plant resistance not as two component systems, but instead as a three-component system, namely the interactions of plant–insect–pathogen. The role of the host-plant, particularly phytochemicals, in the epizootiology of insect disease is in need of further investigation.

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SEMIOCHEMICAL ATTRACTANTS OF *Diabrotica undecimpunctata howardi* BARBER, SOUTHERN CORN ROOTWORM, AND *Diabrotica virgifera virgifera* LECONTE, THE WESTERN CORN ROOTWORM (COLEOPTERA: CHRYSOMELIDAE)

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Abstract—During the summers of 1984 and 1985, a variety of structurally related benzenoid compounds was evaluated in sweet corn plots as attractants for adult southern corn rootworms (SCR), western corn rootworms (WCR), and northern corn rootworms (NCR). Field response to the volatiles was measured by beetle counts on baited cylindrical sticky traps placed inside the corn plots at a height of 1 m above ground level. SCR adults were attracted late in the season (last week of August through September, 1984 and 1985) to numerous aromatic compounds, including phenylacetaldehyde, benzyl acetone, phenethyl alcohol, phenyl acetate, indole, veratrole, methyl eugenol, methyl isoeugenol, eugenol, and isoeugenol. Although many compounds attracted SCR adults late in the season, only veratrole, phenylacetaldehyde, and chavicol were significantly active in early and middle August 1985. WCR adults were attracted to a different group of compounds, namely estragole, *trans*-anethole, and indole. Estragole (4-methoxy-l-allylbenzene) was an effective WCR attractant from corn tasseling in early August 1985, until the end of the trapping period in late September and early October 1985. Indole and *trans*-anethole (4-methoxy-l-propenylbenzene) were less effective attractants than estragole and were most active at the beginning and/or end of the corn season. Traps baited with 100 mg of estragole caught an average of 20 times more WCR adults than unbaited control traps, and the females outnumbered the males in the baited traps. Estragole dosage tests were conducted in three sweet corn plots on different dates in 1985 and the minimum effective dose ranged between 5 and 30 mg/trap. Field tests with structural analogs revealed the importance of the site of unsaturation in the allylic side chain of

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estragole and the effect of different ring substituents on WCR response. The phenylpropanoids, eugenol and isoeugenol, significantly attracted NCR adults, even though these beetles were in low abundance in the test corn plots. Field tests indicate there is no cross-species response by WCR and NCR adults to their related phenylpropanoid attractants. However, in late August, SCR adults do respond to some WCR and NCR attractants (indole and several eugenol analogs). Electroantennographic analysis of SCR males revealed they can perceive peripherally a wide range of benzenoid compounds.

Key Words—*Diabrotica*, Coleoptera, Chrysomelidae, western corn rootworm, southern corn rootworm, northern corn rootworm, attractants, semiochemicals, veratrole, estragole, *trans*-anethole, indole, eugenol, phenylacetaldehyde.

INTRODUCTION

Larvae and adults of the northern corn rootworm (NCR), *Diabrotica barberi* Smith and Lawrence, and the western corn rootworm (WCR), *D. virgifera virgifera* LeConte, are serious root and silk pests of field and sweet corn in the corn belt. The univoltine NCR and WCR overwinter as diapausing eggs in North America and the oligophagous larvae develop only on the roots of grasses (Poaceae), primarily corn (Branson and Krysan, 1981; Branson and Ortman, 1967, 1971; Smith, 1966). In contrast to these two *Diabrotica* species, the multivoltine southern corn rootworm (SCR) = spotted cucumber beetle, *D. undecimpunctata howardi* Barber, overwinters in southern areas as an adult, annually reinvades the Midwest, and both larvae and adults are polyphagous (Smith, 1966; Branson et al., 1978). SCR bettles are pests of cucurbits, peanuts, corn, and a variety of vegetable and field crops (Campbell and Emery, 1967).

Although the New World diabroticites (Chrysomelidae: Galerucinae: Luperini: Diabroticina) have greatly expanded their host distribution and range from their origins, they apparently retain an ancestral compulsive feeding response to the bitter-tasting cucurbitacins found in the cotyledons, foliage, fruit, and flowers of many species of the Cucurbitaceae (Chambliss and Jones, 1966; Sharma and Hall, 1973; Howe and Rhodes, 1976; Metcalf et al., 1982; Ferguson et al., 1983; Andersen, 1984). Cucurbitacins are undoubtedly potent kairomones for the diabroticites, as well as potent allomones for many other herbivores and for some diabroticite predators (Metcalf, 1979; Tallamy, 1985; Ferguson and Metcalf, 1985), yet their high molecular weights and low vapor pressures make them ineffective as volatile semiochemicals (Branson and Guss, 1983).

Considerable progress has been made recently in understanding *Diabrotica* response to volatile attractants. The sex pheromone of WCR virgin females, 8-methyl-2-decyl propanoate, attracts both WCR and NCR males; however, the

two species demonstrate temporal differences in response and high doses of the racemic synthetic mixture inhibits NCR response (Guss et al., 1982, 1985). SCR females release 10-methyl-2-tridecanone and the *R* enantiomer is the most active attractant for male SCR and *D. u. undecimpunctata*, the western spotted cucumber beetle (Guss et al., 1983). Furthermore, the nonpheromonal phenylpropanoids eugenol, isoeugenol, and 2-methoxy-4-propylphenol strongly attract NCR adults, whereas WCR adults do not respond to these compounds (Ladd et al., 1983; Ladd, 1984). Andersen and Metcalf (1986) isolated the shikimic acid derivative indole from the male blossoms of *Cucurbita maxima* cultivars, where *Diabrotica* and *Acalymma* adults often congregate in large numbers, and found adult WCR and striped cucumber beetles, *Acalymma vittatum* (Fabr.), are attracted early and late in the season to as little as 5 mg of indole. Although indole is clearly a kairomonal attractant of WCR adults, a host-plant relationship has not been established in the literature for the attractants of NCR adults (Ladd, 1984).

During the summers of 1984 and 1985, we investigated the response of WCR, SCR, and NCR adults to a wide variety of benzenoid compounds using baited, one-quart (0.95-liter) white cylindrical sticky traps in sweet corn plots. Chemical analogs of attractive compounds were used to determine the specificity of response by the corn rootworm species. This report summarizes the data obtained with more than 20 aromatic compounds, many of which have been detected in corn or cucurbits (Buttery et al., 1978, 1980, 1982; Andersen, 1984). A preliminary electroantennographic (EAG) study with SCR males evaluates their response to several benzenoid compounds.

METHODS AND MATERIALS

Field Tests. During the summers of 1984 and 1985, various volatile compounds were evaluated for corn rootworm attraction on the South Farms of the University of Illinois in Urbana, Illinois. In 1984, tests were conducted in a single sweet corn plot which measured 52 × 22 m with eight rows each of cultivars, "Florida Stay Sweet" and "Gold Cup." The corn plot was bordered on one side by a grass lane and on the other side by a cucurbit plot. The adjacent cucurbit plot included several common squash varieties (predominantly *Cucurbita maxima* cv. "Blue Hubbard"), as well as high cucurbitacin-containing species and hybrids (*Cucurbita andreana, C. texana,* and *C. andereana* × *C. maxima* hybrids) (Rhodes et al., 1980). Large numbers of SCR adults (>20,000 beetles) aggregated on the leaves, blossoms, and fruits of the bitter cucurbits and "Blue Hubbard" cultivar in 1984 and 1985. In both years, SCR adults were ca. 10–20 times more abundant than WCR and *A. vittatum* adults during the test period. The experimental design for the main plot used in 1985 was essentially the same as in 1984, except the corn was a monoculture of 20 rows

of cultivar "Illini Xtra Sweet." Tests were also conducted in 1985 in two non-adjacent sweet corn plots located approximately 150 m and 250 m from the main plot. These auxilliary plots were not adjacent to any cucurbits and WCR adults were the predominant rootworm species present.

The 1984 test plot was planted on June 12 and initial tests were conducted in late August after tasseling. After the first frost (last week of September 1984), field tests were discontinued. In 1985, trapping began in mid-July, before tasseling, and continued until the corn foliage was totally dry in late September. The two auxiliary sweet corn plots were used to test WCR attractants and were planted approximately two and four weeks, respectively, after the main plot (June 6, 1985) and were used until the first week of October. Pretest whole-plant counts were taken every other week in the main plot and once in the two auxiliary plots by counting beetles in the rows used for testing treatments.

All compounds tested, except two, were from commercial sources and verified as being ≥95% pure by gas chromatographic analysis. Chavicol (4-hydroxy-l-allylbenzene) was prepared (R.L.M.) by the method of Buu-Hoi et al. (1954) and 4-methoxy-l-propylbenzene by reacting the sodium salt of 4-propylphenol with dimethyl sulfate. The products were glass distilled and purity and identity confirmed by boiling points, infrared analysis, and gas chromatography–mass spectrometry. Compounds were field tested for attraction of rootworms by treating dental cotton wicks with 200 mg or less of each compound (see Tables). In order to prolong volatilization of the treatment compound, the wicks (ca. 15 mm long × 6 mm diameter) were soaked in polyethylene glycol and the excess squeezed out prior to treatment with each test compound. The treated wicks were attached to the tops of one-quart (0.95-liter) white paper cartons, similar to those described by Hein and Tollefson (1984). The paper cartons were 15.2 cm high and had a circumference of 27.9 cm and were evenly coated with clear Tangletrap® in the field. The baited sticky traps were placed in the corn plots in the early afternoon (1–4 PM) and beetle counts were taken periodically for one to three days.

In 1984, the traps were placed ca. 1 m high by breaking off the corn stalk at ear level and placing the hollow carton trap on top of the stalk. In 1985, the traps were placed in corn rows on 1 m-high wood stakes. In both years, the baited traps were arranged in a randomized complete block design with treatments 3 to 5 m apart and blocks 2 to 3 m apart, depending on the number of compounds and replicates in a test. Results were analyzed by an ANOVA program and, in the case of multiple comparisons, means were separated by Duncan's multiple-range test with significance levels set at $P = 0.05$ (Nie et al., 1975).

Electroantennographic Analysis. Several SCR attractants with variable field activity were selected for EAG analysis (see Table 4 for a list of compounds). Antennae of male SCR adults were excised at the head following removal of the distal segment. The antennae were placed between two glass capillaries

filled with 0.9% NaCl solution. Silver–silver chloride wires were used as electrodes and connected by shielded leads to a Grass P-18 preamplifier. Sample compounds were dissolved in paraffin oil (Fisher) at 100- and 1000-fold dilutions, and filter paper triangles were impregnated with 5 μl of each solution. The treated triangles were then inserted into Pasteur pipets and a glass syringe used to pass 0.4 ml of air through the pipet into a charcoal-filtered airstream (100 ml of air per minute) which flowed over the beetle antennae. Since EAG response diminishes through time, the response of the antenna to each treatment is expressed as a percent relative response in comparison to a standard compound (*cis*-3-hexen-l-ol) at a 1 : 100 dilution in paraffin oil (similar to that used by Visser, 1979). Six replicates were conducted for each compound at both dilutions, and the laboratory-reared beetles ranged in age from 2 to 4 weeks postemergence. Each treatment was compared to the control by a single-classification ANOVA (Sokal and Rohlf, 1969).

RESULTS

SCR Attractants. In the first field test, August 28, 1984, the mean 24-hr trap catches (± standard error) for phenylacetaldehyde (PAA), indole, eugenol, and control traps were 238.0 ± 14.5, 95.8 ± 3.3, 55.5 ± 5.9, and 16.0 ± 2.6 SCR adults per trap, respectively (N = 6; dosage = 200 mg/trap). All three treatments were significant attractants; however, PAA traps significantly attracted 2.5 and 4.3 times more SCR adults than indole- and eugenol-baited traps. The downwind surface of all baited traps had the greatest concentration of beetles, suggesting an anemotactic response by the insects. Since a pretest whole-plant count in the corn plot yielded a mean (± standard error) of 3.0 ± 0.4 SCR adults per corn stalk (N = 50), the ca. 1500 beetles captured on the six PAA traps were more than could have been present on the surrounding corn stalks. In fact, SCR adults were often observed, in both years, flying from the adjacent cucurbit plot into the corn plot and orienting to the treated traps from several meters downwind.

Since PAA was such an active semiochemical for SCR adults, structurally related compounds were evaluated in the same corn plot over a 4-hr period for potential activity (September 9, 1984) (Table 1). Although PAA-baited traps had the highest mean trap catch, ca. 8 times greater than the control traps, they were not significantly more attractive to SCR adults than benzyl acetone-baited traps. Phenethyl alcohol and benzyl acetate were significantly less active than PAA-baited traps; however, both compounds were still 5.6 and 3.9 times more attractive, respectively, than controls. The structural similarity of these active compounds suggests that they may interact with the same sensory receptors. A substantial decrease in SCR attraction was observed between compounds which differed only by one carbon and two hydrogens. For example, PAA traps were

TABLE 1. MEAN TRAP CATCH OF SOUTHERN CORN ROOTWORM ADULTS ON STICKY TRAPS BAITED WITH 200 mg PHENYLACETALDEHYDE AND RELATED COMPOUNDS IN CORN PLOT ON SEPTEMBER 9, 1984 (SOUTH FARMS, URBANA, ILLINOIS)

Treatment	Chemical formula	$\bar{X}$/trap ± SE[a]	RA (%)[b]
Phenylacetaldehyde	$C_6H_5CH_2C(O)H$	93.0 ± 15.0 a[c]	100
Benzyl acetone	$C_6H_5CH_2CH_2C(O)CH_3$	71.0 ± 4.2 ab	76
Phenethyl alcohol	$C_6H_5CH_2CH_2OH$	63.3 ± 2.7 bc	68
Phenyl acetate	$C_6H_5OC(O)CH_3$	43.7 ± 16.3 cd	47
Benzyl alcohol	$C_6H_5CH_2OH$	36.7 ± 9.0 de	39
Methyl benzoate	$C_6H_5C(O)OCH_3$	28.0 ± 8.5 def	30
Benzyl acetate	$C_6H_5CH_2OC(O)CH_3$	26.7 ± 6.2 def	29
p-Anisaldehyde	$4\text{-}CH_3O,C_6H_4C(O)H$	26.3 ± 1.5 def	28
Acetophenone	$C_6H_5C(O)CH_3$	25.3 ± 5.8 def	27
p-Anisyl alcohol	$4\text{-}CH_3O,C_6H_4CH_2OH$	24.0 ± 8.1 def	26
Benzaldehyde	$C_6H_5C(O)H$	14.0 ± 3.2 ef	15
Control		11.3 ± 1.0 ef	12
Anisole	$C_6H_5OCH_3$	9.3 ± 1.5 f	10

[a]Mean number of beetles per trap after a 4-h period ± standard error of the mean (SE); three replicates per treatment.
[b]RA (%) = percent relative attractancy (mean of treatment/mean of PAA) × 100.
[c]Means followed by the same letter are not significantly different at the 5% level by Duncan's multiple-range test.

6.6 times more attractive than benzaldehyde traps; phenethyl alcohol traps, 1.7 times more than benzyl alcohol traps; and phenyl acetate traps, 1.6 times more than benzyl acetate-baited traps.

Eugenol-related compounds were also investigated (Table 2) in 1984. This evaluation was conducted in mid-September and SCR activity was reduced due to lower day and night temperatures (compare controls in Tables 1 and 2). The 24-hr mean beetle catches of veratrole-, isoeugenol-, methyl isoeugenol-, and methyl eugenol-baited traps were significantly greater than the control beetle catch. Veratrole (orthodimethoxybenzene) consistently attracted more beetles than the other compounds in this series; however, the metadimethoxybenzene and paradimethoxybenzene isomers of veratrole were not significantly different from the control trap catch. This indicates the ortho ring position of the methoxy groups is important for activity. Eugenol and indole had higher mean trap catches than controls in this test but were not significantly different from controls. Apparently, the propenylbenzenoid compounds are slightly more attractive than the allylbenzenoid compounds (Table 2).

Field tests with SCR adults were discontinued in late September 1984 after the first frost. In 1985, veratrole- and PAA-baited traps were reevaluated in the main corn plot from July 18 to September 13. Veratrole-baited traps attracted

TABLE 2. MEAN TRAP CATCH OF SOUTHERN CORN ROOTWORM ADULTS ON STICKY TRAPS BAITED WITH 200 mg VERATROLE AND RELATED COMPOUNDS IN CORN PLOT ON SEPTEMBER 15, 1984 (SOUTH FARMS, URBANA, ILLINOIS)

Treatment	Chemical formula	$\bar{X}$/trap + SE[a]	RA (%)[b]
Veratrole	$1,2\text{-}(CH_3O)_2C_6H_4$	45.0 ± 6.7 a[c]	100
Isoeugenol	$3\text{-}CH_3O,4\text{-}HO,C_6H_3CH{=}CHCH_3$	27.3 ± 7.1 b	60
Methyl isoeugenol	$3,4\text{-}(CH_3O)_2C_6H_3CH{=}CHCH_3$	23.3 ± 3.8 bc	52
Methyl eugenol	$3,4\text{-}(CH_3O)_2C_6H_3CH_2CH{=}CH_2$	21.7 ± 1.7 bcd	48
Eugenol	$3\text{-}CH_3O,4\text{-}HO,C_6H_3CH_2CH{=}CH_2$	17.7 ± 3.7 bcde	39
Indole	$C_6H_4 \cdot C_2H_2NH$	16.3 ± 5.5 bcde	36
m-Dimethoxybenzene	$1,3\text{-}(CH_3O)_2C_6H_4$	15.3 ± 0.3 cde	34
Anethole	$4\text{-}CH_3O,C_6H_4CH{=}CHCH_3$	12.7 ± 1.7 cde	28
Safrole	$3,4\text{-}(OCH_2O){-}C_6H_3CH_2CH{=}CH_2$	10.0 ± 2.9 de	22
3,4-Dimethoxypropylbenzene	$3,4\text{-}(CH_3O)_2C_6H_3CH_2CH_2CH_3$	9.3 ± 3.5 e	21
Control		7.3 ± 1.2 e	16
p-Dimethoxybenzene	$1,4\text{-}(CH_3O)_2C_6H_4$	7.3 ± 1.9 e	16
Estragole	$4\text{-}CH_3O,C_6H_4CH_2CH{=}CH_2$	7.3 ± 1.9 e	16

[a] Mean number of beetles per trap after a 24-hr period ± standard error of the mean (SE); three replicates per treatment.
[b] RA (%) = percent relative attractancy = (mean of treatment/mean of veratrole) × 100.
[c] Means followed by the same letter are not significantly different at the 5% level by Duncan's multiple-range test.

an average of two times more SCR adults than PAA traps and eight times more than control traps (Table 3). Initial baited trap catches of beetles in 1985 were much lower than the late season beetle catches in 1984; however, trap catches dramatically increased at the end of August and early September for both treatments and controls (in Table 3 compare mean trap catches from July 18 to August 13 to those from August 25 to September 13).

Analysis of the 1985 data by two-way ANOVA (blocks versus treatments) yielded significantly different block mean squares for SCR trap catches in all late-August and September tests; therefore, there was a substantial field effect. The beetle trap catches in the main plot were 3–10 times greater in the block closest to the cucurbit plot than the trap catches in the other blocks. The whole-plant count in the corn plot on August 20 revealed there were 4.9 ± 0.54 SCR adults per stalk (mean ± standard error, $N = 20$) on the cucurbit side of the plot and 1.5 ± 0.36 SCR per stalk ($N = 20$) on the opposite, open field side of the plot. Within a four-day period from August 16 to August 20, the whole-plant count doubled. Mean (± standard error) whole-plant counts in 1985 were 0.1 ± 0.05 (July 23, $N = 79$), 0.7 ± 0.12 (August 7, $N = 70$), 1.5 ± 0.16

TABLE 3. MEAN TRAP CATCH OF SOUTHERN CORN ROOTWORM ADULTS ON STICKY TRAPS BAITED WITH VERATROLE AND PHENYLACETALDEHYDE (PAA)

		Treatment		
Date (1985)	Dose (mg)/trap	Veratrole	PAA	Control
July 18	100	5.00 ± 0.71a[a]	1.00 ± 0.41b	0 ± 0 c
August 2	100	6.75 ± 3.28a	2.50 ± 0.96b	0.75 ± 0.25c
August 13	20	8.25 ± 3.77a	4.75 ± 1.19a	0.75 ± 0.48b
August 18	100	23.25 ± 6.75a	3.50 ± 1.19b	1.50 ± 0.50b
August 25	30	—	26.75 ± 6.34a	6.75 ± 1.55b
August 27	200	92.50 ± 9.90a	72.20 ± 7.86b	4.50 ± 2.30c
September 13	100	33.00 ± 9.70a	—[b]	13.00 ± 5.24b

[a]Mean trap catch per 24 hr ± S Error; $N = 4$. Means in the same row followed by the same letter are not significantly different at the 5% level by Duncan's multiple-range test.
[b]Compound not tested on that date.

(August 16, $N = 54$), and 3.2 ± 0.28 (August 20, $N = 40$) SCR adults per stalk. The field effect and late season increase in SCR trap catches was apparently the result of an increase in SCR dispersal from the cucurbit plot into the corn test plot.

Other field tests conducted in August 1985 showed chavicol (4-hydroxy-l-allylbenzene) also significantly attracted SCR adults (Table 5). Related compounds like estragole (4-methoxy-l-allylbenzene), *trans*-anethole (4-methoxy-l-propenylbenzene), and 4-methoxy-l-propylbenzene, as well as several eugenol-related compounds, were not significant attractants of SCR adults in early and mid-August, 1985. However, the late August and September results in the main plot were similar to those obtained in 1984, with eugenol, isoeugenol, PAA, veratrole, and indole having significantly greater trap catches than controls. SCR trap catch increases late in the season and previously unattractive compounds become significant attractants. This suggests SCR adults in central Illinois exhibit an enhanced response to volatiles in late August and early September.

Electroantennograms of SCR Males. The relative EAG response (compared to *cis*-3-hexen-l-ol) for male SCR was determined for several aromatic compounds (Table 4). Males were used for the EAG analysis since they often outnumber females in high cucurbitacin-containing cucurbit plots (Andersen, 1981), and our plot was adjacent to such a cucurbit plot. The EAG response to indole, phenethyl alcohol, PAA, benzyl acetate, benzyl acetone, eugenol, and veratrole, at 100- and 1000-fold dilutions in mineral oil, was seven or more times greater than that of control air puffs. Indole, phenethyl alcohol, and PAA had EAG responses 20–40 times greater than that of the control. This indicates

TABLE 4. MEAN RELATIVE ELECTROANTENNOGRAPHIC RESPONSE[a] OF MALE SOUTHERN CORN ROOTWORMS TO SEVERAL AROMATIC COMPOUNDS

Treatment	Dilution in mineral oil	
	1:1000	1:100
cis-3-Hexenol	—	100
Indole	80.3 ± 6.1[b]	154.5 ± 8.2
Phenethyl alcohol	80.3 ± 4.5	146.7 ± 8.0
Phenylacetaldehyde	81.3 ± 2.5	106.5 ± 3.9
Benzyl acetate	46.6 ± 9.3	74.5 ± 2.3
Benzyl acetone	33.1 ± 4.5	55.5 ± 3.1
Eugenol	26.4 ± 4.3	58.4 ± 4.0
Veratrole	29.1 ± 5.1	47.3 ± 6.5
Control	4.0 ± 2.5	4.0 ± 2.5

[a]EAG response is relative to a 1:100 dilution of *cis*-3-hexenol.
[b]Mean response ± SD; $N = 6$.

SCR males are able to perceive peripherally a wide variety of aromatic compounds. Although all the compounds evaluated yielded significant responses, veratrole, one of the most active field attractants, had the lowest EAG response, and indole, a moderately active field attractant, had the highest EAG response.

WCR Attractants. In a late season experiment, October 7, 1984, *trans*-anethole- and indole-baited traps significantly attracted WCR adults; therefore, these and related compounds were intensively evaluated in 1985. Estragole (4-methoxy-l-allylbenzene), a structural analog of *trans*-anethole (4-methoxy-l-propenylbenzene), was the most active WCR attractant throughout the 1985 field evaluation of volatiles. Estragole was initially tested in the main plot on August 9, 1985, approximately one week after the sweet corn tassels had started to shed pollen. The treated (100 mg) mean trap catch (± standard error) was 34.25 ± 6.86 and the control, 1.5 ± 0.65 WCR adults per trap per 24 hr ($N = 4$). The test was replicated on August 11, and estragole traps had ca. 40 times more WCR adults than control traps (estragole, 32.5 ± 6.60 WCR per trap and control, 0.8 ± 0.96 WCR per trap per 24 hr; $N = 4$). Fifty WCR adults per trap were removed 48 hr later (August 13) and sexed in the laboratory. The estragole traps had sex ratios (female–male) of 4.3 : 1, 1.4 : 1, 2.1 : 1, and 4.1 : 1; a mean sex ratio of 2.98 females to 1.0 male per trap. Whether the predominance of females reflected field, chemical, or trap bias was not determined.

Estragole (100 mg/trap) and control traps were also tested in the main plot on August 16 and 28 and September 3, 1985, with mean trap catches (± standard error) of 23.4 ± 2.93, 31.0 ± 6.94, and 7.25 ± 2.53 WCR per trap per

24 hr for estragole-baited traps and 3.5 ± 0.65, 2.25 ± 0.75, and 1.5 ± 0.50 WCR per trap per 24 hr for control traps ($N = 4$ for each trap date). On all dates, estragole was significantly different from controls ($P < 0.05$) by single classification ANOVA. Whole-plant counts in the main corn plot yielded means (± standard errors) of 2.1 ± 0.22 (August 7, $N = 54$), 0.8 ± 0.15 (August 16, $N = 37$), and 0.5 ± 0.07 (August 28, $N = 45$) WCR per stalk. Although the WCR population decreased throughout August (unlike the SCR population), estragole traps consistently attracted WCR adults.

The attraction of WCR adults to estragole and several phenylpropanoid compounds was investigated in the main plot on August 9 and August 16, 1985 (Table 5). Estragole trap catches were 3.64 ± 0.90 (mean ± standard error) times greater than that of the propenyl isomer *trans*-anethole and 4.6 ± 1.50 times greater than the propyl analog, 4-methoxy-l-propyl-benzene (MPB) ($N =$ 28 for the estragole–anethole comparison). Furthermore, estragole with a para-methoxy ring substituent was eight times more attractive to WCR adults than chavicol, an estragole analog with a parahydroxy ring substiutent (see Table 5). Other allyl and propenyl compounds, such as eugenol, methyl eugenol, isoeugenol, and methyl isoeugenol, as well as veratrole and PAA, were not significantly different from control WCR trap catches. WCR attraction to estragole is characterized by a highly specific structure–activity relationship; the position of the double bond and the methoxy ring substituent are critical for activity.

A nitrogen containing phenylpropanoid derivative, indole, was attractive to WCR adults early and late in the test period. On July 18, 1985, in the main plot prior to corn tasseling, indole (100 mg/trap) attracted a mean (± standard error) of 10.8 ± 5.57 WCR per trap per 48 hr compared to a control trap catch of 4.0 ± 0.82 WCR per trap per 48 hr, ($N = 4$). After this test (August 2, 13, and 16, 1985), indole was not significantly different from controls until August 23, 1985 (indole traps, 8.8 ± 2.87 WCR per trap per 24 hr and control traps, 1.5 ± 1.00 WCR per trap per 24 hours; $N = 4$). When indole (20 mg/trap) was tested on September 28, 1985 in the auxiliary plot 250 m from the main plot, 22.0 ± 8.29 WCR were caught per trap per 24 hr versus a control of 2.8 ± 2.36 WCR per trap per 24 hr. The early and late season attraction of WCR adults by indole agrees with the results of Andersen and Metcalf (1986).

Dosage–activity tests with estragole were conducted in all three experimental plots on different dates (Table 6). In the auxiliary field 150 m from the main plot, the minimum dose per trap which significantly attracted WCR adults was 5 mg, and the highest dose, 100 mg/trap, attracted ca. 31 times more beetles than the control (tested from August 20 to 22, 1985). In the main plot the minimum effective dose was 30 mg and the highest dose, 100 mg/trap, attracted 18 times more beetles than the control (tested from August 28 to 30, 1985). In the plot 250 m from the main experimental plot, the minimum effective dose was 10 mg and the highest dose, 30 mg/trap, attracted four times more WCR adults than the control (tested from September 19 to 20, 1985). In the last dos-

TABLE 5. MEAN TRAP CATCH OF WESTERN (WCR), SOUTHERN (SCR), AND NORTHERN (NCR) CORN ROOTWORM ADULTS ON BAITED (100 mg/TRAP) STICKY TRAPS

Bait	August 9, 1985			August 16, 1985		
	WCR	SCR	NCR	WCR	SCR	NCR
Control	1.50 ± 0.65a[a]	3.00 ± 1.08a	0.25 ± 0.25a	3.50 ± 0.64ac	1.50 ± 0.50a	0.00 ± 0.00a
Estragole	34.25 ± 6.86b	4.25 ± 0.85ab	0.00 ± 0.00a	23.50 ± 5.86b	6.50 ± 3.33ab	0.25 ± 0.25a
trans-Anethole	6.25 ± 1.60c	2.75 ± 0.85a	0.25 ± 0.25a	7.75 ± 1.79c	2.00 ± 0.82a	1.00 ± 0.41a
Chavicol	3.75 ± 0.85ac	8.00 ± 1.20bc	1.00 ± 0.71a	3.50 ± 0.96ac	10.25 ± 4.13b	0.75 ± 0.25a
4-Methoxy-1-propylbenzene	—[b]	—	—	2.75 ± 1.03a	2.53 ± 1.71a	0.50 ± 0.50a
Eugenol	—	—	—	2.25 ± 0.95a	4.75 ± 1.11ab	6.25 ± 1.60b
Methyl eugenol	—	—	—	2.25 ± 1.03a	4.00 ± 1.73ab	0.75 ± 0.25a
Isoeugenol	—	—	—	3.25 ± 0.48ac	5.25 ± 2.25ab	3.75 ± 0.75b
Methyl isoeugenol	—	—	—	3.25 ± 0.85ac	3.00 ± 0.82a	0.50 ± 0.50a
Safrole	—	—	—	1.25 ± 1.25a	2.25 ± 0.48a	0.25 ± 0.25a
Phenylacetaldehyde	4.00 ± 1.68ac	9.25 ± 1.97c	0.00 ± 0.00a	—[b]	—	—

[a]Mean ± SE; $N = 4$. Means in the same column followed by the same letter are not significantly different at the 5% level by Duncan's multiple-range test.
[b]Not tested on that date.

TABLE 6. DOSAGE TESTS WITH ESTRAGOLE FOR ATTRACTION OF WESTERN CORN ROOTWORM ADULTS IN THREE NONADJACENT FIELDS[a] (SOUTH FARMS, URBANA, IL)

Dose (mg)/trap	Field 1 (Aug. 20–22, 1985)	Field 2 (Aug. 28–30, 1985)	Field 3 (Sept. 19–20, 1985)
0	1.27 ± 0.75[b]a[c]	1.75 ± 0.48a	17.0 ± 2.51a
1	—[d]	—	13.25 ± 1.56a
3	—	—	22.5 ± 3.37ab
5	7.25 ± 1.44b	—	—
6	—	2.50 ± 0.87ab	—
10	11.50 ± 2.73bc	—	50.0 ± 3.05b
20	28.00 ± 6.80cd	—	—
30	—	6.0 ± 1.36b	71.75 ± 16.56b
50	47.50 ± 13.86d	—	—
60	—	14.0 ± 7.15ab	—
100	39.43 ± 7.30d	31.0 ± 6.94c	—
Pretest WCR/stalk	0.84 ± 0.84[e]	0.50 ± 0.53	1.78 ± 2.45
	$N = 37$	$N = 23$	$N = 64$

[a] Field 2 was the main test plot; field 1, 150 m from field 2; field 3, 250 m from field 2.
[b] Mean WCR trap catch ± SE; $N = 4$ for all treatments.
[c] Means in a column followed by the same letters are not significantly different from each other at the 5% level by Duncan's multiple-range test.
[d] Not tested on that date.
[e] Mean number WCR adults per stalk ± SE.

age test, the 24-hr control trap catch was ca. 10 times greater than the 48-hr control trap catches in the two previous tests. Since this field was planted one month after the main plot, it had the only fresh host available in the test area on that date. The high pretest whole-plant count (Table 6) suggests beetles were attracted to this field.

NCR Attractants. Northern corn rootworms were significantly attracted to eugenol- and isoeugenol-baited traps in mid-August (Table 5), with eugenol-baited traps having a higher mean trap catch than the isoeugenol baited traps. A pretest whole-plant count found only one NCR per 45 stalks, and the control traps caught no NCR adults; therefore, both compounds must be very active attractants. In the auxiliary plot 250 m from the main plot, 10 mg eugenol-baited traps had a mean (± standard error) of 3.8 ± 2.06 NCR per trap per 24 hr and 30 mg eugenol-baited traps, 8.0 ± 0.82 NCR per trap per 24 hr, with control trap catch of 0.8 ± 0.96 NCR per trap per 24 hr ($N = 4$, tested on September 19, 1985). None of the related compounds, such as estragole, anethole, chavicol, 4-methoxy-l-propylbenzene, methyl eugenol, methyl isoeugenol, or safrole significantly attracted NCR adults. Veratrole and PAA, the two major SCR attractants, also did not attract NCR adults. The results of this study in-

dicate both the methoxy and hydroxy ring substituents and the propanoid side chain in eugenol and isoeugenol are critical for NCR attraction, which agrees with the results of Ladd (1984).

DISCUSSION

Southern, western, and northern corn rootworm adults exhibited a species-specific pattern of response to sticky traps baited with various benzenoid compounds. Southern corn rootworm (SCR) adults were attracted in early and mid-August to traps baited with veratrole (1,2-dimethoxybenzene), phenylacetaldehyde, and chavicol (4-hydroxy-1-allylbenzene). In late August and September, 1984 and 1985, SCR trap catches dramatically increased for veratrole and phenylacetaldehyde, as well as for some previously unattractive compounds, such as indole, several eugenol-related compounds, benzyl acetone, and phenethyl alcohol. The rapid change in trap catch was at least partially due to an increase in SCR beetles dispersing from an adjacent cucurbit plot into the sweet corn test plot. Environmental conditions and/or host quality in mid- to late August apparently enhanced SCR response to volatiles. Regardless of the seasonal variability in beetle response, SCR adults demonstrated a relatively specific structure–activity relationship for veratrole and phenylacetaldehyde, since closely related compounds were always less attractive to beetles. An electroantennographic analysis (EAG) of SCR males revealed the beetles can perceive peripherally a variety of benzenoid compounds; however, the level of EAG response did not reflect the magnitude of field response. Subsequent field tests indicate that indole, which yielded a high EAG response but low field response, interacts synergistically with veratrole which had a low EAG response, but a high field attraction of beetles (unpublished data). A more detailed EAG analysis of all three *Diabrotica* species with single compounds and mixtures is in progress to determine whether species differences in field attraction to volatiles can be related to different characteristics of the EAG response.

Western corn rootworm (WCR) adults were significantly attracted to the phenylpropanoid estragole in three nonadjacent corn plots from early August to the end of the trapping period in late September 1985. In the main experimental plot WCR trap catches with 100 mg of estragole per trap were ca. 20 times greater than that of blank controls, and the minimum amount of estragole resulting in a significant trap catch was 5 mg/trap. Furthermore, estragole attracted more females than males, comparable to the results recorded by Ladd et al. (1985) for eugenol and NCR adults. The paramethoxy ring substituent and the position of the double bond in the propanoid side chain are critical for maximum WCR response; compounds differing in either aspect were less attractive. For example, estragole caught an average of 3.6 times more beetles than its structural analog *trans*-anethole. Despite the specificity of estragole for

WCR attraction, another shikimic acid derivitive, indole, was active early and late in the season. Andersen and Metcalf (1986) had previously noted that indole showed a decrease in activity during corn tasseling and silking, which may reflect multiple sensory cues from the corn plants outcompeting the attraction to single-component traps or it may indicate a seasonal change in beetle response to volatiles. A greatly enhanced response to volatiles, comparable to that of the SCR adults under the late season conditions, was not recorded for WCR adults.

Northern corn rootworm (NCR) adults were relatively rare in this test; control trap catches never exceeded one to two beetles per trap, and the mean number of NCR per plant was typically less than 0.1 adult throughout the season in all plots. Despite the low bettle population, eugenol and isoeugenol were significant attractants and 10 mg of eugenol per trap caught a significant number of beetles. None of the other test compounds, such as estragole, *trans*-anethole, chavicol, and 4-methoxy-1-propylbenzene, attracted NCR adults. Ladd (1984) found NCR response is optimal for phenylpropanoids with a metamethoxy and parahydroxy substituent. In our study conducted in mid-August using 100 mg/trap, more NCR adults were attracted to eugenol, an allylbenzenoid, then isoeugenol, a propenylbenzenoid, analogous to the greater attraction of estragole to WCR adults than *trans*-anethole. Ladd (1984), using 1500 mg/trap, found NCR attraction to isoeugenol was 1.6 times greater than eugenol in early August, roughly equivalent in mid-August, and the results reversed in late August. NCR adults apparently display a seasonal change in response to attractants similar to that previously discussed for SCR and WCR adults in this study. Whether this seasonal variability in beetle response to volatiles is due to changes in the environment, host plant, and/or beetle biology is undetermined.

A comparison between the rootworm species indicates the ecologically similar NCR and WCR adults are attracted to structurally related phenylpropanoids and both species respond to changes in the ring substituents of the major attractants. However, WCR and NCR adults are not attracted to the same phenylpropanoids, although SCR adults are occasionally attracted to indole, a WCR attractant, and eugenol and isoeugenol, NCR attractants. An increase in SCR trap catch has also been recorded for either eugenol or veratrole added to insecticide-impregnated cucurbitacin baits (unpublished data). Apparently, SCR adults cross-respond (especially late in the season) to some WCR and NCR attractants, although the main attractants for each species is highly specific.

Perhaps one of the most intriguing aspects about the newly described *Diabrotica* semiochemicals is the establishment of an allelochemical basis for their activity. Indole has been isolated from natural aggregation sites, cucurbit blossoms, as well as corn whorls (Andersen and Metcalf, 1986; Thompson et al., 1974). The major SCR attractants, veratrole and phenylacetaldehyde, are present in corn husk and silk volatiles (Cantelo and Jacobson, 1979b; Buttery et al., 1978), and phenylacetaldehyde and several di- and trimethoxybenzenes have

been detected in *Cucurbita maxima* blossoms (Andersen, 1984). The presence of veratrole, phenylacetaldehyde, and indole in larval or adult host plants suggests they act as kairomonal attractants. Other NCR, WCR, and SCR attractants (such as eugenol, isoeugenol, estragole, and *trans*-anethole) have not been described from the essential oils of the primary larval host, *Zea mays*, or cucurbits (Thompson et al., 1974; Flath et al., 1978; Buttery et al., 1980; Buttery and Ling, 1984; Andersen, 1984). However, vinyl guiacol, which is structurally similar to eugenol and isoeugenol, has been isolated from corn husks (Buttery et al., 1978).

Ladd et al. (1983) originally described eugenol as a "food-type" attractant present in a number of alternative NCR adult hosts. SCR and NCR adults are often found on composites, grasses, legumes, and cucurbits not adjacent to corn, especially late in the season, whereas WCR adults are considered to have a closer association with corn and weeds in the immediate area (Cinerski and Chang, 1968; Ludwig and Hill, 1975; Branson and Krysan, 1981; Rhodes et al., 1980; Messina and Root, 1980; Sholes, 1984). In Illinois, NCR, SCR, and *D. cristata* are often found feeding on the pollen in flower heads of Canada thistle (*Carduus arversis*), white heath aster (*Aster pilosis*), goldenrod (*Solidago* spp.), and sunflowers (*Helianthus* spp.) in late August and September (personal observation). Since eugenol and phenylacetaldehyde are volatile constitutents of sunflowers (Etievant et al., 1984), these phenylpropanoids may be the cues SCR and NCR adults utilize to locate pollen sources. This would suggest that eugenol and/or phenylacetaldehyde may also attract *D. cristata*, as it is occasionally found on the same plant species. It also suggests the other pollen hosts previously mentioned may release volatile compounds that attract Diabroticites. Phenylacetaldehyde, which attracts numerous Lepidoptera, Diptera, Coleoptera, and Hymenoptera and which is widely distributed among the plant families, may act as a general insect attractant to numerous pollen or nectar sources (Cantelo and Jacobson, 1979a,b, references therein, and unpublished data). The major WCR attractant, estragole, has not been detected in cucurbits, corn, or any of the aforementioned wildflowers; therefore, a host-plant relationship remains to be established.

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SEX PHEROMONE OF ORIENTAL ARMYWORM *Mythimna separata* WALKER

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Abstract—In the mainland of China, the male Oriental armyworm was not attracted to the sex pheromone components (Z)-11-hexadecenyl acetate and (Z)-11-hexadecenol identified by Takahashi et al. in 1979. By means of EAG, GC, and GC-MS techniques, (Z)-11-hexadecenal, hexadecenal, and (Z)-11-hexadecenol were found in female gland washings, and encouraging captures were obtained in preliminary field trapping.

Key Words—Sex pheromone, *Mythimna separata,* Lepidoptera, Noctuidae, 11-hexadecenal, hexadecenal, 11-hexadecenol.

INTRODUCTION

The sex pheromone of the female Oriental armyworm (*Mythimna separata* Walker) was identified as a blend of (Z)-11-hexadecenyl acetate (Z11–16:Ac) and (Z)-11-hexadecenol (Z11–16:OH) with a ratio of 8:1 by Takahashi et al. (1979). The activity of this pheromone composition had been reexamined in the island of Taiwan (Lin et al., 1983), but few males were caught on the mainland of China with pheromone component ratios ranging from 9:1 to 1:9. Field screening tests using compounds of "one structure change" (Steck et al., 1982) from Z11-16:Ac indicated that (Z)-11-hexadecenal (Z11–16:Ald) is an attractant. Occurrence of pheromone polymorphism or different species was assumed, and the chemical constituents of the sex pheromone of the Oriental armyworm in mainland China was investigated.

METHODS AND MATERIALS

Extraction of Pheromone. Laboratory-reared and emerged females were fed on 10% honey solution for seven days, and then the abdominal tips were

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excised at 2 or 3 AM. The glands were soaked twice in methylene chloride, 20 min for the first portion and 4 hr for the second. The extracts were combined for structure identification.

TLC Separation. The extract of 50 FE was concentrated and dropped onto silica gel plates of 0.2 mm thick. After developing with *n*-hexane–ethyl ether (4:1), the adsorbate was cut into seven fractions, followed by washing with ethyl ether. Twenty FE of the washing concentrates were used for EAG assay, and two active fractions were selected for structure identification.

Structure Identification. Electroantennograms were obtained by Ag–AgCl electrodes with antennae plucked from laboratory-reared males three days after emerging. Preliminary GC analysis was conducted on a Shimadzu GC-RIA chromatograph with polar or nonpolar glass columns and a hydrogen flame detector. GC-MS analysis was conducted on a Finnigan-MAT 4510 quadrupole mass spectrometer with EI source or methane CI source at 0.86 torr. Ozonolysis procedures described by Beroza and Bierl (1967) were used to locate double bonds. Geometrical configuration of active component was examined on a GC capillary column by comparison with synthetic isomers. Chromatographic conditions are shown in Table 1.

Field Trapping. Preliminary field trappings were carried out in Jintan (first flight, March 29–April 23, 1984) and Beijing (second flight, June 2–15, 1984). Water basin traps were used, and chemicals synthesized at the institute were applied to rubber septa as dispensers with a dosage of 100 μg, which were not renewed throughout the test period. Three replicates were arranged for each treatment.

RESULTS AND DISCUSSION

Fractions of R_f 0.52–0.71 corresponding to long-chain aldehydes with an EAG response of 1.8 mV, and of R_f 0.10–0.23 corresponding to long-chain alcohols with an EAG response of 0.7 mV were selected by TLC separation.

Identification of Aldehyde Fraction. The retention time of the aldehyde fraction was coincident with that of Z11–16:Ald on columns A, B, and C (see Table 1) in a preliminary GC test. In GC-MS with EI source, a major peak (peak 1) of 16 min 16 sec and a less remarkable one (peak 2) of 17 min 4 sec were eluted. The fragment ions and their abundances in the mass spectrum of peak 1 were identical with those of synthetic Z11–16:Ald, but the molecular ion *m/e* 238 and the characteristic ion 220 were not detected. For further confirmation, a concentrated sample with a larger amount was introduced, and methane CI source at 0.86 torr was used. The ions *m/e* 239 (MH^+), 238 (M^+), 237 (M−1), 221 (MH−18), and 267 (M+29), which occurred in the mass spectrum of peak 1 indicated that a straight-chain hexadecenal is the active component. The ions *m/e* 241 (MH^+), 239 (M−1), 223 (MH−18), and 269

TABLE 1. CHROMATOGRAPHIC CONDITIONS

Sample	Column	Temperature	Carrier gas
Preliminary test A	2.3 m × 2 mm, 2% CHDMS, 101 White support	150° 3 min, 2°/min to 170°	26 ml/min N_2
B	2.6 m × 3 mm, 1.5% OV-255, Chromosorb W	200°	50 ml/min N_2
C	1.6 m × 3 mm, 10% PEG-20M, Chromosorb W	160°	60 ml/min N_2
GC-MS	2.8 m × 2 mm, 3% OV-101, Chromosorb W	180°	20 ml/min He
Ozonolysis product	2.8 m × 2 mm, 3% OV-101, Chromosorb W	80° 5 min, 8°/min to 180° holding for 30 min	15 ml/min N_2
Capillary GC	35 m × 0.4 mm DEGS	164°	0.7 kg/cm^2 N_2

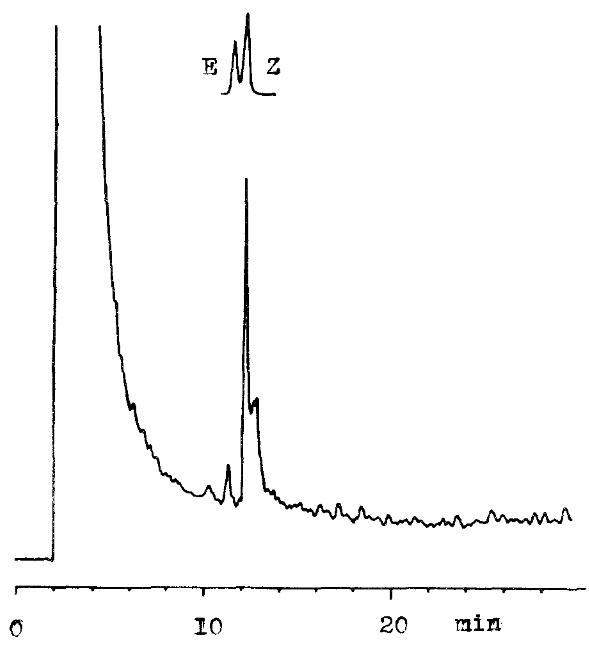

FIG. 1. Capillary chromatogram of aldehyde fraction

TABLE 2. FIELD TRAPPING OF FIRST FLIGHT ARMYWORM[a]

Compound	Ratio	Total captures per trap
Z11-16:Ald		471
Z11-16:Ald + 16:Ald	3:1	392
	10:1	284
Z11-16:Ald + Z11-16:OH	3:1	118
	10:1	149

[a] March 29–April 23, 1984, Jintan; 100 μg/lure.

(M+29) of peak 2 indicated that saturated hexadecanal (16:Ald) may occur in the gland extract. The ozonolysis product was eluted at 19 min 33 sec and ions *m/e* 185 (MH^+), 183 (M−1), 167 (MH−18), 149 (MH−36), and 213 (M+29) occurred in the mass spectrum. They are identical with those of 1,11-undecadial, thus supporting the assumption that 11-hexadecenal is the major component of the pheromone. The capillary gas chromatogram of the aldehyde fraction, in comparison with those of synthetic *Z*- and *E*11–16:Ald, is shown in Figure 1. Synthetic *Z*- and *E*11–16:Ald were eluted at 12 min 12 sec and 11 min 40 sec respectively, while a peak at 12 min 10 sec was displayed by the aldehyde fraction, and no signal occurred at 11 min 40 sec. Z11–16:Ald was then identified as major component of the sex pheromone of the Oriental armyworm.

Identification of Alcohol Component. In GC-MS analysis, the alcohol fraction displayed a peak at 21 min 32 sec and characteristic ions *m/e* 241 (MH^+), 239 (M−1), 269 (M+29), and 223 (MH−18). These results indicated that hexadecenol is the active component. Owing to its extremely small amount, we could not get more information about the location and geometrical configuration

TABLE 3. FIELD TRAPPING OF SECOND FLIGHT ARMYWORM[a]

Compound	Ratio	No. captures per trap: Total	No. captures per trap: Daily max.
Z11-16:Ald		76	20
Z11-16:Ald	100:10:1	59	15
+ 16:Ald	100:10:0.1	108	21
+ Z11-16:OH	100:10:0.5	102	14
Z11-16:Ald	100:10	42	11
+ 16:Ald	100:1	47	8

[a] June 3–15, 1984, Beijing, 100 μg/lure.

of the double bond other than EAG tests of known isomers of Δ7 to Δ11 hexadecenols. An EAG response of 1.6 mV was obtained from Z11-16:OH, while no response greater than 0.8 mV was obtained from the other isomers.

Field Trapping. The captures by various component ratios are shown in Tables 2 and 3. Although the data are rather insufficient and scattered, we believe that Z11-16:Ald + 16:Ald + Z11-16:OH (100:10:0.1) is the preferred blend.

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SEX PHEROMONE COMPONENTS OF PURPLE STEM BORER *Sesamia inferens* (WALKER)

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Abstract—The pheromone of the female purple stem borer, *Z*-11-hexadecenyl acetate, identified by Nesbitt, was not active in field trapping in China. From EAG, TLC, GC, and GC-MS tests, we found that *Z*-11-hexadecenyl acetate, *Z*-11-hexadecenol, and *Z*-11-hexadecenal were the pheromone components, and the preferred blend ratio in field tests was 4 : 1 : 0.1. The number of captures of the later generations was substantially lower than that of the overwintered generation.

Key Words—*Sesamia inferens*, Lepidoptera, Noctuidae, purple stem borer, sex pheromone, *Z*-11-hexadecenyl acetate, *Z*-11-hexadecenol, *Z*-11-hexadecenal.

INTRODUCTION

Recently, various crops, particularly in the paddies of southern China, were heavily damaged by the purple stem borer *Sesamia inferens* (Walker) (Li and Wen, 1983). The pest insect develops three or four generations, occasionally five generations a year, and the generations overlap. The sex pheromone of the female purple stem borer was identified by Nesbitt et al. in 1976 as *Z*-11-hexadecenyl acetate (Z11–16 : Ac); however, few males were lured with this compound in field trapping in China (1977). Several enols, acetates, and their mixtures were tested as lures in field trapping, and Z11–16 : Ac blended with *Z*-11-hexadecenol (Z11–16 : OH) elicited a high activity to the overwintered generation (Jiangsu Institute, 1981). Lately, Takahashi et al (1983) reported that male *S. inferens* had been captured in their traps baited with 9 : 1 or 4 : 1 mixtures of Z11–16 : Ac and Z11–16 : OH for the armyworm. Z11–16 : Ac and Z11–16 : OH were identified as the main components of *S. inferens*, and a trace amount of *Z*-11-hexadecenal (Z11–16 : Ald) was also detected.

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METHODS AND MATERIALS

Extract from Female Glands. Full-grown larvae were collected from the field. After pupation and sexual separation, the females were put into screen cages. Up to 90% of the moths raised their abdominal tips for calling during the period from midnight to 1:00 AM of the third day after emerging, and this is the time to excise. The excised glands were soaked twice in *n*-heptane, 1 hr for the first portion and 7 hr for the second. The extracts were combined, sealed, and stored in a refrigerator for structure identification.

TLC Separation. Sixty FE of the above extract were concentrated and dropped onto a silica gel plate 0.2 mm thick. After developing with a 4:1 mixture of *n*-hexane and ethyl ether, the plate was cut into five fractions and washed with treated acetone.

Instrumentation. Electroantennograms were obtained by Ag-AgCl electrodes with antennae cut from males of the same field source as described above during the 24–48 hr after emergence. Preliminary GC analysis was conducted on a Shimadzu GC-RIA with a hydrogen flame detector. GC-MS was done with a Finnigan-MAT 4510 quadruple mass spectrometer using an EI source of 0.25 mA and 70 eV. The chromatographic conditions are shown in Table 1.

Ozonolysis and Acetylation. Ozonolysis was done as described by Beroza and Bierl (1967) and acetylation by the procedure of Nesbitt et al. (1980).

Preliminary Field Trapping. Preliminary field trapping was carried out at monitoring stations of several counties in Jiangsu Province from 1981 to 1985 under the direction of the Cooperative Group for Application of the Sex At-

TABLE 1. CHROMATOGRAPHIC CONDITIONS

Sample	Column	Temperature	Carrier gas
Preliminary test	1.6 m × 3 mm, 10% PEG-20M, Chromosorb W	170°	50 ml/min, N_2
Carbon skeleton			
Acetate	2 m × 2 mm, 10% SE-30, Chromosorb W	[a]	35 ml/min, He
Alcohol	2 m × 2 mm, 10% SE-30, Chromosorb W	190°	35 ml/min, He
Aldehyde	2 m × 2 mm, 4% FFAP, Chromosorb AW-DHCS	180°	10 ml/min, He
Configuration			
Acetate	35 m × 0.4 mm, DEGS	168°	0.7 kg/cm^2, N_2
Alcohol	35 m × 0.4 mm, DEGS	168°	0.7 kg/cm^2, N_2
Aldehyde	25 m × 0.2 mm, PEG-20M	145°	1 ml/min, He

[a] 220° for the TLC separated fraction; 80° 5 min, 5°/min to 220°, holding 50 min for ozonolysis product.

TABLE 2. TOTAL NUMBER OF CAPTURES PER TRAP THROUGH A GENERATION IN 1984 AT VARIOUS LOCALITIES[a]

	Generation								
	Overwintered			Second			Third		
Lure	A	B	C	A	B	C	A	B	C
Wuxian	827	1627	1635	787	510	767	24	32	57
Wujin	836	826	1467	414	249	431	29	22	68
Yangzhong	789	814	1359	93	81	120	12	24	21
Jiangning	632	1129	848	49	57	38	84	39	48
Danyang	686	660	938	23	29	44	26	7	32
Average	754b	1011ab	1249a						

[a]Dosage: 150 μg. Blending ratio: A, Z11-16:Ac, Z11-16:OH, 4:1; B, Z11-16:Ac, Z11-16:OH, 8:1, C, Z11-16:Ac, Z11-16:OH, Z11-16:Ald, 4:1:0.1. Average captures followed by the same letter are not statistically different at the 5% level.

tractant of Purple Stem Borer. Water basin traps were placed 30 m apart in a grid system and 1 m above wheat stems. Pheromone compounds were synthesized in this institute and dispersed from rubber septa at a dosage of 150 μg/lure and blending ratios as shown in Table 2. The lures were used throughout a generation without renewing them.

RESULTS AND DISCUSSIONS

Preliminary Tests. Two active fractions were elicited by TLC separation. The more active fraction evoked an EAG response of 1.8 mV and occurred at R_f 0.54–0.75, which corresponds to long-chain acetates and aldehydes, while the less active one evoked 1.0 mV at R_f 0.15–0.35, corresponding to long-chain alcohols.

The chromatogram of the preliminary GC analysis in comparison with synthetic chemicals is shown in Figure 1. The retention time, 11.35 min of peak 3, was close to that of Z11-16:Ac; peak 5 (13.38 min) was close to that of Z11-16:OH; peak 4 (12.28 min) was close to that of 16:OH, and peak 2 (9.26 min) might be hexadecenal.

Identification of Acetate Fraction. The acetate fraction from TLC separation was eluted at 23 min 52 sec in GC-MS analysis, and the major fragment ions *m/e* 222 ($M^+ - CH_3COOH$), 194 ($C_{14}H_{26}^+$), 166 ($C_{12}H_{22}^+$), 152 ($C_{11}H_{20}^+$), 138 ($C_{10}H_{18}^+$), 124 ($C_9H_{16}^+$), 110 ($C_8H_{14}^+$), 96 ($C_7H_{12}^+$), 82 ($C_6H_{10}^+$), 61, and 43 were identical with the characteristic fragment ions of synthetic Z11-16:Ac reported by Ando et al. (1980). The ozonolysis product of the acetate was eluted

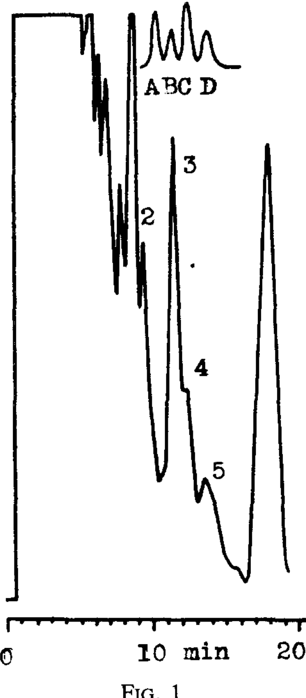

FIG. 1.

at 35 min 52 sec; the major fragment ion *m/e* 185 (M^+-43), 150 (M^+-78), 140 (M^+-88), 125 (M^+-103), 111 ($C_8H_{15}^+$), 97 ($C_7H_{13}^+$), 83 ($C_6H_{11}^+$), 61, and 43, as well as their relative abundances were in agreement with those of synthetic 11-acetoxyundecanal (Bierl-Leonhardt et al, 1980). Thus, the double bond location of 11 was further confirmed. The retention time of the acetate on the capillary column was 15 min 54 sec, while those of the synthetic *Z*- and *E*11-16:Ac were 15 min 54 sec and 15 min 16 sec, respectively. Thus, the configuration of the pheromone acetate double bond was *Z*.

Identification of Alcohol Fraction. The alcohol fraction was acetylized and purified before GC-MS analysis. Three peaks were eluted at 26 min 13 sec, 63 min 27 sec and 72 min 49 sec, respectively. The characteristic fragment ions of peak 1, *m/e* 196 (M^+-CH_3COOH), 168 ($C_{12}H_{24}^+$), 154 ($C_{11}H_{22}^+$), 140 ($C_{10}H_{20}^+$), 125 ($C_9H_{17}^+$), 111 ($C_8H_{15}^+$), 97 ($C_7H_{13}^+$), 83 ($C_6H_{11}^+$), 61, and 43 were in agreement with those of *n*-tetradecyl acetate; those of peak 2, *m/e* 224 (M^+-CH_3COOH), 154 ($C_{11}H_{22}^+$), 61, and 43 were in agreement with those of *n*-hexadecyle acetate; and those of peak 3, *m/e* 222 (M^+-CH_3COOH), 194 ($C_{14}H_{26}^+$), 180 ($C_{13}H_{24}^+$), 166 ($C_{12}H_{22}^+$), 152 ($C_{11}H_{20}^+$), 138 ($C_{10}H_{18}^+$), 124 ($C_9H_{16}^+$), 110 ($C_8H_{14}^+$), 96 ($C_7H_{12}^+$), 82 ($C_6H_{10}^+$), 61, and 43 were in agreement with those of *n*-hexadecenyl acetate.

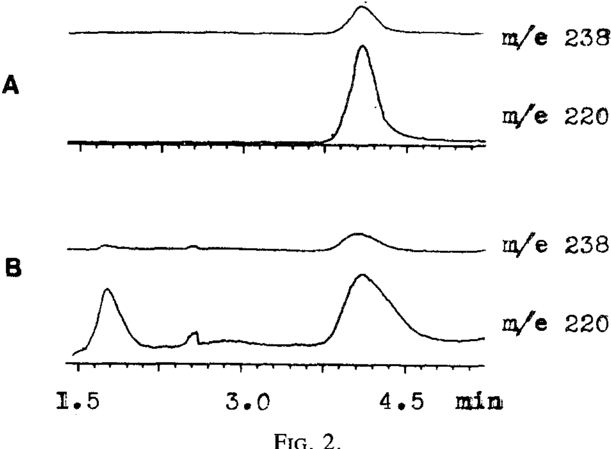

FIG. 2.

Three peaks were eluted in GC-MS of the ozonolysis product of acetylized alcohol, two of which were 14:Ac and 16:Ac. The third peak at 74 min 55 sec showed characteristic fragment ions *m/e* 185 (M^+-43), 150 (M^+-78), 140 (M^+-88), 125 (M^+-103), 111 ($C_8H_{15}^+$), 97 ($C_7H_{13}^+$), 83 ($C_6H_{11}^+$), 61, and 43, which were in agreement with those of 11-acetoxy-undecanal. This confirms the C-11 location of the double bond of pheromone hexadecenol. The retention times of synthetic *E*- and *Z*11-16:OH and the pheromone alcohol on the capillary column were 19 min 29 sec, 20 min 20 sec, and 20 min 20 sec, respectively. Thus, *Z*11-16:OH, 16:OH, and 14:OH may occur in the alcohol fraction from TLC separation of the purple stem borer abdominal tip extract.

Identification of Aldehyde Component. Owing to the trace content of aldehyde occurring in the pheromone, no peak could be detected in the total ion chromatogram. A multiple ion detection technique was adopted, and the molecular ion *m/e* 238 and the characteristic ion 220 (M^+-18) of hexadecenal were chosen as detecting ions. The chromatograms of the acetate-aldehyde fraction from TLC separation and of the synthetic hexadecenal are compared in Figure 2. Occurrence of hexadecenal in the pheromone may be demonstrated by the coincidence between the peaks. Capillary column MID chromatograms of the pheromone fraction and of the synthetic hexadecenal isomers are shown in Figure 3; the *Z* configuration of pheromone aldehyde may thus be presumed.

CONCLUSIONS

Although the field trapping was preliminary, and no statistical data are available, a preferred blend ratio of 4:1:0.1 of *Z*11-16:Ac + *Z*11-16:OH + *Z*11-16:Ald may be assumed. No evident synergic or inhibitory effect was

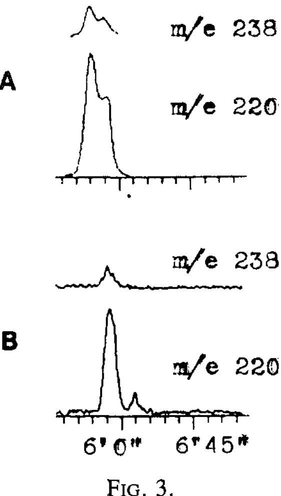

FIG. 3.

shown in field trapping by adding 16 : OH or 14 : OH to this blend. The captures during later generations were lowered substantially as shown in Table 2, and this phenomenon needs further study.

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IDENTIFICATION OF SEX PHEROMONE COMPONENTS FROM PHEROMONE GLAND VOLATILES OF THE TOMATO LOOPER, *Plusia chalcites* (Esp.)[1,2]

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Abstract—The sex pheromone glands of *Plusia chalcites* release, dodecyl acetate, (Z)-7-dodecenyl acetate, (Z)-9-dodecenyl acetate, 11-dodecenyl acetate, (Z)-8-tridecenyl acetate, and (Z)-9-tetradecenyl acetate. A combination of capillary GC, GC-MS, and dimethyl disulfide derivatization enabled a rigorous identification of all these compounds, some of which were previously found in gland extracts. Bioassays in a flight tunnel showed that a ternary blend of (Z)-7-dodecenyl acetate, (Z)-9-dodecenyl acetate, and (Z)-9-tetradecenyl acetate elicited directed flights from 85 to 100% of the males tested and elicited copulation attempts, at the end of the flights, from 44 to 74% of the males tested. This blend was equal in activity to the natural gland extract. Addition of the other acetates had only a slight influence on the activity of this mixture. Substitution of either (Z)-9-dodecenyl acetate or (Z)-9-tetradecenyl acetate in this blend by 11-dodecenyl acetate gave two ternary mixtures which also elicited high levels of courtship activity, almost as high as that of the original blend. Addition of (Z)-7-dodecenyl alcohol inhibited almost totally the flight activity of males.

Key Words—*Plusia chalcites*, Lepidoptera, Noctuidae, sex pheromone, identification, flight-tunnel bioassay, (Z)-7-dodecenyl acetate, (Z)-9-dodecenyl acetate, (Z)-9-tetradecenyl acetate.

[1]Lepidoptera: Noctuidae.
[2]Contribution from the Agricultural Research Organization (ARO) No. 1701-E, 1986 series.

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INTRODUCTION

A common sex pheromone component of several moths of the Plusiinae subfamily has been identified as (Z)-7-dodecenyl acetate (Z7-12:Ac) (Berger, 1966; Tumlinson et al., 1972a; Kaae et al., 1973a). At that time, it seemed as if different species of Plusiinae utilize the same compound for their sexual communication. Different concentrations of Z7-12:Ac and temporal factors (Kaae et al., 1973a, b), were proposed as means for reproductive isolation among species utilizing the same pheromone.

With the advance of sensitive analytical instrumentation, in particular, efficient capillary GC columns, additional compounds to Z7-12:Ac were identified in the sex pheromone blends of various species of Plusiinae. At the present time, partial or complete blends are known for *Plusia chalcites* (Dunkelblum et al., 1981), *Chrysodeixis eriosoma* (Benn et al., 1982), *Autographa gamma* (Dunkelblum and Gothilf, 1983; Toth et al., 1983), and for *Trichoplusia ni*, which has been studied thoroughly for many years (Berger, 1966; Bjostad et al., 1980, 1984). Extensive use of the wind tunnel, as a reliable tool for bioassays, was of crucial significance in evaluating the biological importance of various components (Linn et al., 1984).

Two components of the sex pheromone of the tomato looper, *Plusia chalcites*, were previously identified as Z7-12:Ac and (Z)-9-tetradecenyl acetate (Z9-14:Ac). These compounds were found in gland extracts, and their activity was assessed in field tests (Dunkelblum et al., 1981). Several additional potential pheromone components were identified later (Dunkelblum et al., 1985).

The present study reports the chemical identification of sex pheromone components collected as gland volatiles and gland extracts of *P. chalcites*. Tests in a wind tunnel were conducted for the behavioral evaluation of these compounds.

METHODS AND MATERIALS

Insects and Extracts. The insects were reared on an artificial medium (Shorey and Hale, 1965), and females were separated from males as pupae. Adults were kept in separate rooms on a light cycle of 14:10 hr light-dark. Pheromone glands were dissected from the ovipositors of 4- to 5-day-old females during their calling period, i.e., 1-3 hr into scotophase (Snir et al., 1986) and immersed in hexane. After 15 min, the supernatant solution was transferred into small conical vials sealed with a Teflon-lined screw cap and concentrated to the desired volume by letting the samples stand in a fume hood.

Gland volatiles were obtained from ligated glands in a modified four-port, glass-wool collector similar to that described by Baker et al. (1981) and Gaston (1984). The trapped volatiles, collected for approximately 20 min, were rinsed

from the glass wool with 500 μl hexane, and samples from 10–20 glands were pooled in a conical vial sealed with a Teflon-lined screw cap and concentrated as described for gland extracts.

Chemical Identification. Capillary GC was conducted on a Varian 3700 model equipped with a FID detector and a splitless injector system. A 30 m × 0.25 mm bonded cyanosilicone column (DB 225, J&W) was kept at 60°C for 2 min and then was programmed from 60° to 130°C at 10°/min for the routine analyses of the C_{12}–C_{14} compounds.

For the analysis of only the C_{12} complex a program of 60°C to 120°C at 10°/min and for the complete range (C_{12}–C_{18}) a program of 60° to 180°C at 5°/min was used. For all programs, the purge valve was opened 1 min after injection, and the helium pressure was 15 psi. Quantification of peak areas was performed by a Varian 4270 electronic integrator.

Capillary GC-MS analysis was conducted on a Finnigan 5100 EI system, at 70 eV, coupled with a Superincos data system. A 30-m × 0.25-mm SE 54 fused silica column (J&W) was programmed from 60° to 250°C at 15°/min, after a 3-min delay, for pheromone components and from 60° to 250°C at 20°/min, after a 2-min delay, for dimethyl disulfide adducts. The column was operated in the splitless mode, with a purge 30 sec after injection and helium pressure of 10 psi.

Dimethyl disulfide (DMDS) adducts were prepared from several standards (Z7–12:Ac, Z9–12:Ac, 11–12:Ac, and Z7–12:OH), gland extracts, and gland volatiles. Samples in hexane (20–50 μl) were treated with DMDS (70–100 μl) and one drop of iodine solution (60 mg iodine in 1 ml diethyl ether). Reaction mixtures were kept at 40°C for 20 hr, cooled, and worked up as described by Buser et al. (1983) and Dunkelblum et al. (1985).

Behavioral Tests. The design of the wind tunnel has been described recently (Snir et al., 1986). Males 6- to 8-day-old, 1–4 hr into scotophase, were used for flight tests. They were kept in cages (30 × 30 × 30 cm) in the tunnel room. Individual moths were transferred carefully into small cages (7 × 7 × 7 cm) and placed on a tripod in the center of the tunnel, 20 cm from the downwind side. After 2–4 min when the males had settled down, the cage was opened and baits were introduced into the tunnel. The tests were performed at a light intensity below 0.5 lux, with a wind speed of 50 cm/sec, at 26° ± 3°C.

Baits were prepared by impregnating 1-cm^2 filter paper squares (Whatman No. 1) with 20 μl of a hexane solution containing the synthetic compounds. They were kept in Teflon-lined 20-ml screw cap vials on pins and fixed on the inside of the caps above an empty rubber septum under the Teflon lining. The baits were placed on a mesh tray, hanging from the roof of the tunnel, 20 cm from the upwind side with the vial cap down and the paper square up. Each bait was used for only one to three tests, within 60 min after preparation. All compounds used in the behavioral tests were from our collection and had an isomeric purity of 98–99% (verified by capillary GC).

RESULTS

Chemical Identification. Analysis of gland extracts by GC indicated the presence of several compounds in small amounts in addition to the major components Z7–12:Ac, Z9–14:Ac, and hexadecyl acetate (16:Ac) which were previously identified (Dunkelblum et al., 1981). The GC profile of the gland volatiles was similar to that of the gland extracts in the C_{12}–C_{14} range only;

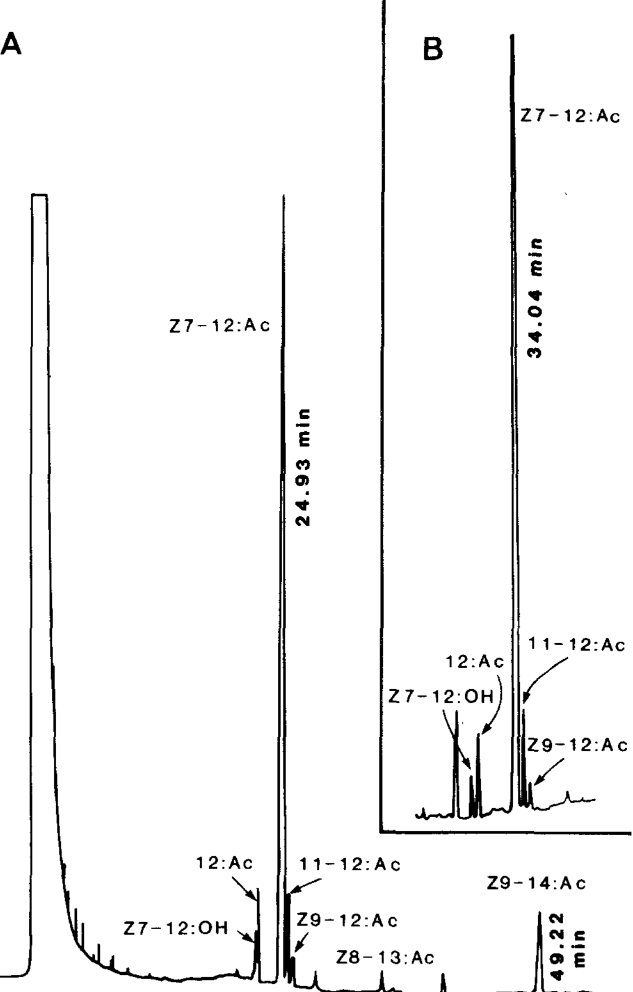

FIG. 1. Capillary GC traces of extracts of *Plusia chalcites*: (A) gland volatile, column programmed from 60° to 130°C; (B) C_{12} complex in gland extract, column programmed from 60° to 120°C. Representative retention times are indicated on some peaks.

higher acetates were almost nonexistent. Analysis at 60° to 130°C gave a satisfactory, but lengthy, separation of all C_{12}, C_{13}, and C_{14} compounds, and the analysis at 60° to 120°C gave an excellent separation of the C_{12} complex (Figure 1). The DB 225 column was able to resolve efficiently all *E/Z* pairs and most positional isomers, including the separation of (Z)-9-dodecenyl acetate (Z9–12:Ac) and 11-dodecenyl acetate (11–12:Ac), which were not resolved before on various capillary columns (Bjostad et al., 1984; Dunkelblum et al., 1981, 1985). A comparison of the relative amounts of the identified compounds in gland extracts and volatiles is presented in Table 1.

The GC-MS analysis (SE 54 column) confirmed the presence of most components in the gland volatiles, although difficulties were encountered with the separation of the C_{12} acetates. Derivatization of both the gland extracts and gland volatiles with DMDS and subsequent GC-MS analysis confirmed the presence and established the position of the double bond in all unsaturated compounds which were detected on the DB 225 column. The separation of the DMDS adducts was much better than that of the parent compounds. Although the amount of some of the minor compounds was extremely low and the peaks of their DMDS adducts on the reconstructed GC profile were barely visible, monitoring of the intensive A^+ or B^+ fragments enabled a reliable identification. The separation of the six DMDS adducts of (Z)-7-dodecenol (Z7–12:OH) and the unsaturated acetates, in the gland volatiles, is presented in Figure 2.

TABLE 1. RELATIVE PROPORTIONS OF COMPOUNDS IDENTIFIED FROM SEX PHEROMONE GLAND EXTRACTS AND GLAND VOLATILES OF *Plusia chalcites*[a]

	Gland extracts[b]		Gland volatiles	
Compound	$\bar{X}$	SE	$\bar{X}$	SE
Z7–12:OH	1.1	0.31	1.4	0.05
12:Ac	3.0	0.21	3.7	0.32
Z7–12:Ac	100.0		100.0	
Z9–12:Ac	1.3	0.05	1.6	0.13
11–12:Ac	3.6	0.27	3.3	0.20
Z8–13:Ac	1.4	0.19	1.2	0.13
Z9–14:Ac	15.8	1.03	7.3	0.66
Z11–14:Ac	0.2[c]	0.08		
16:Ac	23.2	2.01	0.7	0.09
Z9–16:Ac	1.2	0.31		
Z11–16:Ac	1.0	0.08		

[a]Relative proportions were calculated with respect to the main component Z7–12:Ac; each proportion is the mean value $\bar{X} \pm$ SE from four samples, each of 10–20 glands.
[b]The gland extracts contained also small amounts of higher acetates and trace amounts of 14:Ac.
[c]Detected only in three samples; its presence was confirmed by DMDS derivatization.

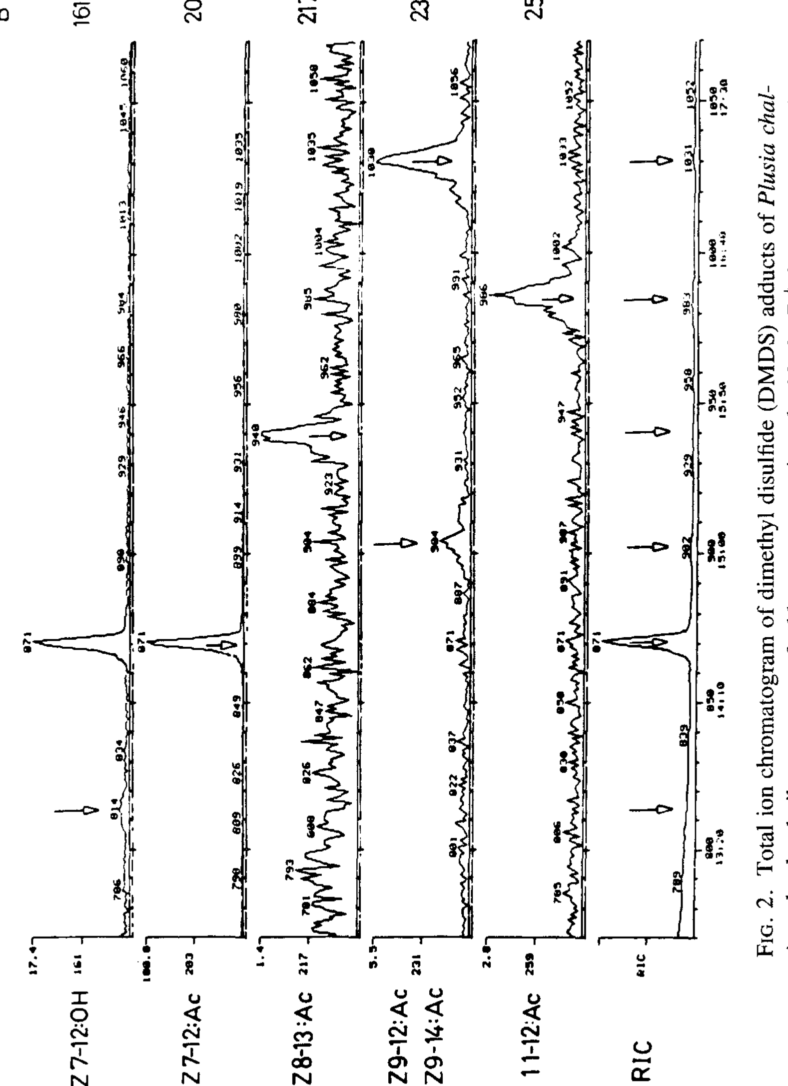

FIG. 2. Total ion chromatogram of dimethyl disulfide (DMDS) adducts of *Plusia chalcites* gland volatile; presence of adducts was monitored with the B^+ fragment (Table 2).

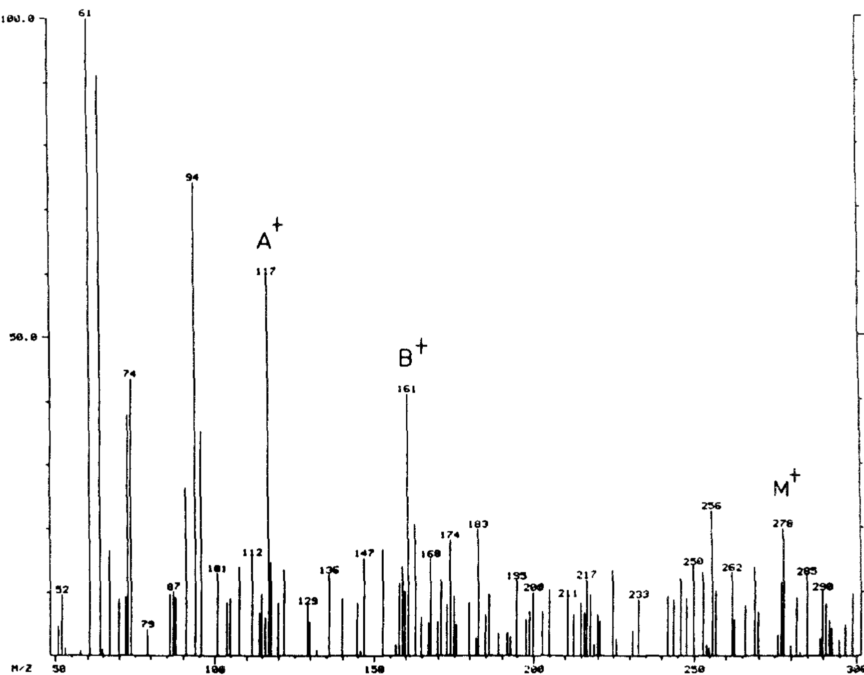

FIG. 3. Mass spectrum of dimethyl disulfide (DMDS) adduct of *Z*7–12:OH from the analysis presented in Figure 2.

Good mass spectra were obtained for all adducts including *Z*7–12:OH · DMDS, which was present in trace amounts only (Figure 3). The mass spectrometric data from all DMDS derivatives are summarized in Table 2.

Behavioral Tests. From field experiments (Dunkelblum et al., 1981; Dunkelblum et al., in preparation) and from preliminary wind tunnel work, we knew that the most active components in the extracts were *Z*7–12:Ac, *Z*9–12:Ac, and *Z*9–14:Ac. These compounds were tested individually in the wind tunnel, and it was found that only *Z*7–12:Ac initiated flights of males. Four concentrations were tested, and the results indicated that 200 ng/bait gave the highest flight response of the males, but with a few exceptions they did not reach the bait (Table 3). In view of these findings, we decided to test mixtures containing 200 ng of *Z*7–12:Ac and 20 ng each of 12:Ac, *Z*9–12:Ac, 11–12:Ac, and *Z*9–14:Ac. Complete gland extracts, calibrated by GC to contain 200 ng *Z*7–12:Ac, were also tested in order to compare the natural blend with the synthetic mixtures. Four behavioral stages were recorded—directed flight, landing, hairpencilling, and copulation. The copulation attempts were readily recognized by the bending of the abdomen; some males repeated this action several times consecutively.

TABLE 2. MASS SPECTROMETRIC DATA OF DMDS DERIVATIVES OF UNSATURATED COMPOUNDS FROM PHEROMONE GLANDS OF *Plusia chalcites*

$$\underbrace{CH_3(CH_2)_mCH(SCH_3)-}_{A^+} \vdots \underbrace{-CH(SCH_3)(CH_2)_nOR}_{B^+} \quad M^+ \quad R = H \text{ (alcohol)}, \; R = COCH_3 \text{ (acetate)}$$

DMDS adduct	Diagnostic ions[a]			
	M^+	A^+	B^+	B^+-60
Z7-12:OH	278(21)	117(60)	161(42)	
Z7-12:Ac	320(7)	117(66)	203(25)	143(30)
Z9-12:Ac	320(18)	89(38)	231(41)	171(18)
11-12:Ac	320(11)	61(100)	259(42)	199(38)
Z8-13:Ac	334(5)	117(83)	217(47)	157(29)
Z9-14:Ac	348(10)	117(75)	231(53)	171(25)
Z11-14:Ac	348(25)	89(50)	259(64)	199(30)
Z9-16:Ac	376(11)	145(100)	231(80)	171(21)
Z11-16:Ac	376(5)	117(68)	259(43)	199(15)

[a] Numbers in parentheses are relative intensities of ions. There are variations in these values, but fragments A^+ and B^+ are very intense in all DMDS adduct spectra.

TABLE 3. RESPONSES OF MALE *Plusia chalcites* IN WIND TUNNEL TO DIFFERENT CONCENTRATIONS OF Z7-12:Ac[a] ($N = 20$)[b]

Z7-12:Ac concentration (ng)	Taking flight	Directed flight	Source contact	Distance source (cm)[c]
2	8b	0c	0a	
20	15a	6b	3a	20
200	17a	13a	1a	40
2000	18a	8ab	1a	120

[a] Baits were used only once.
[b] Percentages followed by the same letter are not significantly different according to χ^2 test of independence ($P \leq 0.05$).
[c] The length of the flight track from the release cage to the source was 150 cm. The numbers represent the approximate distance from the source at which the males lost the plume and stopped the directed flight. This distance was measured with the help of the black and white stripes (10 cm wide) on the stationary floor of the tunnel.

TABLE 4. RESPONSE (%) OF MALE *Plusia chalcites* IN WIND TUNNEL TO MIXTURES OF COMPOUNDS IDENTIFIED FROM FEMALE SEX PHEROMONE GLAND VOLATILES[a]

Z7-12:Ac	Z9-14:Ac	Z9-12:Ac	11-12:Ac	12:Ac	Number tested	Directed flight	Source contact	Hairpencil display	Copulation attempt
x	x				39	95a	90a	21b	15b
x		x			15	93a	93a	40ab	20b
x	x	x			25	87a	84a	48a	44a
gland extract					32	84a	84a	50a	44a
x	x				23	91a	78a	44b	44b
x				x	12	92a	0b	0c	0c
x	x	x			19	100a	95a	79a	74a
x	x	x		x	36	97a	86a	72a	72a
x	x	x			26	96a	85a	62a	54a
x			x		21	100a	0b	0b	0b
x	x	x	x		34	94a	82a	50a	44a
x	x	x			49	100a	96a	88a	67ab
x	x		x		69	100a	94a	81a	52b
x		x	x		64	100a	97a	84a	56b
x	x	x	x	x	43	100a	98a	91a	77a

[a]Sources contained 200 ng of Z7-12:Ac and 20 ng of each of the other compounds. The gland extract was calibrated to contain 200 ng Z7-12:Ac. Percentages followed by the same letter are not significantly different according to χ^2 test of independence ($P < 0.05$).

The tests in the wind tunnel were conducted over several months, and there was considerable variation in male response during these experiments. To overcome this difficulty, the ternary mixture of Z7-12:Ac + Z9-12:Ac + Z9-14:Ac was included in each test as a behavioral internal standard. This ternary blend gave a high response: in one test about 70% of the males completed the courtship sequence. Addition of the other components to this mixture had only a moderate influence on its activity (Table 4). The alcohol Z7-12:OH was tested separately only at a high dosage of 200 ng in a mixture of Z7-12:Ac + Z9-14:Ac. Significant inhibition of the directed flight was observed; out of 20 males tested, only three reached the bait as compared with 18 in the absence of the alcohol.

DISCUSSION

The sex pheromone of *P. chalcites* was investigated by our group several years ago (Dunkelblum et al., 1981), and a number of saturated and unsaturated

acetates were identified in gland extracts. Field tests confirmed the essential role of at least two components: Z7–12 : Ac and Z9–14 : Ac (Dunkelblum et al. 1981, 1982). Recently, when studying the fatty acids in pheromone glands of four lepidopterous species, additional unsaturated acetates were discovered in *P. chalcites* (Dunkelblum et al., 1985). The presence of a relatively large number of saturated and unsaturated acetates in the gland extracts made it clear that females of *P. chalcites* utilize a multicomponent sex pheromone. Therefore, it was of importance to identify the compounds actually emitted by the female and to assess their role in wind-tunnel tests.

A combination of a very efficient capillary column, DMDS derivatization and mass spectrometry made it possible to identify the components of the gland volatiles to a level of 0.2% relative to the main component Z7–12 : Ac. The gland extracts contained additional components, in particular, large amounts of 16 : Ac (Table 1). It is plausible that some of the compounds, present only in the gland extracts, are biosynthetic intermediates or side products (Bjostad and Roelofs, 1983; Bjostad et al., 1984). The trace amounts of (Z)-11-tetradecenyl acetate (Z11–14 : Ac) could be an indication of the presence of an intermediate compound in the biosynthesis of Z9–12 : Ac (Bjostad et al., 1985).

The gland volatiles contained 12 : Ac, Z7–12 : Ac, Z9–12 : Ac, 11–12 : Ac, Z8–13 : Ac, Z9–14 : Ac, and Z7–12 : OH (Table 1). The very small amount of 16 : Ac present in the volatiles is probably a residue from the large pool of this compound in the interior of the pheromone gland (Table 1). For this reason 16 : Ac was excluded from the flight tests. The exceptional compound in this mixture is Z8–13 : Ac, which is very rare as a pheromone component. Recently it has been detected in the sex pheromone glands of *Chilo auricilius* (David et al., 1985), and it is also known to be a sex attractant for some species of the genus *Chrysochrista* (Ando et al., 1977). A few other Δ13 : Acs are known to be pheromone components in Lepidoptera (Inscoe, 1982). This compound (Z8–13 : Ac) might arise via a shortening by one carbon or a lengthening by one carbon of either a Z9–14 or Z7–12 precursor, respectively. Trapping experiments using Z8–13 : Ac, as a substitute for either Z7–12 : Ac or Z9–14 : Ac, in lures gave negative results. Traps baited with these lures did not catch *P. chalcites* males, indicating that this compound is inactive (Dunkelblum et al., in preparation).

It was also surprising that both the gland extracts and the gland volatiles consistently contained small amounts of Z7–12 : OH. This alcohol, at higher concentrations, is a potent inhibitor for the trapping of *P. chalcites* (Dunkelblum et al., 1982). This is also true for *T. ni* (Bjostad et al., 1984; Tumlinson et al., 1972b), in which it was found in small amounts in the gland volatiles (Bjostad et al., 1984). The alcohol was tested only briefly in the wind tunnel. Addition of 200 ng of Z7–12 : OH to a lure of Z7–12 : Ac + Z9–14 : Ac inhibited almost totally the flight activity of males. The possibility of Z7–12 : OH

being an artifact in pheromone glands of *T. ni* was raised by Bjostad et al. (1984); the same might be true for *P. chalcites*. A plausible source of the small amounts of Z7–12 : OH in both insects could be some hydrolysis of Z7–12 : Ac catalyzed by tissue from the female. Such a process was reported recently for the hydrolysis of (*E*)-11-tetradecenyl acetate to the corresponding alcohol (Silk et al., 1985).

The behavioral tests concentrated on the C_{12} and C_{14} acetates, in particular, Z7–12 : Ac, Z9–12 : Ac, and Z9–14 : Ac, which were used previously as trap baits in field experiments. Only Z7–12 : Ac elicited directed flights of males when used alone, although none of these males reached the source. Most of the moths lost the plume after a short distance and flew to the ceiling of the tunnel (Table 3). The strongest response was observed to sources containing 200 ng Z7–12 : Ac, and this concentration was therefore used in the mixture. Since field tests (Dunkelblum et al., 1981) indicated that *P. chalcites* males were not sensitive to the ratio of the components in baits, we decided at this stage to test only one ratio (10 : 1) between Z7–12 : Ac and the other acetates. Addition of one compound, either Z9–12 : Ac or Z9–14 : Ac to Z7–12 : Ac, had a significant effect on the response of the males. A considerable percentage of the insects (15–44%) completed the courtship sequence and attempted to copulate with the sources. On the other hand, addition of either 12 : Ac or 11–12 : Ac to Z7–12 : Ac enhanced only the percentage of males performing flights, although without landing on the sources. The ternary mixture of Z7–12 : Ac + Z9–12 : Ac + Z9–14 : Ac elicited a very high response, with 44–74% of the males performing a complete courtship sequence. The activity of this blend equaled that of the natural gland extract. Substitution of Z9–12 : Ac or Z9–14 : Ac in this blend, by 11–12 : Ac yielded two additional ternary blends which were almost as active as the mixture of Z7–12 : Ac + Z9–12 : Ac + Z9–14 : Ac (Table 4). Addition of either 12 : Ac or 11–12 : Ac to this mixture had no effect, whereas the addition of both slightly enhanced the activity of the five-component blend (Table 4).

Although a relatively large number of components were identified in the gland volatiles, it may be that only a few of them are essential for the complete male response. The purpose of the present behavioral tests was to assess the activity of the major components released by *P. chalcites* females. The results indicate that Z7–12 : Ac is responsible for initiating flight activity in males, but addition of Z9–12 : Ac + Z9–14 : Ac or Z9–12 : Ac + 11–12 : Ac or 11–12 : Ac + Z9–14 : Ac is essential for the performance of the entire courtship sequence, including a high percentage of copulatory attempts. Since male responses to ternary mixtures were already high, a comprehensive set of behavioral tests such as response to different ratios of components and measurement of time of flight is necessary in order to determine whether all the released compounds are indeed essential as sex pheromone components.

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ROLE OF FEMALE-PRODUCED SEX PHEROMONE IN BEHAVIORAL REPRODUCTIVE ISOLATION BETWEEN *Trichoplusia ni* (HÜBNER) AND *Pseudoplusia includens* (WALKER) (LEPIDOPTERA: NOCTUIDAE, PLUSIINAE)

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Abstract—In laboratory flight tunnel bioassays, response rates of male cabbage looper, *Trichoplusia ni* (Hübner), to female soybean looper, *Pseudoplusia includens* (Walker), were similar to response rates of male *T. ni* to conspecific females for plume tracking and source contact. Male soybean loopers, however, exhibited a greatly reduced response to female cabbage loopers compared to conspecific females. Similar differences were observed in male responses to extracts of female abdominal tips. Studies of flight tunnel responses of male soybean loopers to the different chemicals known to be components of the female cabbage looper sex pheromone indicated that the reduction in response was due to inhibitory effects of (Z)-5-dodecen-1-ol acetate and (Z)-9-tetradecen-1-ol acetate, when added singly to (Z)-7-dodecen-1-ol acetate (major component of both species) at release rates and at ratios close to those observed in female cabbage loopers.

Key Words—*Trichoplusia ni*, *Pseudoplusia includens*, Lepidoptera, Noctuidae, (Z)-5-dodecen-1-ol acetate, (Z)-9-tetradecen-1-ol acetate, (Z)-7-dodecen-1-ol acetate, pheromone, pheromone blends, cross-attraction, inhibition, reproductive isolation.

INTRODUCTION

Many moth species in the noctuid subfamily Plusiinae have chemically overlapping female sex pheromones, characterized by the presence of (Z)-7-dodecen-1-ol acetate (Z7–12:Ac) as a major component. This compound has been found to be a sex pheromone of the cabbage looper (CL), *Trichoplusia ni* (Hüb-

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ner) (Berger, 1966), the soybean looper (SBL), *Pseudoplusia includens* (Walker) (Berger and Canerday, 1968; Tumlinson et al., 1972), *Autographa biloba* (Stephens), *Rachiplusia ou* (Guenee) (Berger and Canerday, 1968), and *Plusia chalcites* (Espinosa) (Dunkelblum et al., 1981), and a sex attractant for *Trichoplusia oxygramma* (Geyer) (Mitchell, 1972), *Autographa californica* (Speyer), and *Syngrapha falcifera* (Kirby) (Butler et al., 1977), among others.

While many closely related moth species are known to maintain premating and behavioral reproductive isolation through chemical differences in their female sex pheromones, if not through other means such as geographic, seasonal, or temporal isolation (Roelofs and Cardé, 1974; Greenfield and Karandinos, 1979), such mechanisms are unknown or undocumented within the Plusiinae, despite the similarities of known pheromones within the subfamily. The CL and SBL are sympatric over much of eastern North America (Eichlin and Cunningham, 1978) and can occur at the same time within the same field, as indicated by trap catches in pheromone-baited traps (Mitchell, 1972; Kaae et al., 1973). Both species have very broad and widely overlapping diel periods of sexual activity, insufficient to achieve sexual isolation (Mitchell, 1973). Leppla (1983) reported attraction of both SBL and CL males to heterospecific females in a laboratory flight chamber bioassay, and Mitchell (1972) and Kaae et al. (1973) observed attraction of CL males to traps baited with SBL females, although SBL males were not appreciably attracted to CL females. Although mating between these two species should be impossible due to genitalic differences (Callahan, 1961), the degree of behavioral reproductive isolation between these two species and any preventive role played by sex pheromone chemistry have not been elucidated.

Behavioral reproductive isolation through differences in the sex pheromone chemistry may be accomplished by differences in the component makeup of the sex pheromones, in the ratios of shared components, or in the pheromone release rates. The sex pheromones of the female CL and SBL apparently are qualitatively different. A six-component blend has been reported as the sex pheromone of the CL (Bjöstad et al., 1980a, 1984), comprised of the saturated dodecen-1-ol acetate (Sat-12:Ac), (Z)-5-dodecen-1-ol acetate (Z5–12:Ac), (Z)-7-dodecen-1-ol acetate (Z7–12:Ac), 11-dodecen-1-ol acetate (Δ11–12:Ac), (Z)-7-tetradecen-1-ol acetate (Z7–14:Ac), and (Z)-9-tetradecen-1-ol acetate (Z9–14:Ac). Only Z7–12:Ac has been reported as a SBL female sex pheromone (Tumlinson et al., 1972). Estimates of the release rate of the major component of the CL pheromone, Z7–12:Ac, were reported by Sower et al. (1972), Bjöstad et al. (1980b), and Baker et al. (1981). Comparable information is not available for female SBL.

We report here the results of studies of the interspecific sex pheromone responses between SBL and CL moths. The investigations were designed to determine if any differences in the release rates or chemical composition of the

female sex pheromones contribute to the maintenance of premating reproductive isolation between the two species. Quantitative determinations were made of pheromone emitted from calling female CL and SBL moths to ascertain the release rates of known components of the natural pheromone blends. Male responses to heterospecific females, female abdominal tip extracts, and two-component synthetic sex pheromone blends were evaluated in laboratory flight-tunnel bioassays and in a field-trapping experiment.

METHODS AND MATERIALS

Insect Rearing and Handling. All moths used were obtained as pupae from colonies maintained at the USDA Insect Attractants, Behavior, and Basic Biology Research Laboratory, Gainesville, Florida, using methods described by Guy et al. (1985) for the CL. The SBL colony was established in November 1984 from pupae collected in a soybean field near Alachua, Alachua County, Florida.

Pupae were sorted by sex, with males and females kept in separate environmental chambers on a 14:10 light–dark reversed-light cycle, at 24°C and 60% relative humidity. Pupae and emerging moths were held in 25 × 25 × 25-cm aluminum frame and plastic screen cages with a 40-ml cup of honey-sucrose solution on cotton. Pupae were moved periodically to new cages to provide moths of discrete ages.

Volatile Collections. Pheromone release rates and the ratios of sex pheromone components emitted by female CL and SBL moths were determined by gas chromatographic (GC) analysis of moth volatiles collected on charcoal filters described by Tumlinson et al. (1982). The glass chamber used to contain the moth(s) was 5 cm OD × 30 cm in length. Air was purified prior to its introduction into the chamber of the volatile collection system by passage through charcoal filters and was humidified using a split air-flow system. The split flow system allowed a variable portion of the air flow to be humidified to near saturation (95%) by its passage through a gas diffusion chamber that contained distilled water. By altering the ratio of humidified to dry air, system humidity could be adjusted from 10 to 95%. A glass frit near the upwind end of the volatile collection chamber provided laminar air flow over the insect which typically perched on the frit. A stainless-steel mesh cone confined the insect to the chamber area near frit. Air flow through the system was maintained at 0.5 liters/min with a slight positive pressure established in the volatile collection chamber (incoming air to vacuum). This was monitored with a vent flowmeter measuring air flow (0–20 ml/min) from a vent in the chamber. During these experiments, vent flow was ca. 2% of air flow through the filters. Pressure drop across the charcoal collection filters was reduced to near zero by

the use of an air-flow regulator on the vacuum line downwind of the collection filters. After collection, the charcoal filters were extracted for analysis using 3 × 20-μl portions of CH_2Cl_2 as described by Tumlinson et al. (1982).

Moths were placed in the chambers of the volatile collection system 0.5 hr before observations and collections were begun during the fifth and sixth hours of the scotophase. A dim, red light was used to illuminate the holding chambers for observations. Room temperature was 24 ± 1°C. Moths were 4–6 days old. Twelve separate 15-min volatile collections were obtained from individual female SBL moths. Ten separate 10-min volatile collections were obtained from individual female CL moths.

Chemical Analyses. Gas chromatographic (GC) analyses were conducted on a Varian model 3700 GC and a Hewlett-Packard model 5890 GC equipped with splitless capillary injectors and flame ionization detectors. A Nelson 4000 data system was used for chromatogram storage and analysis. Helium was used as the carrier gas at a linear flow of 18 cm/sec. Columns used for analysis were a 50 m × 0.25 mm ID CPS-1 (high-polarity cyano column equivalent obtained from Quandex), operated at 60°C for 2 min, then temperature programmed at 30°/min to 180° or a 20 m × 0.25 mm ID Supelco wax (bonded Carbowax), operated at 60°C for 2 min, then temperature programmed at 30/min to 160°C. Both of these columns afforded complete resolution of the four dodecenol and the two tetradecenol acetates reported previously as the pheromone components of the CL (Bjöstad et al., 1980a, 1984).

Samples were analyzed by GC–mass spectrometry with a Nermag model R1010 mass spectrometer in the chemical ionization mode. The CPS-1 capillary column used in previous analyses was used in the GC–mass spectrometric analyses using helium carrier gas. Either methane or isobutane was used as the reagent gas in the mass spectrometer. Spectra of the natural products were compared with those of candidate synthetic compounds.

All synthetic standards used in this study were obtained from commercial sources and 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). These compounds were analyzed on the capillary GC columns described previously and determined to be greater than 99% pure.

Formulation. Synthetic blends and individual synthetic compounds were formulated on 5 × 9-mm rubber septa (A.H. Thomas Co.) for all biological tests. Septa were Soxhlet extracted with CH_2Cl_2 for 24 hr and air dried prior to loading. Desired release ratios of the components of blends were obtained by loading the septa with mixtures containing calculated percentages by weight of each component of the blend. The percentage of a component in the loading mixture was calculated on the basis of its relative volatility determined from retention indices on liquid crystal capillary GC columns (Heath and Tumlinson, 1986) and a method developed to predict release ratios of components of a blend

from rubber septa (Heath et al., 1986). Each septum was loaded with 100 μl of a hexane solution of the blend pipetted into the well on the large end of the septum. Septa were aired for two days at room temperature before use.

Determination of the ratio and rate of release of the pheromone from a septum consisted of placing the septum in a stainless-steel chamber in which purified air was introduced at a wind velocity of 0.225 m/sec over the septum. The volatilized pheromone was collected on a charcoal trap (3–5 mg of charcoal), extracted with ca. 60 μl CH_2Cl_2 and 20 μl hexane, concentrated, and analyzed by capillary gas chromatography using the 50-m CPS-1 column. Collection time varied from 0.5 to 1 hr, depending on the amount of material emitted from the septum for a given load.

Flight-Tunnel Bioassays. A comparison was made in a flight-tunnel bioassay of the responses of male CL and SBL moths to a range of release rates of Z7–12:Ac encompassing those determined from volatile collections from females of both species. Desired release rates were obtained by altering the flow of air over a rubber septum containing Z7–12:Ac. A pheromone-loaded septum was placed inside a small stainless-steel chamber (positioned near the center of the upwind end of the tunnel) through which regulated air from a compressed air cylinder was forced. Determinations of the pheromone loads and air-flow rates required to provide the desired range of release rates from the septum were made as described above (Figure 1). Air flow within the tunnel (1 × 1 × 4-m Plexiglas box) was calibrated at 0.22 m/sec. Air was drawn into the tunnel from

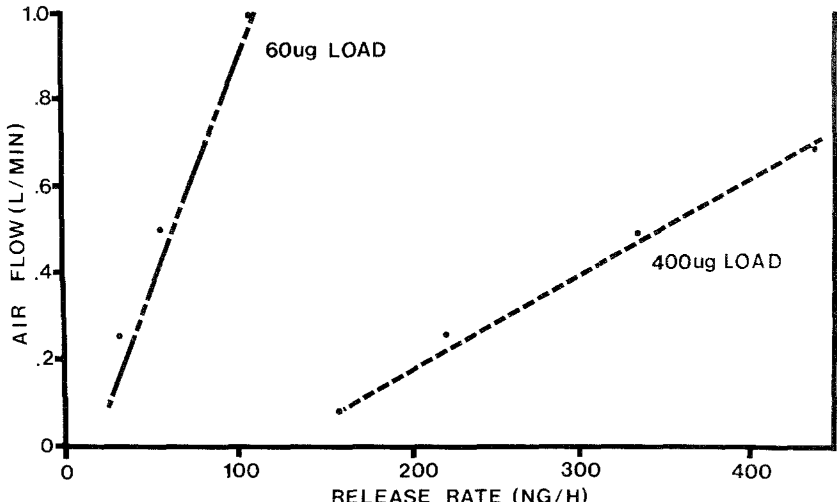

Fig. 1. Measured release rates of Z7–12:Ac from septa at different flow rates of air over septa, for a 60-μg load and a 400-μg load per septum.

within the environmental chamber housing the tunnel by a blower motor and fan. The environmental chamber was kept at 24 ± 1°C and 65 ± 10% relative humidity.

Moths tested were released singly from a 60-ml plastic vial hung on a metal stand 20 cm from the downwind end of the tunnel and near the center of the long axis of the tunnel. Test moths were then observed for a 2-min period following release, or until the maximum response occurred, and were scored for flight from the release vial, for upwind-oriented flight within the pheromone plume (plume tracking), and for contacting the pheromone emitter (source contact). When a male contacted the source, the time from release to source contact was also recorded. Responses of male CL and SBL to Z7-12:Ac were evaluated at 0, 10, 20, 40, 100, 200, and 500 ng/hr, as determined by measurements of release rates (Figure 1). On each of five days, each release rate was tested with two moths of each species in an ascending release rate order to avoid contamination problems. All bioassays were conducted in the fourth and fifth hour of the scotophase.

Differences between CL and SBL plume tracking response data were determined by regression analysis of percent response on log release rate.

The responses of male CL and SBL moths to calling heterospecific females were evaluated in a similar flight-tunnel bioassay. For each species, three 3- to 6-day-old females were confined in a 5 × 7-cm screen cylinder placed on a wire hook near the center of the upwind end of the tunnel 1 hr prior to the bioassays. Male response bioasays were conducted during the fourth hour of the scotophase for CL females and fifth hour of the scotophase for SBL females. Males were released singly from 50-ml vials near the downwind end of the tunnel and observed for 2 min following release or until they contacted the cage of females. Males were scored for flight from the vial, for upwind-oriented flight, for contacting the cage of females, for hairpenciling (CL), and for copulatory attempts (SBL). Twenty-five conspecific and 25 heterospecific males were tested with females of each species, conducted as five sets of 10 bioassays (five conspecifics followed by five heterospecifics). All other bioassay conditions were as described previously.

Male response to extracts of abdominal tips of conspecific and heterospecific females was also evaluated in a flight tunnel bioassay. Abdominal tips (segments 8-10) were cut and placed in a vial containing 300 μl hexane for 30-60 min, and the amount of Z7-12:Ac was determined by quantitative capillary gas chromatography. Dosages of abdominal tip extracts containing 100 ng Z7-12:Ac in 100 μl hexane were compared to 100 ng synthetic Z7-12:Ac in 100 ml hexane as a control. These were applied to 6-cm-diam. filter papers and air dried for 2 min prior to bioassay. Papers were suspended from a wire hook near the center of the upwind end of the flight tunnel. Males were released singly near the downwind end and their behavior observed for 2 min following release. Males were scored for flight from the vial, upwind-oriented flight, con-

tacting the filter paper, and the time from release to source contact. Bioassays were conducted as sets of five males exposed consecutively to the abdominal tip extracts followed by five males exposed consecutively to synthetic Z7–12:Ac. Each data set (10 males) was replicated five times, each on a different day. Bioassays were conducted during the fourth and fifth hours of the scotophase.

Male SBL responses to synthetic female CL sex pheromone components were evaluated in a flight tunnel bioassay. Each of the five minor components of the female CL sex pheromone reported by Bjöstad et al. (1984) (Sat-12:Ac, Δ11–12:Ac, Z5–12:Ac, Z7–14:Ac, and Z9–14:Ac) was tested with the major component of the CL female pheromone (Z7–12:Ac) in load ratios determined to produce release ratios similar to those reported by Bjöstad et al. (1984) from female CL. These five two-component blends: Z7–12:Ac/Sat-12:Ac (100:9 load ratio), Z7–12:Ac/Z5–12:Ac (100:9), Z7–12:Ac/Δ11–12:Ac (100:4), Z7–12:Ac/Z7–14:Ac (100:6), Z7–12:Ac/Z9–14:Ac (100:3), and Z7–12:Ac alone were tested at dosages of 100, 500, and 1000 μg on rubber septa. Bioassays were conducted in sets of 30, with five male SBL tested for each of the six blends at a given dosage. Each set was replicated five times per dosage (on 15 days) for a total of 450 males. Males were released singly as described, were observed for 2 min following release, and were scored for upwind-oriented flight, contacting the septum, and reorienting to the septum (after first contacting it and then leaving the plume entirely). The time from release to first contact with a septum was also recorded. Moths were 3–6 days old and were tested for the fourth and fifth hours of the scotophase. For each behavioral response, treatment means were separated using Duncan's new multiple-range test at $P \leq 0.05$.

Field Test. The sex pheromone blends tested in the flight-tunnel bioassay (Z7–12:Ac plus synthetic female CL pheromone minor components) were also evaluated (along with a six-component synthetic blend) for attractancy in a six-day field-trapping test. The blends were formulated as before in rubber septa at 1 mg/septum and were tested in International Pheromones Moth Traps (bucket trap), with 5 × 4 × 1-cm pieces of Vapona to kill captured moths. Traps were mounted on wooden stakes ca. 1 m above ground and were placed 45 m apart in two north–south rows 320 m apart. Each row consisted of seven traps, each with one of the seven blends tested. Traps were checked and treatments were randomized daily within each row and septa were replaced after three days.

Treatment means for trap catch data were compared by Duncan's new multiple-range test at $P \leq 0.05$ for treatment differences.

RESULTS

Amounts of Z7–12:Ac collected from air passed over female SBL moths in the volatile collection apparatus averaged 1.6 ± 0.48 ng ($\bar{X}$ ± SD) per min

of collection (range 0.32–1.66 ng/min). Amounts of Z7–12 : Ac collected from female CL moths averaged 4.26 ± 2.18 ng/min of collection (range 2.42–9.08 ng/min). Ratios of CL pheromone components observed were similar to those reported by Bjöstad et al. (1984).

Responses rates of CL and SBL males to different release rates of Z7–12 : Ac were similar throughout the ranges tested (0–500 ng/hr). Mean release rates of Z7–12 : Ac from calling female CL and SBL moths (256 vs. 96.0 ng/hr averages) were both well within the ranges tested for male response. The observed regressions for plume tracking were both significant but were not significantly different from each other in slope (Figure 2). Response rates by both CL and SBL males for source contact were nearly identical to those for plume tracking.

In the flight-tunnel bioassay, CL males responded to SBL females by orienting within and flying up the pheromone plume to the cage of females. The percentages of CL males responding to SBL females were significantly greater than those of CL males responding to conspecific females (Table 1). Soybean looper males, however, exhibited much lower response rates to CL females than to conspecific females, with significantly fewer males tracking the plume and

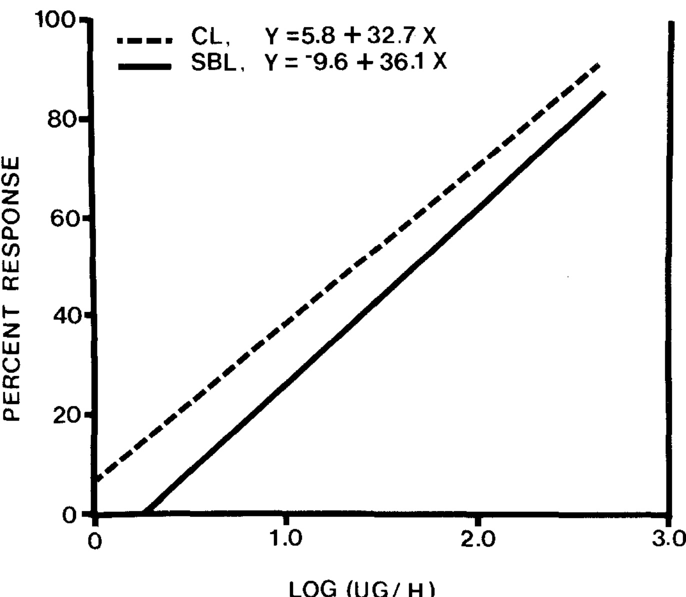

FIG. 2. Percentages of male soybean loopers and male cabbage loopers exhibiting upwind oriented flight to contact the emitter (septum) in a flight tunnel bioassay, for different release rates of Z7–12 : Ac. For cabbage looper, R^2 = 0.81, for soybean looper, R^2 = 0.76.

TABLE 1. MEAN PERCENTAGE RESPONSES ($\bar{X} \pm$ SD) OF SBL AND CL MALES TO THREE CALLING SBL OR CL FEMALES AND MEAN TIMES (SEC) TO CONTACT FEMALE CAGE[a]

	Three CL females		Three SBL females	
Response	CL male	SBL male	CL Male	SBL Male
Plume tracking	80.0 ± 17.9b	20.0 ± 0.0a	100.0 ± 0.0c	83.3 ± 15.1b
Source contact	76.7 ± 19.7b	13.3 ± 10.0a	96.7 ± 8.2c	76.7 ± 15.1b
Time to contact	24.2 ± 5.5a	64.3 ± 28.2b	27.8 ± 5.3a	34.8 ± 10.6a

[a] Means within a row followed by the same letter are not different significantly by Duncan's new multiple-range test at $P \leq 0.05$.

contacting the cage of CL females. Soybean looper males also took significantly longer to contact the cage of females when they did respond.

There were no differences in CL male flight-tunnel responses to 100-ng dosages of *Z*7–12 : Ac and comparable amounts of extracts of female SBL abdominal tips on filter paper. Male SBL response rates, however, were significantly reduced to extracts of female CL abdominal tip extracts, compared to *Z*7–12 : Ac alone (Table 2). Fewer males oriented with the plume, contacted the filter paper, and hairpenciled.

At 100-μg loads on rubber septa, SBL males exhibited a reduced response to *Z*7–12 : Ac with the CL minor components *Z*5–12 : Ac or *Z*9–14 : Ac added vs. *Z*7–12 : Ac alone (Figure 3A). The addition of *Z*7–14 : Ac had no significant

TABLE 2. MEAN PERCENTAGE RESPONSES ($\bar{X} \pm$ SD) OF SOYBEAN LOOPER (SBL) AND CABBAGE LOOPER (CL) MALES TO 100-NG SAMPLES OF (Z)-7-DODECEN-1-ol ACETATE ON FILTER PAPER VS. EQUIVALENT SAMPLES OF FEMALE ABDOMINAL TIP EXTRACTS ON FILTER PAPER[a]

Behavior	*Z*7–12 : Ac	Heterospecific female abdominal tip extracts
SBL male response		
Plume tracking	75.0 ± 10.0a	20.0 ± 23.1b
Source contact	70.0 ± 11.5a	10.0 ± 20.0b
Copulatory attempts	70.0 ± 11.5a	5.0 ± 10.0b
CL male response		
Plume tracking	68.0 ± 17.9a	76.0 ± 16.7a
Source contact	64.0 ± 16.7a	72.0 ± 22.8a
Hairpenciling	36.0 ± 26.1a	48.0 ± 22.8a

[a] Means within a row followed by the same letter are not different significantly by Student's *t* test, $P \leq 0.05$.

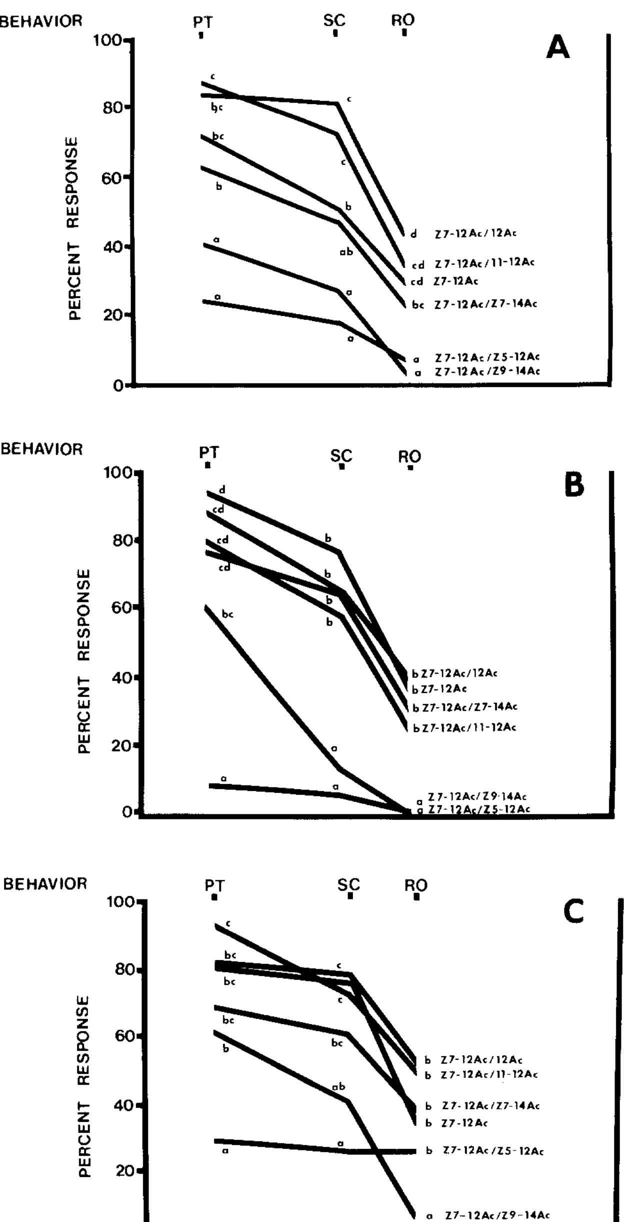

FIG. 3. Percentages of male soybean looper moths responding to pheromone blends formulated at loads of 100 μg (A), 500 μg (B), or 1 mg (C). PT = plume tracking, SC = source contact, and RO = reorientation to the source. Percentages within a behavioral response category followed by the same letters are not significantly different at $P \leq 0.05$, Duncan's new multiple-range test.

effect on numbers of male SBL tracking the plume, contacting the releaser, or reorienting to the releaser. However, with the addition of Sat-12:Ac or Δ11-12:Ac, significantly more males contacted the releaser. Similar results were observed at 500 μg (Figure 3B). At this dosage, SBL male responses to blends containing *Z*5-12:Ac and *Z*9-14:Ac were reduced, while the addition of *Z*7-14:Ac, and in this case Sat-12:Ac and Δ11-12:Ac, had no significant effect on the response. At 1-mg loads (Figure 3C), the presence of *Z*5-12:Ac and *Z*9-14:Ac both reduced numbers reaching the releaser. However, *Z*9-14:Ac did not significantly reduce the number of males tracking the plume but did reduce reorientation to the releaser, while *Z*5-12:Ac reduced the number of males tracking the plume.

Similar results were obtained in the field trapping test. Significantly fewer male SBL were caught in traps baited with either the six-component CL blend ($\bar{X} = 0.0$), with *Z*5-12:Ac/*Z*7-12:Ac (0.0) or with *Z*9-14:Ac/*Z*7-12:Ac (0.8 ± 0.9), than with *Z*7-12:Ac alone (8.6 ± 9.7). Catches in traps baited with Sat-12:Ac/*Z*7-12:Ac (3.7 ± 5.6), Δ11-12:Ac/*Z*7-12:Ac (6.7 ± 8.9), or *Z*7-14:Ac/*Z*7-12:Ac (7.1 ± 11.1) were not different significantly from those baited with *Z*7-12:Ac alone.

DISCUSSION

Since spermatophore transfer between the cabbage looper and soybean looper should be impossible because of genitalic incompatibility (Callahan, 1961), and the production of viable hybrid offspring extremely unlikely, interspecific attraction and courtship interactions would appear to be maladaptive, reducing the fitness of those involved. In the very least, males flying upwind in response to the pheromone of a heterospecific female are wasting time and energy that could be expended searching for potential conspecific mates. Also, close-range heterospecific interactions may interfere with the attractiveness of a calling female to conspecific males. Copulation attempts may cause physical damage or eventual death without reproduction, as reported for male *Heliothis zea* (Boddie) copulating with female *Heliothis virescens* (Fab.) (Stadelbacher et al., 1983).

Our experimental results and observations of male CL and SBL flight-tunnel behavior in response to calling females, extracts of abdominal tips of females, and combinations of synthetic sex pheromone components indicate that inhibition of male SBL sex pheromone response by the female CL sex pheromone, although not absolute, is a principle factor contributing to the maintenance of behavioral reproductive isolation between SBL and CL moths by greatly reducing male SBL attraction to calling female CL. However, no such role is served by the female SBL pheromone in species isolation; male CL are attracted strongly to female SBL. These results are similar to the trap catches reported by Mitchell (1972) with very few male SBL caught in female CL-

baited traps and as many male CL in female SBL-baited traps as in traps baited with synthetic Z7–12:Ac.

Results of our dose–response test (Figure 2) and those of Kaae et al. (1973) indicate no difference between the responses of male CL and SBL to a range of release rates of Z7–12:Ac covering those of females of both species. It is then unlikely that pheromone concentration is a factor in reproductive isolation here, as was reported by Kaae et al. (1973) for the CL and *A. californica*. Our experimental results also demonstrate that the inhibition of the male SBL sex pheromone response by the female CL pheromone is due to the presence of the two minor components, Z5–12:Ac and Z9–14:Ac in the released pheromone blend.

Differences in the observed effects of the addition of Z5–12:Ac vs. Z9–14:Ac to the major component Z7–12:Ac suggest a longer range effect by Z5–12:Ac. At the 500-μg and 1-mg dosages tested, the percentages of male SBL tracking the plume from the septum were significantly higher with Z9–14:Ac added to Z7–12:Ac, compared to Z5–12:Ac and Z7–12:Ac (Figure 3). Since the release rate of Z5–12:Ac was considerably higher than that of Z9–14:Ac (because of the different load ratios and higher volatility of Z5–12:Ac compared to Z9–14:Ac, the Z5–12:Ac may have been more readily detected at the release site in the flight tunnel.

The increased response rates of male SBL to the synthetic blends containing Sat-12:Ac and Δ11–12:Ac at the 100-μg dosage were unexpected. These compounds may be as yet undetermined minor components of female SBL sex pheromone. Despite the presence of these two compounds in the six-component blend tested in the field, it was still unattractive to male SBL, compared to Z7–12:Ac alone, presumably because of the inhibitory effects of Z5–12:Ac and Z9–14:Ac in the blend.

Interactions between female CL and male SBL are minimized by inhibition of the male SBL sex pheromone response by Z5–12:Ac and Z9–14:Ac in the female CL pheromone and by ecological factors (Leppla, 1983). Male SBL that do respond and fly to calling CL females reportedly are aggressively rejected (Leppla, 1983), precluding any further interactions. However, since male CL are attracted strongly to calling female SBL, close-range interactions are in this case likely, and it is not known how reproductive isolation is maintained. Although Leppla (1983) reported attempts by male CL to copulate with female SBL, nothing is known of the likelihood of such coupling occurring in nature or of any effects on the ability of a conspecific male to locate and subsequently copulate with such a female. Male CL may be better able to discriminate conspecific females from SBL females in a choice situation, which may minimize these heterospecific sexual interactions in the field.

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REINVESTIGATION CONFIRMS ACTION OF Δ11-DESATURASES IN SPRUCE BUDWORM MOTH SEX PHEROMONE BIOSYNTHESIS

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Abstract—The biosynthesis of a large number of sex pheromone components of various moth species has been shown to start with common fatty acids and involve chain shortening by two carbons and introduction of a double bond at the 11–12 position. A recent report indicates that one of these common components, (*E*)-11-tetradecenyl acetate, is present in the eastern spruce budworm, *Choristoneura fumiferana*, but is not made by this pathway. Reinvestigation of this insect using in vivo and in vitro techniques indicates that the acetate indeed is made by a sequence of reactions similar to that used in other leafroller moths. In fact, evidence was found for the presence of several Δ11-desaturase systems in spruce budworm. One produced a large quantity of (Z)-11-hexadecanoic acid, and another produced (*E*)-11-tetradecanoic acid. It is not known if the small amount of (*Z*)-11-tetradecanoic acid is produced by either of those two systems or by a third system. A comparison with other species showed that cabbage looper moths have only the first system, red-banded leafroller moths use the last two systems, and European corn borer moths have all three.

Key Words—Spruce budworm, *Choristoneura fumiferana*, sex pheromone biosynthesis, Δ11-desaturase, *Trichoplusia ni*, *Argyrotaenia velutinana*, *Ostrinia nubilalis*, Lepidoptera, Tortricidae, Noctuidae, Pyralidae.

INTRODUCTION

The biosynthesis of many lepidopterous sex pheromone components has been shown to involve the reaction of common fatty acids with two key enzyme systems (Roelofs and Bjostad, 1984). These include microsomal β-oxidation to give limited chain shortening by two carbons, and a Δ11-desaturase to yield

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acids with unsaturation at the 11–12 position. A variety of compounds is produced by varying (1) the length of the starting fatty acids (normally between 12 and 18 carbons), (2) the order in which the chain shortening and desaturation steps occur, (3) the number of times chain shortening occurs, (4) the stereochemistry of the unsaturated products, and (5) the functionality (alcohol, acetate, aldehyde) of the pheromone components, which are finally produced by reducing the acid precursors.

A good example of these key steps is given by the biosynthetic routes for the cabbage looper moth (CL), *Trichoplusia ni*, pheromone components. In this species the Δ11-desaturase system produces large quantities of Z11–16 : Acid[1] and Z11–18 : Acid from palmitic and stearic acid, respectively. These are chain shortened to give pheromone precursor acids Z9–14 : Acid and Z7–12 : Acid from the former, and Z7–14 : Acid and Z5–12 : Acid from the latter (Bjostad and Roelofs, 1983). The Δ11-desaturase enzyme from CL was partially purified from the microsomal fraction and found to have a substrate specificity for 16- and 18-carbon acids (Wolf and Roelofs, 1986). However, in the redbanded leafroller moth (RBLR), *Argyrotaenia velutinana*, the Δ11-desaturase enzyme produces unsaturated 14-carbon acids from myristic acid in an *E*/*Z* ratio of ca. 3/2 (Bjostad and Roelofs, 1981). These are reduced to give a specific 8 : 92 ratio of the pheromone components, *E*11– and *Z*11–14 : OAcs.

Recently, Morse and Meighen (1984, 1986) indicated that the eastern spruce budworm (SBW), *Choristoneura fumiferana*, does not utilize the Δ11-desaturase enzyme system for production of the *E*11– and Z11–14 : OAcs found in their pheromone glands as precursors to the corresponding aldehyde pheromone components. Their data did not support a specific desaturation of myristic acid to give Δ11–14 : Acid, and so they suggested that the pheromone was produced by some unspecified pathway, perhaps starting directly from acetate.

We found it surprising that there were two different biosynthetic pathways for *E*11– and Z11–14 : OAcs in two leafroller moth species. It was difficult to accept an alternate route in the case of SBW also because Dunkleblum et al. (1985) had shown that the SBW pheromone glands contain both 14- and 16-carbon Δ11-unsaturated fatty acyl moieties, indicating the presence of a Δ11-desaturase in their pheromone gland. Thus we decided to reinvestigate the biosynthesis of the *E*11– and Z11–14 : OAcs in SBW by both in vivo and in vitro techniques.

Since the SBW apparently desaturated both 14- and 16-carbon acids, we also conducted a comparative study with the Δ11-desaturase system of other species, such as the CL moth (specific to 16- and 18-carbon acids), the RBLR moth (specific to 14-carbon acids), and two strains of the European corn borer

[1] Specific compounds will be referred to by an abbreviated naming system, where a letter (*Z* or *E*) indicates the stereochemistry, the first number gives the site of unsaturation, the second number the chain length, and symbols indicate the oxygen function. Thus Z9–18 : Acid is the abbreviation for oleic acid.

moth (ECB), *Ostrinia nubilalis*, that use *E*11- and *Z*11-14:OAc pheromone components (Roelofs et al., 1985) but produce Δ11 products of both 16- and 14-carbon acids. We tested the ability of pheromone gland extracts of each of these species to desaturate 14- and 16-carbon fatty acyl coenzyme A derivatives.

METHODS AND MATERIALS

Female SBW pupae were obtained from Dale Grisdale, Forest Pest Management Institute, Sault Ste. Marie, Canada, and maintained in our laboratory on a 16:8 light-dark regime. Experiments (gland removal or compound topical application) were conducted on 2-day-old (24–48 hr) insects approximately 1 hr before the onset of scotophase. CL, RBLR, and ECB pupae were obtained from cultures raised in our laboratory and were treated in the same manner.

[1-^{14}C]Palmitoyl-coenzyme A (59 mCi/mmol), [1-^{14}C]myristoyl-coenzyme A (31 mCi/mmol), [U-^{14}C]palmitic acid (403 mCi/mmol), [1-^{14}C]lauric acid (36 mCi/mmol), [1-^{14}C]myristic acid (38 mCi/mmol), and [1-^{14}C]-palmitic acid (57 mCi/mmol) were obtained from Amersham Corporation, Arlington Heights, Illinois. Solutions of the acids in dimethyl sulfoxide were prepared, with each containing aproximately 100,000 dpm/μl.

Extracts were prepared by adding the whole glands or the cell-free reaction mixture to 3 ml of 2:1 chloroform-methanol. After 15 min, 1 ml water was added, the organic layer was removed, and the water extracted with chloroform. The organic layers were combined and concentrated (Folch et al., 1957).

Fatty acids were analyzed as their methyl esters. The extracts were concentrated and placed in a solution containing 2 ml methanol, 1 ml benzene, and 0.1 ml sulfuric acid. The mixture was heated for 1 hr at 85°, cooled, and 1 ml water added. The organic layer was removed, washed three times with water and dried. The transmethylated extract was concentrated and 0.2 ml of acetyl chloride added to regenerate the acetates from the pheromone alcohols. After 10 min, the residual acetyl chloride was removed with N_2.

Gas-liquid chromatography (GLC) separation of the mixtures into fractions of differing chain length (C_{12}, C_{14}, and C_{16}) was performed on a column containing 3% OV-1 (methyl silicone) on 100–120 mesh Gas Chrom Q, with fractions being collected in 30-cm capillary tubes when appropriate. Further separation of *E* and *Z* isomers was accomplished on a column containing 10% XF-1150 (50% cyanoethyl, methyl silicone) on 100- to 120-mesh Chromosorb W-AW-DMCS. Capillary GLC was carried out using a 30-m Supelcowax column, 0.25 mm ID, on a Hewlett-Packard 5890 gas chromatograph.

Thin-layer chromatography (TLC) was performed on Whatman K5 plates that had been sprayed with a solution of 10 g silver nitrate in 65 ml 80% ethanol until damp. The plates were activated for 1 hr at 110° before use. The plates

were developed with a 9:1 mixture of Skelly B and ether, and the areas of radioactivity located by autoradiography. Areas of the plate were scraped into vials, scintillation fluid added, and radioactivity determined in a Packard Tri-Carb scintillation counter.

Ozonolysis was accomplished by adding a solution of the compound in CS_2 to a solution of ozone in CS_2 at $-70°C$. The reaction was allowed to slowly warm to room temperature and was concentrated and directly subjected to GLC analysis.

Topical application was performed in a manner similar to that previously reported (Bjostad and Roelofs, 1983). Female insects were anesthetized with carbon dioxide and their pheromone glands held everted by means of smooth-jawed alligator clips. A droplet of radiolabeled material dissolved in DMSO was applied to the glands. After 1 hr, the insects were removed from the clips and maintained in the dark for 3 hr. At this time they were cooled to 0° for 10 min and the glands removed and extracted as above.

Cell-free reactions were conducted similarly to that reported in Wolf and Roelofs (1986). Pheromone glands were removed and placed in a cold buffer containing 0.10 M phosphate, 0.35 M sucrose, and 5 mM dithiothreitol, pH 7.6. The glands were homogenized in a Potter Elvehjem tissue grinder and centrifuged at 12,000*g* for 15 min at 4°C. The supernatent was combined with 0.1 μCi of labeled fatty acid CoA ester and cofactors in a total volume of 0.20 ml. Cofactor concentrations were: NADH, 5 mM; 5 mM; NADPH, 1 mM; BSA, 1 mg/ml. After 1 hr at 25°C, the assays were extracted as above.

For capillary GLC, five glands were excised, extracted, and transmethylated. An aliquot was subjected to capillary GLC and compared to standards. The rest of the material was subjected to GLC on OV-1, and the relevant peaks subjected to ozonolysis to confirm the position of the double bond.

For in vivo experiments, 3.0 μl of radiolabeled fatty acid in DMSO was topically applied to the glands of 10 insects. After extraction and transmethylation, the products were subjected to GLC on OV-1. Trapped fractions were counted or subjected to further GLC on XF-1150 or ozonized.

In vitro experiments were conducted by preparing the cell-free extract, adding 0.1 μCi of labeled fatty acid, and subsequent extraction and transmethylation. The resultant mixture was analyzed by TLC, GLC, and ozonolysis.

RESULTS

Five SBW glands were extracted, transmethylated, and analyzed by capillary GLC as well as by ozonolysis of the compounds collected from nonpolar and polar GLC columns. This confirmed the presence of $\Delta 11$-unsaturated acids in SBW extracts (Table 1). Both the *E* and *Z* isomers of the 14-carbon acid were found, but only the *Z* isomer of the 16-carbon acid was detected.

TABLE 1. FATTY ACIDS IN SBW EXTRACTS[a]

Chain length (carbons)	Saturated	Δ-9	*E*11	*Z*11
14	1.81	0.53	1.13	0.19
16	26.17	1.19	[b]	0.90

[a] As percent of total fatty acids.
[b] Not found.

In Vivo Experiments. The results of topical application of radiolabeled saturated fatty acids are presented in Table 2. Using uniformly labeled palmitic acid as the starting material, significant radioactivity was obtained in both the 14-carbon acid and 14-carbon acetate fractions. When palmitic acid labeled in only the 1-carbon was used, the amount of label found in the 14-carbon acid fraction decreased more than 60%, and very little radioactivity was detected in the unsaturated 14-carbon acetates. Similarly, lauric acid labeled in the 1-position gave rise to some labeled 14-carbon acids, but no radioactivity was detected in the acetate fraction.

When myristic acid labeled in the 1-carbon was topically applied, the greatest amount of incorporated radioactivity was detected in the 14-carbon acetates. The 14-carbon acid fraction in this reaction was not counted directly, but was collected from the XF-1150 GLC column to separate the saturated acid (starting material) and the isomers of the Δ11-unsaturated acids. Typical results, presented as percent of initial radioactivity incorporated into the fraction, were: saturated, 0.52%; *E* isomer, 0.54%; *Z* isomer, 0.07%. The ratio of *E*/*Z*

TABLE 2. INCORPORATION OF RADIOLABELED COMPOUNDS USING TOPICAL APPLICATION TO SBW PHEROMONE GLANDS

Starting material	Product[a]	
	14:Acid[b]	*E*/*Z*11-14:OAc
[U-^{14}C]16:Acid	0.65	0.36
[1-^{14}C]16:Acid	0.24	0.03
[1-^{14}C]12:Acid	0.34	[c]
[1-^{14}C]14:Acid	[d]	0.48

[a] As percent of starting material.
[b] Total (saturated and unsaturated).
[c] Not found.
[d] Further separated; see text.

TABLE 3. INCORPORATION OF RADIOLABELED COMPOUNDS USING CELL-FREE EXTRACTS PREPARED FROM SBW

Starting material	Product[a]	
	*E*11 acid	*Z*11 acid
[1-^{14}C]14:CoA	6.25	0.72
[1-^{14}C]16:CoA	0.35	1.82

[a] As percent of starting material.

incorporation is very close to that found for the total amounts of the acids present (Table 1).

In Vitro Experiments. Pheromone glands from SBW were excised, homogenized in buffer, centrifuged, and the resulting extracts reacted with radiolabeled CoA esters. After transmethylation and TLC separation, the isomers of the desaturated acids were located by autoradiography and quantified. The results are given in Table 3. Both 14- and 16-carbon acids were desaturated. The 14-carbon acids showed a high reactivity (over 7% of the starting saturated acid was isolated in the products), and the *E/Z* ratio was similar to that observed in the topical application experiments. The 16-carbon acids, however, were much less reactive in this system (just over 2% total) and gave predominantly the *Z* isomer.

These same experiments were repeated using extracts prepared from other species. As shown in Table 4, the results show a pattern similar to the acid

TABLE 4. INCORPORATION OF RADIOLABELED COMPOUNDS USING CELL-FREE EXTRACTS PREPARED FROM SEVERAL INSECTS

	Product (pmol)[a]			
	11–14:Acid		11–16:Acid	
Species	*E*	*Z*	*E*	*Z*
CL	[b]	4	[b]	67
RBLR	17	25	[b]	[b]
SBW	200	24	6	31
ECB (*Z* strain)	114	45	1	189
ECB (*E* strain)	41	18	<1	142

[a] Products were *E*- and *Z*11 isomers of the 14- and 16-carbon acids. Starting materials were 14:CoA and 16:CoA, respectively.
[b] Not found.

precursors in each species. For CL only the *Z* isomer of the 11-desaturated product was observed, predominantly from the 16-carbon acid. In RBLR, only the 14-carbon acid underwent desaturation, but both the *E* and *Z* isomers of the product were labeled. In two strains of ECB, one having a predominantly *E*11 pheromone and the other having mostly *Z*11, the pattern of unsaturated products was similar. In both strains, the 14-carbon starting material gave about 70% *E* product, whereas the 16-carbon acid produced less than 1% *E* product. Also in both, the 16-carbon substrate was preferred (11.2% incorporation total compared to 5.0% for the 14-carbon acid in the *Z* strain).

DISCUSSION

Investigations of the biosynthesis of sex pheromones in moths have involved techniques such as analysis of the fatty acid content of the pheromone gland and in vivo and in vitro incorporation of radiolabeled precursors to gain insight into possible pheromone intermediates or precursors (Lofstedt and Roelofs, 1985; Roelofs and Bjostad, 1984). When we applied these techniques to the SBW, we obtained strong evidence that this insect uses the previously proposed (Roelofs and Bjostad, 1984) biosynthetic pathway involving chain-shortening of palmitic to myristic acid, followed by Δ11-desaturation and then reduction to *E*11- and *Z*11-14 : OAcs.

First, we confirmed the presence of 11–12 unsaturated 14- and 16-carbon acids in the gland (Dunkelblum et al., 1985). Since previous studies with moth sex pheromone glands demonstrated that acids unsaturated in this position are made directly by a Δ11-desaturase (Wolf and Roelofs, 1986), our findings indicate the presence of such an enzyme in SBW.

We then topically applied several radiolabeled fatty acids to SBW sex pheromone glands. If the *E*/*Z*11-14 : OAcs were being made directly from acetate, we would expect similar results from all of the starting materials, as they each would be degraded to this common intermediate. As can be seen in Table 2, this was not the case. Both lauric and palmitic acids labeled in the 1-carbon gave rise to little or no labeled *E*/*Z*11-14 : OAcs. This is the expected result if the first step in the biosynthetic pathway involves chain shortening of palmitic acid, resulting in the loss of the first two carbons, including the labeled one. Lauric acid cannot participate in this reaction. Note that in both cases some activity is incorporated in the myristic acid fraction, perhaps by degradation and recycling through the fatty acid cycle.

Radiolabel from uniformly labeled palmitic acid and from myristic acid labeled in the 1-carbon is incorporated into the *E*/*Z*11-14 : OAcs. In addition, the myristate gives rise to labeled *E* and *Z*11-14 : Acids in a ratio close to that of the total Δ11-14 : Acid found in the gland. Again, this supports the pathway involving chain shortening to myristic acid followed by 11-desaturation.

More direct evidence for the existence of a Δ11-desaturase was provided by in vitro experiments. Cell-free preparations from SBW sex pheromone glands were capable of introducing an 11–12 double bond into CoA esters of myristic and palmitic acids. No radioactivity was found in any compound other than the starting material and this product, indicating that degradation–resynthesis was not occurring under these conditions.

These data demonstrate that the biosynthesis of the *E*/*Z*11–14:OAcs in SBW follows a pathway similar to that in other moths, involving chain shortening and Δ11-desaturation. It differs only in that in SBW these acetates, which are found as major components in the pheromone gland, are converted to the corresponding aldehydes when emitted as a pheromone components.

While collecting the data to prove the pheromone biosynthetic pathway for SBW, we were surprised at the results found in Table 3 showing the stereoselective production of Z11–16:Acid. This had not been found in RBLR, which also produces an *E*/*Z* mixture of Δ11–14:Acids. We conducted studies similar to those of Table 3 with other species and found evidence for the presence of several Δ11-desaturase enzyme systems. The results in Table 4 show that there is a system that produces the *Z* isomer and is specific to 16-carbon chain acids. This system is found in all the insects studied except RBLR. Another system is specified to 14-carbon chain acids and produces the *E* isomer. This is found in all the species except CL. There also may be a third enzyme system that is specific to 14-carbon chain acids and produces the *Z* isomer. Although some of the Z11–14:Acid could be produced by the enzyme specific to 16:Acid, such as in CL, this does not appear to be the case with RBLR or SBW. The presence of an isomerase to yield the *E*/*Z* mixtures of 14:Acids has been ruled out by previous studies using radiolabeled Δ11–14:Acids (Bjostad and Roelofs, 1981). Further purification and characterization of these Δ11-desaturase systems will be needed to clarify the number of variations involved.

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AN INTERACTIVE VIDEO-COMPUTER TRACKING SYSTEM FOR QUANTIFICATION OF LOCOMOTOR BEHAVIOR

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Abstract—We have developed a flexible, moderately priced, behavioral analysis system which has been used to determine the response of salmonids to certain olfactory stimulants. The system, which we call ITS for interactive computer-video tracking system, consists of a 128K Apple IIe computer with software, a video camera and videocassette recorder, and a special-effects generator. Experiments are video taped and then, during playback, the special effects generator is used to simultaneously display the video image and the graphics output of the computer on a monitor. The user tracks the animal of interest using an electronic pen, and the position of that animal in the test chamber, in the form of x–y coordinates, is determined by the computer at user-defined time intervals. When tracking is complete, a plot of the track of the animal is printed within the outline of the test chamber. The following data can also be calculated: swimming velocity, distance from a predetermined point in the chamber (for example, olfactory stimulant source), and time spent in a given area. These variables can be calculated over any chosen time periods and/or for the entire experiment. ITS has numerous advantages over commercially available devices that perform similar tasks. First, it is relatively inexpensive, especially if one already owns video equipment and a computer. Second, it can analyze many types of experiments that can be stored on video tape, including field observations or manipulations. Third, because it is not automated, it is easy to track multiple objects, even if their tracks cross or are not easily located against a low-contrast background. Finally, because whole images do not have to be digitized, and data collection intervals can be adjusted by the user, it is possible to analyze very long experiments with a microcomputer. In this paper we describe ITS and then we demonstrate how we have used it to demonstrate that changes in ambient pH alter the behavioral response of juvenile Atlantic salmon to olfactory stimuli.

Key Words—computers, video, tracking, locomotion, fish behavior, acid precipitation, olfaction.

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INTRODUCTION

One of the most difficult challenges encountered when investigating animal behavior is developing a way to express data quantitatively. In recent years, behavioral analysis of an animal's reaction to environmental or toxicological manipulation has become much more quantitative, due in large part to technological advances in remote monitoring and manipulation of data by computers. Several commercially available systems now enable researchers to track both aquatic and terrestrial animals and to determine behavioral parameters such as distance traveled, time spent in particular areas, and even the number of social interactions (Crawley et al., 1982; Miller et al., 1982; Kaufman, 1983). These modern laboratory tools have enabled investigators to analyze and manipulate behavioral data in increasingly more effective and efficient ways and thus perform experiments that previously would have been too tedious or complex to undertake.

While these systems have proven to be quite useful, they have three major disadvantages for the average investigator. First, they are expensive, costing on the order of $10,000–80,000. Second, the investigator is often separated from the raw data. Many subtle, but important, behavioral responses might not be noticed by the computer. Finally, sophisticated systems are often not flexible enought to allow for widespread applications, such as field work. In the field the areas in which behaviors are being monitored are often not standarized and have varied backgrounds, which would confound most automated tracking systems. What is needed is an inexpensive and flexible, yet sophisticated, technique that allows for close investigator interaction with the data from the initial stage to plotting of the final results.

We have developed a system that uses a standard video camera and video cassette recorder (VCR) to record the movements of an animal, and an Apple IIe computer, with peripherals, to digitize these tracks. We have written software to perform data analysis and statistical calculations, store data in appropriate files, and, if desired, plot the final values. The only unique piece of hardware in the system is a special-effects generator that allows the user to display a video image along with the computer's graphics output on a single monitor. The user can then use the computer-generated targeting crosshairs to track an animal and, thereby, create a digitized record of the animal's movements.

The experimental techniques and methodology described herein have been applied to an investigation of the effect of pH on the olfactory-related behavior of fish. However, this system can be, and has been, used for a wide variety of experiments. A number of different types of quantitative analyses can be performed with it if one simply modifies the controlling software.

METHODS AND MATERIALS

The interactive tracking system (ITS) consists of three parts: (1) recording components; (2) a video–computer interface and digitizer; and (3) integrated data analysis software.

The recording module consists of the behavioral chamber, video recording components, and video integration hardware. The behavioral chamber we used was the Y-maze shown in Figure 1 (Greer and Kasolsoski, 1978; Royce-Malmgren, 1985). The video recording equipment included a color video camera and video recorder (Figure 1). The video integrating hardware enabled us to overlay, on the recording of the fishes' behavior, data pertinent to the experiment, which in this case included pH, temperature, date, and time (Figure 1). The video recording components of the ITS are all portable, enabling data collection in the field and analysis in the lab.

The computer components of the ITS consist of an Apple IIe 128K computer equipped with an Apple digitizer tablet, dual disc drives, internal clock, x/y plotter, and dot matrix printer (Figure 2A). Coupled to these is the video integration hardware that allows for simultaneous projection of the output of the video recorder and computer graphics onto the display monitor (Figure 2B,C). The user tracks one animal at a time using the targeting crosshairs controlled by the electronic pen of the graphics tablet. The computer samples the x–y coordinates at user-determined time intervals and writes these to data files on disc for later analysis.

The software consists of 25 interactive programs, all under the control of a master menu program. The master menu program leads the user through the tracking, data analysis, and plotting programs using a question and answer format. A complete analysis of a 2-hr experiment involving 720 x–y coordinate locations (one every 10 sec) takes approximately 30 min.

Components

Video Equipment. The color camera we used was a moderately low light model with high sensitivity and resolution (Hitachi model 8A). It has a 12:1, 50- to 200-mm telephoto zoom lens with macro capabilities and a minimum focal range of 8.4 mm. The video recorder was a Cannon VR-20. It uses a four-head system with separate recording and playback circuits for low-distortion recording and playback of high-resolution images at variable speeds. A high-resolution Sanyo monitor was used during the experimental recording phase. During the playback and subsequent data digitization phase, we used either an Amdek model I, 13-inch color monitor or a 25-inch high-resolution RCA RGB monitor (model 2526). Recording speed was super long play (SLP) for 6 hr of data collection per video tape (Scotch Color Plus T-120).

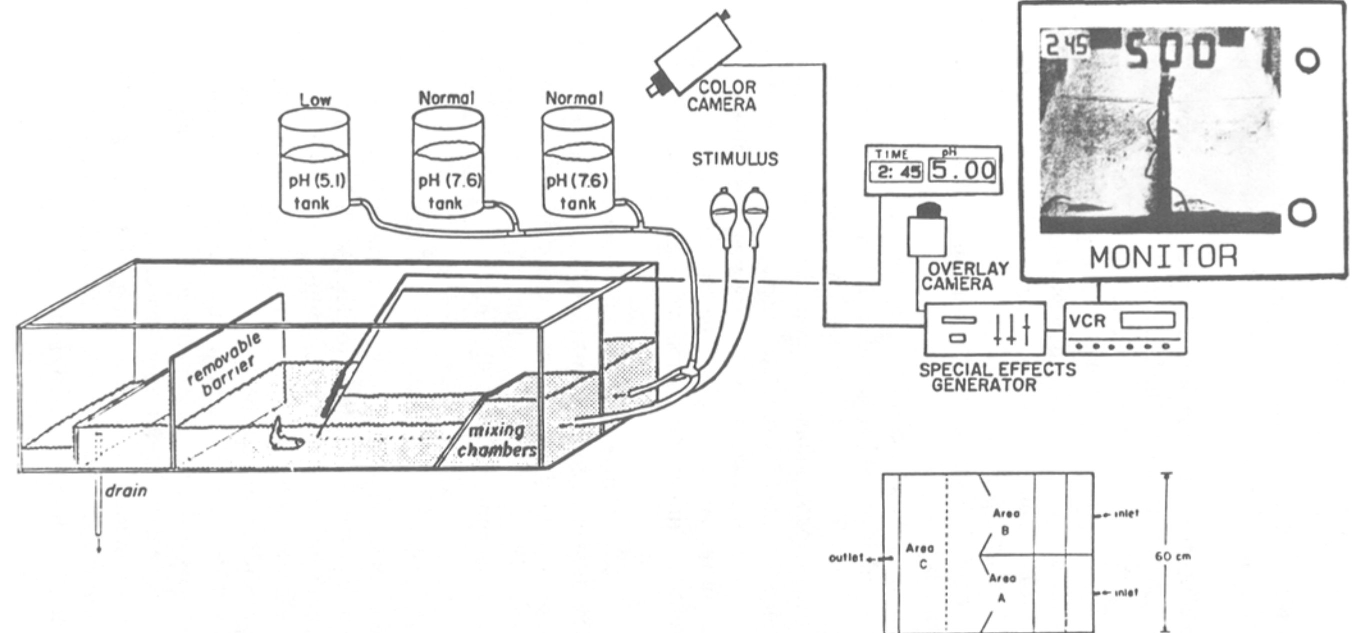


FIG. 1. Test chamber. Fish are introduced into area C behind the removable barrier. Dechlorinated, aerated tap water from overhead storage tanks flows by gravity through the chamber at a rate of 3 liters/min. Following an acclimation period, the removable screen is lifted to start the experiment. During control experiments, no olfactory stimulus is added. During the test experiments, stimulus is added to area A or B from the stimulus reservoirs. The concentration of stimulus falls off as the water from area A and B mix in area C. This creates a concentration gradient from the stimulus input to the opposite side of the tank. This gradient has been confirmed with dyes with fish present. A color video camera mounted above the chamber is used to record the experiment. The video image of the test chamber and a video display of the pH, temperature, and time throughout the experiment is combined using the special-effects generator, recorded by the VCR, and displayed on the monitor. The photograph on the monitor is a view of the experimental chamber from an actual experiment, showing the digitized display of pH and time overlayed on the video image. Two fish are visible in the lower left corner of the test chamber in area A and another in the lower left corner of the right channel in area B. The wire in the middle of the chamber comes from the pH electrode.

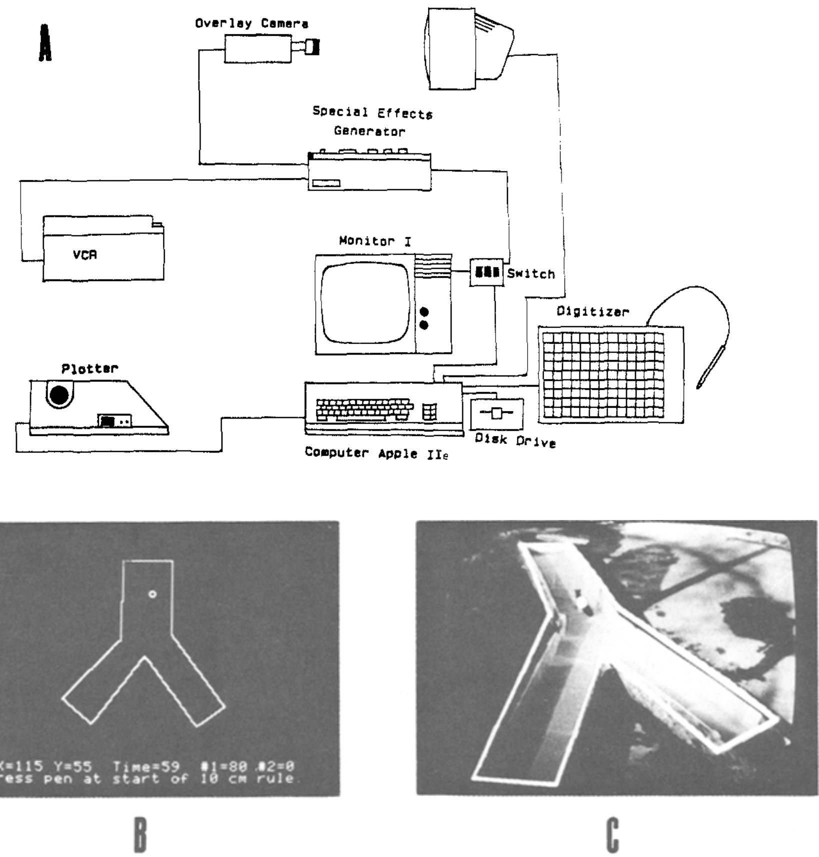


FIG. 2. Interactive computer–video tracking system (ITS). (A) Schematic showing equipment for data analysis. Experiments are video taped, and this record along with the graphics output of the computer is combined for analysis using a special-effects generator to create a composite video of the two signals. (B) Computer generated graphics output consisting of flashing cursor and a test chamber outline. The text at the bottom includes *x–y* coordinates, time in seconds, and additional key system levels. The user is being told to place the pen at the start of the 10-cm calibration rule and press the button on the tablet. (C) Photo of an actual screen during analysis. Computer generated flashing cursor (presently over a fish near the top of the chamber) is used to track individual fish, and *x–y* coordinates from the digitizer table are sampled at user determined time intervals, and then transferred to the data files on the disc.

Our video equipment is more sophisticated than necessary for most purposes. A simple black and white camera, VCR, and monitor can be purchased for as little as $700. It is likely, however, that most investigators will have suitable video components available to them, which can be adapted for use with this ITS. This eliminates the need to purchase any new video equipment, other than the special-effects generator.

The most unique piece of hardware in the ITS is the Ambico special effects generator that comes equipped with a special monochrome camera sensitive to the red/orange range of the visual spectrum. This unit costs approximately $700. It allows us to perform two important procedures: (1) overlay output of instruments such as a pH meter, clock, and thermometer on the original video signal, and (2) combine the graphics output of the computer (Figure 2B) with the prerecorded video images of the experiment to yield a composite image for data analysis (Figure 2C).

Computer and Associated Equipment. An Apple IIe with 128K memory was utilized for track digitization, data analysis, and presentation of results. Two disc drives were used, one for the controlling software, and the other for storage of data and/or results. A RAM (random access memory) disc was utilized to store temporary work files for rapid access. Two output devices were used, a Panasonic dot-matrix printer and a Hewlett-Packard x–y plotter. An Apple digitizer tablet ($800) with electronic pen was used to control the on-screen targeting crosshairs. The computer determined the x–y coordinates at user-defined time intervals as determined by an onboard clock. The precision of the digitizer tablet was much greater than needed and a Koala pad ($125) or a light pen ($150–300) was used when the high precision of the tablet was not required.

Software. All controlling programs for data acquisition and analysis were written by one of the authors (C.R.M.). The integrated data acquisition consists of three discrete programs, all controlled from a master menu program (Figure 3). These include: (1) the experiment protocol program, (2) the chamber outline and distance calibration program, and (3) the track digitization program.

The experiment protocol program records information such as data; technicians initials; number, species, and life stage of animals involved; type of experiment; physical parameters (including water temperature, pH, water hardness, and calcium concentration); video tape code name and number; tape counter at the beginning and end of the experiment; and other pertinent information (Figure 3B). Analysis information for track digitization, such as video playback speed, sample interval, experimental length, and time periods to be analyzed are then recorded. These data are stored in two files, an INFO/ on data disc and PRMTRS file on RAM disc.

The master menu program then passes the user to the chamber outline and distance calibration program (Figure 3C). The user is asked to use the electronic pen to outline the test chamber, using the video-overlay system and to designate

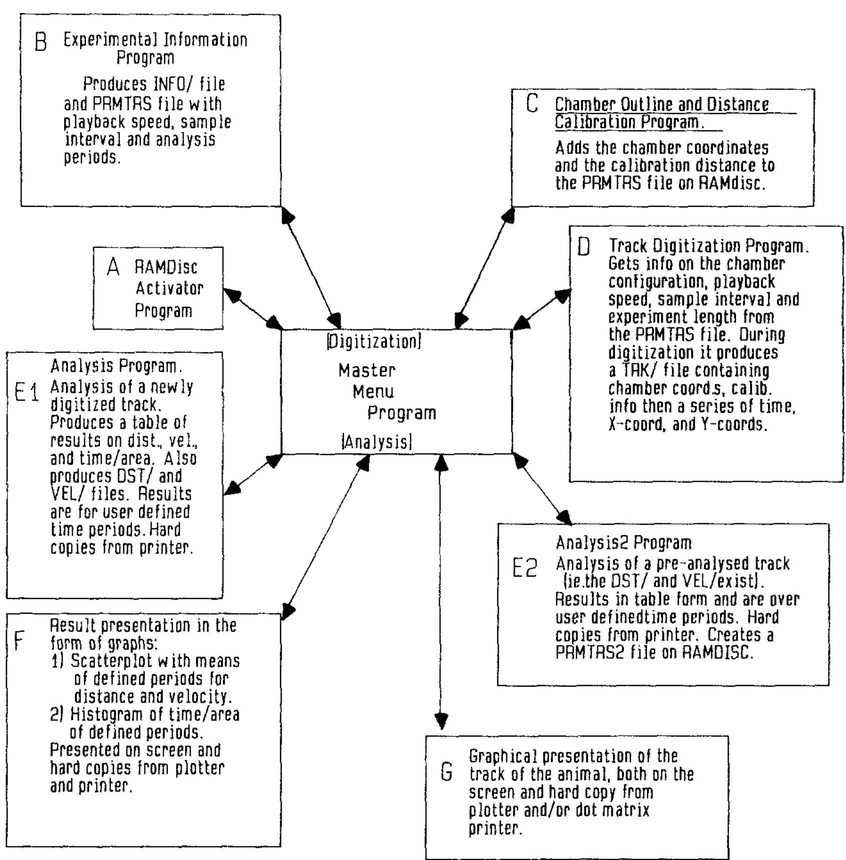


FIG. 3. ITS software block diagram. (A) The RAMdisc Activator Program first determines the availability of memory and correct system configurations. If adequate, it installs a 64K electronic disc drive (RAMdisc). (B) The Experimental Protocol Program documents the experimental and analysis parameters, then saves these to both RAMdisc and data disc. (C) Using the video overlay system, the chamber outline and calibration distance is recorded and stored on RAMdisc. (D) The Track Digitization Program, using information stored in the PRMTRS file on RAMdisc, will perform the actual track digitization with the user controlling the electronic pen. At the end of tracking, the TRK/ file is written to RAMdisc and data disc. (E1) The Analysis Program uses the TRK/ file on RAMdisc and the analysis parameters in the PRMTRS file to analyse the track. (E2) The Analysis2 Program can reanalyze a dignitzed track over new (or different) time periods. (F) The results determined in E1 or E2 can be presented graphically using the Results Plotting Program. (G) The Track Display Program can show all or any part of a digitized track on the monitor screen and hard copies can be produced on either the printer or plotter.

the ends of a 10-cm calibration rule placed in the test chamber. This information is added to the PRMTRS file on RAMdisc.

The user is then passed to the track digitization program by the master menu program (Figure 3D). Using information from the PRMTRS file, the tracking program outlines the test chamber and supplies targeting crosshairs overlayed on the video image of the experiment. The user starts the video tape and collects data simply by keeping the targeting crosshairs over the image of the fish for the duration of the experiment. Time and x–y coordinates are displayed at the bottom of the screen during data acquisition and stored in RAM (not on RAMdisc). At the end of track digitization, the user enters the stimulus source(s) position(s). The calibaration distance and the stimulus source(s) location(s), along with the time and x–y coordinates, are now written to a TRK/ file on both data disc and RAMdisc.

There are two types of analysis programs. The first (Figure 3E1) is for a newly digitized track that will create distance (DST/) and velocity (VEL/) files. The second, the reanalysis program (Figure 3E2), analyses a track for which the DST/ and VEL/ files already exist. Following analysis (either program), tabular results are displayed on the screen.

Above the table is a summary of the analysis and tracking parameters, including (1) the code name of the track analyzed and files produced and/or read, and (2) the tracking information, such as VCR playback speed, sample interval, and experiment length. A sample of such a table is shown in Figure 7. Following analysis, the data from the table can be graphically presented (Figure 7B–E). Distance and velocity are presented as a scatterplot of all data with means ($\pm$ SEM) of each period analyzed (Figure 7D,E). Time per area data appear as a histogram with separate clusters for each analysis period (Figure 7C). Finally, the actual track of the fish can be plotted over any time period(s) (Figure 7B). Hard copies of all tables, graphs, and tracks can be produced on the dot-matrix printer, and the graphs and tracks can be produced on the x–y plotter.

Analysis of Sample Populations and Statistics. Data from individual animals can be combined to form a sample population of animals responding to a given situation. For example, in our experiments, data recorded for individual fish identified by color-coded tags was stored in data files on floppy discs. Distance and velocity files for individual fish which had undergone similar experimental procedures were then combined, and composite files were created which contained the mean and variance at each sample interval. These represent the response of the sample population to the stimulus. Analysis of the results included a chi-square test to determine homogeneity of variance of replicate means. Two-way ANOVAs were used to determine if activity levels varied between treatments for individual fish.

The pH throughout each experiment was determnined from the video tape

record and transcribed into pH data files for graphic presentation along with the behavioral data (see Figure 4, bottom).

Sample Experiment

To illustrate how the system functions, a sample experiment will be outlined from its beginning to the final data presentation. Our objective is to determine the effect of pH on the response of juvenile salmonids to olfactory stimuli.

Protocol.

1. The test chamber receives water (3 liters/min) from overhead reservoirs (Figure 1), and an olfactory stimulus, in this case L-glutamine, is added to one of the inputs (chosen randomly).
2. Four fish are placed in the common area behind a barrier for a 15-min acclimation period.
3. The video camera is placed overlooking the test chamber and the pH electrode, automatic temperature compensation (ATC) probe, and pH meter are all calibrated with the probe and electrode in place at the center of the chamber. Output of the pH meter, including calibration and temperature, are overlayed on the video record of the experiment (Figure 1).
4. The experiment commences with the removal of the barrier (time = 0 sec).
5. A pH of 7.6 is maintained in the chamber for 30 min.
6. At 1800 sec (30 min), a pH change is initiated using a mixture of 50% H_2SO_4 and 50% HNO_3 (both 0.5 M). It takes 15 min to change from pH 7.6 to pH 5.1. A pH of 5.1 is held for the second 30 min.
7. At 4500 sec (75 min), the second pH change is initiated, returning the pH from 5.1 back to the original level of 7.6, over a 15-min period. A pH of 7.6 is maintained for the final 30 min.

All fish are identified with color-coded tags. At the beginning of the video tape, the experiment is given a code number, which is all the analysis technician will know about the experiment (for double-blind analysis). Pertinent data, such as stimulus type, stimulus concentration, and stimulus source location are recorded verbally at the end of the tape and in a separate notebook containing the key to the experiment's code number.

Track Digitization and Analysis of Data. Following entry of experimental information and analysis parameters, a series of calibrations begins. The first is the chamber outline procedure. The user is required to press the digitizer pen down at the corner boundaries of the chamber whose outline is then drawn on the screen and the user is asked if it is correct. If so, the distance calibration procedure follows. In this, the user is required to press the pen down at opposite ends of a 10-cm rule present in the test chamber. Again, the user is asked if

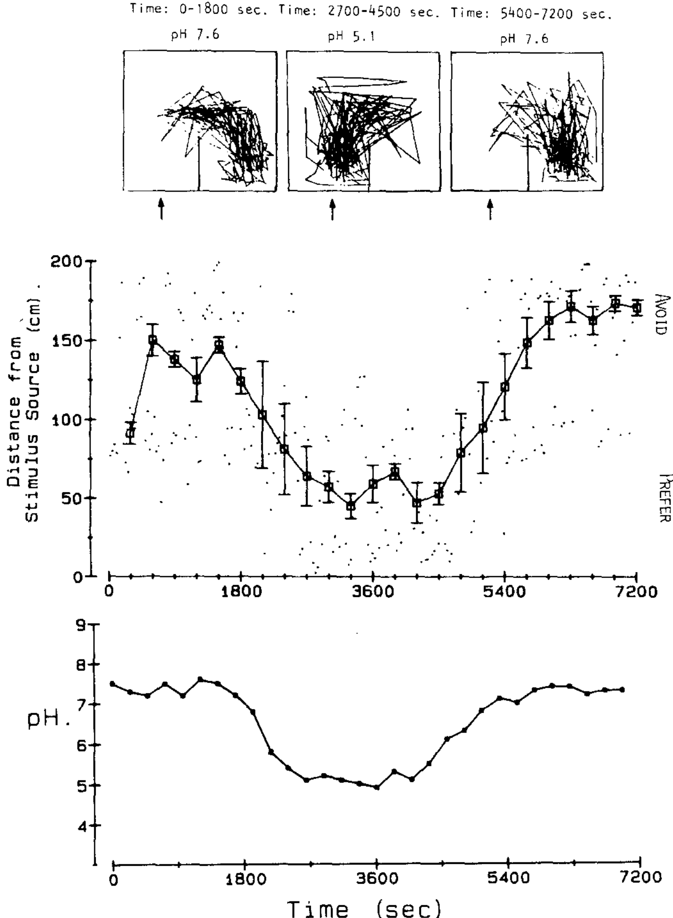


FIG. 4 Modulation of the behavioral response of twenty salmon to L-glutamine by changes in pH. Each animal was tracked for the entire experiment and mean distances (cm) from the stimulus source was calculated (see text). Top: The track of a fish during three different time periods. The arrows indicate the area receiving the L-glutamine (10^{-4} M). At pH 7.6 the fish spent most of its time away from the source of L-glutamine, but as the pH was lowered (see bottom plot), the fish's response changed to an attraction to L-glutamine. The effect was reversible with a return to pH 7.6. Middle: The mean distance from the stimulus source was determined every 300 sec for the twenty fish (squares, ± SEM). The dots represent means determined over every 30 sec, although sampled at 10-sec intervals. The closer the fish are to the stimulus, the more they prefer it. As the pH changes from pH 7.6 to pH 5.1, the avoidance changes to a neutral reaction and finally to an preference. Bottom: Record of the pH during the course of the experiment. Data was read off the video tape and entered into the computer by hand.

this is correct. All this information is placed at the beginning of a digitized track data file that will eventually contain the time, x coordinates, and y coordinates of the animal. Following this, the user begins the playback of the experimental video tape and simply tracks the animal using the digitizer pen to keep the targeting crosshairs on the image of the fish (Figure 2C). The track digitizing program accesses an onboard digital clock for time information and the digitizer tablet for the x–y coordinates of the targeting crosshairs. This information is written to RAM. The digitizer table continuously provides the computer with x and y coordinates during the tracking, but only x and y coordinates at user-defined sample intervals are recorded. This conserves memory and allows considerable flexibility in terms of track resolution. Following track digitization, the stimulus parameters are requested from the user. These include stimulus source(s) location(s), stimulus type and concentration. The user is then given the option of digitizing another track immediately or analyzing the one in memory. If the choice is the latter, the computer confirms the user's choice of time periods to be analyzed and then asks the user to wait while the analysis is conducted. Depending on the length of the experiment and the sampling rate, the analysis can take from less than 1 min for a short 30-min experiment with a data sampling rate of once every 20 sec, to 35 min for long experiments (2–5 hr) with high sampling rates (more than one sample per second). At the end of data analysis, a table summarizing the results appears on the screen. The user can then plot the results graphically. Hard copies of all or part of the data with graph can be produced by the printer and/or plotter (Figures 4–7). Following this, the track of the animal over user-specified time periods is drawn on the screen, with hard copies available (Figure 7).

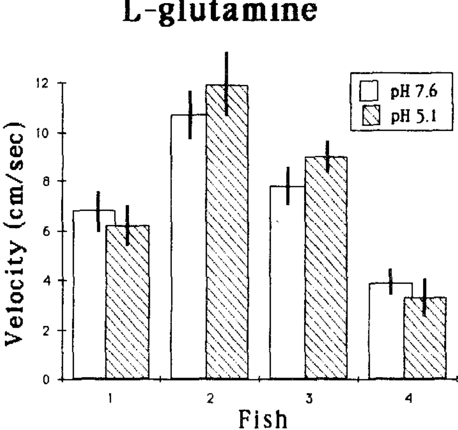


FIG. 5 Mean velocities (± SEM) of four individual fish at different pH levels with L-glutamine (10^{-4} M) present. The velocities were determined by sampling data at 10-sec intervals over 30-min periods of stable pH and then averaging these data.

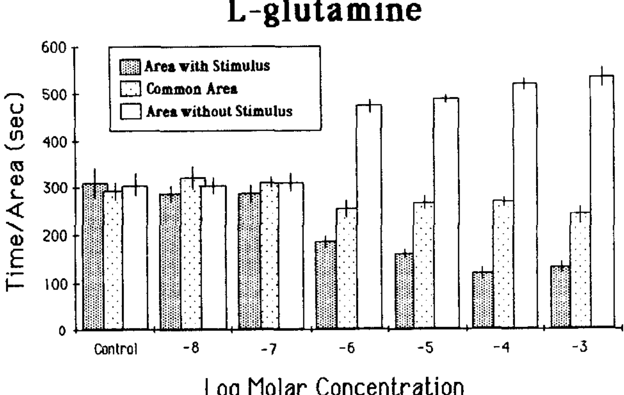


FIG. 6. Mean time (± SEM) spent per area at different concentrations of L-glutamine (N = 8 fish/concentration). The control periods did not have any stimulus present. Clearly, the avoidance of Atlantic salmon parr to L-glutamine is dose-dependent.

RESULTS

The ITS described in this manuscript was used to demonstrate that changes in acidity of water in our test chamber modified the behavioral response of juvenile Atlantic salmon (*Salmo salar*) to olfactory stimuli. We tracked each animal, in each experiment, and calculated its mean velocity, distance from the stimulus source, and time per area, every 10 sec. The data from 20 animals was compiled (15,000 data points), statistical determinations were performed by the computer, and the results plotted (Figure 4). The distance of a fish from the stimulus and time per area were used as an indication of avoidance or attraction to a given substance.

When L-glutamine (10^{-4} M) was perfused into one area of the test chamber (see Figure 1), fish spent most of their time in the area of the chamber farthest away from the stimulus (Figure 4, top), indicating an avoidance response at pH 7.6. This can be illustrated either by plotting the track of an individual fish (Figure 4, top), or by averaging the distance from the stimulus source over every 30 sec for all 20 fish (Figure 4, middle plot). The tracks at the top of Figure 4 also show that it is possible to view any portion of the experiment desired. The middle plot demonstrates that one can plot all, or part, of the data. The plot of the pH at the bottom of Figure 4 was obtained using the pH data as recorded on the original video tape of the experiment.

It is also possible to perform a number of other calculations with our software. For instance, we have determined the velocity of the animals over specific periods during control experiments (Table 1) and stimulus experiments (Figure 5). These data indicate that the acidic pH levels in these experiments do not

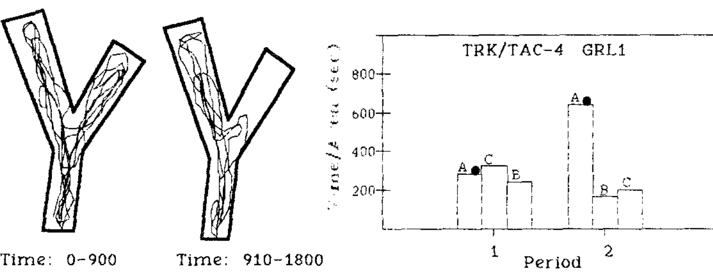
A. Data Table

Track file: TRK/TAC-4 GRL1
Number of periods examined: 2
# of entries in data file: 180

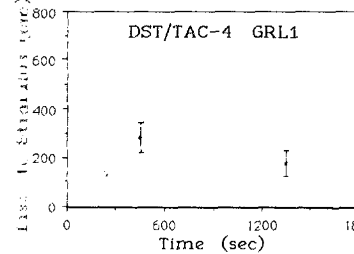
Viewing speed: 10X
Experiment duration: 1800 sec
Distance file: DST/TAC-4 GRL1

Period	Start time	End time	Mean Dist(cm)	SD	Mean Vel(cm/sec)	SD	Time (sec)/area A*	B	C
1	0	900	282.	61.5	1.64	.561	320	240	340
2	910	1800	180.	53.5	1.10	.155	650	130	120
Experimental totals:			231.	125.	1.37	1.26	970	370	460

B. Track C. Time/Area



D. Distance to Stim. E. Velocity



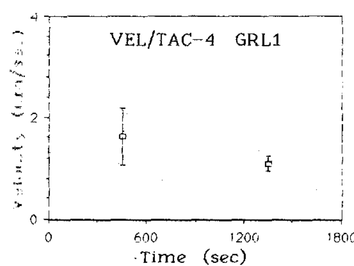


FIG. 7. Hard copy of results generated on a dot-matrix printer. An adult Atlantic salmon was tracked in a Y-maze during a two-part experiment: first a 15-min control period (no stimuli), followed by a 15-min experimental period with taurocholic acid (10^{-4} M) added to area A. (A) Results data table includes analysis parameters and pertinent file names and summary of results arranged by time periods. (B) Track of the fish over the two periods of the experiment (control, 0–900 sec; experimental, 910–1800 sec). Clearly the random movement of the fish during the control period changed to preference of the area containing taurocholic acid during the experimental period. (C) Histogram of time per area for the two time periods indicating a preference for the area with the stimulus only when the stimulus was present. (D) Distance scatterplot including mean (± SEM) of the two periods, again illustrating the preference for the area with stimuli only when it was present. (E) Scatterplot of velocity, including mean (± SEM) of the two periods indicating a fairly constant level of activity between the control and experimental periods.

TABLE 1. MEAN VELOCITY (cm/sec) OF FISH AT DIFFERENT pH LEVELS DURING SINGLE 2-HOUR CONTROL EXPERIMENT[a]

	Fish			
pH	1	2	3	4
7.6	9.14 ± 0.78[b]	7.23 ± 0.70	6.70 ± 0.69	10.37 ± 0.87
5.1	8.59 ± 0.90	6.93 ± 0.66	5.81 ± 0.76	10.75 ± 0.65
7.6	8.31 ± 0.79	6.38 ± 0.66	5.71 ± 0.65	9.45 ± 0.79

[a]These were control experiments in which there was no stimulus present. Means were obtained from data sampled at 10-sec intervals (see text for experimental protocol).
[b]Mean ± standard error mean.

effect the general activity of the animal. In Figure 4 (top), the tracks of the fish show that activity levels stay fairly constant between pH levels. Statistically (ANOVA), there is a significant ($P \leq 0.05$) difference in velocities among different fish, while there is not a significant ($P \geq 0.1$) difference in the velocity of the same fish at different pH levels. We have plotted a dose–response curve illustrating that the response of these salmon to a particular odor is dose-dependent (Figure 6). It is also possible to a do vector analysis over any given time period to determine an animal's orientation and/or directionality. These calculations illustrate that once the digitized tracks of all the animals are stored in appropriate data files, one can rapidly manipulate the data in a variety of different ways, depending on need.

DISCUSSION

The behavioral modification experiments described here, both in the laboratory and in the field, shown the high potential of ITS for quantitative analysis of behavior. There are similar systems on the market which track animals automatically. However, we chose not to fully automate the system for five reasons. First, we did not want to miss subtle, yet important, information that may not be recognized by a fully automated system. Second, it is not necessary to have large differences in contrast between object to be tracked and the background. Often, especially in the field, conditions were not suitable for a system that automatically tracks dark objects against a light background or vice versa. Third, although one has to repeat the analysis for each animal if there is a group of animals present, there is no problem with paths of the animals crossing. Fourth, we wanted sufficient flexibility to be able to analyze any suitable videotaped behavioral experiment. Many researchers are turning to video as a means of recording behavioral experiments. Our system (ITS) can be used to

analyze any appropriate experiments that can be recorded on video tape. Thus, a large number of researchers with varied interests can simply video tape their experiment and bring the video tape to the video–computer station in the laboratory for analysis. Finally, this arrangement allows ITS to be used for analyzing field data. We have already used it successfully to study the role of pheromones in horseshoe crab (*Limulus polyphemus*) mating behavior and the response of adult Atlantic salmon to olfactory stimuli (Figure 7).

The ITS system allows us to store data from six complete 2-hr experiments, sampled at 1-sec intervals, on a single disc, whereas other video digitizers can only store 3 min of video picture frames per disc (Kaufmann, 1983). This is because a single video frame can contain up to 52,000 bits of data and at the normal rate of 60 video frames per second, 3 min of video generates 936,000 bits of data that, when stored, will fill a data disc. While these systems need all this information in order to track a given subject automatically, our system saves memory by having a technician track the animal. The memory thus saved can then be used to store the data from a number of complete experiments in less space.

The analysis program(s) in our system can handle large amounts of data at a moderately high speed and make statistical determinations which previously would have required many man hours. This short turn-around time is beneficial because, after viewing results from one run, the investigator could rapidly modify the experimental protocol to more effectively investigate the behavior.

While there are several comparable systems available commercially or described in the literature, they tend to be much more expensive. The system we have described would cost approximately $5000 for the total assembly. However, most modern laboratories are already equipped with a microcomputer and video equipment. Therefore, the only item required would be the special-effects generator ($700) and the software. Further development of data analysis software should give rise to a wide application package that may lead to routine quantification and standardization of sensitive behavioral bioassays in the environmental monitoring field.

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DIFFERENCES IN ATTRACTION TO SEMIOCHEMICALS PRESENT IN SYMPATRIC PINE SHOOT BEETLES,[1] *Tomicus minor* AND *T. piniperda*[2]

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Abstract—The chemical ecology of host- and mate-finding in the pine shoot beetles, *Tomicus minor* and *T. piniperda*, was studied in southern Sweden. Beetles were collected in the field from defined attack phases on Scots pine. Using gas chromatography–mass spectroscopy, a number of oxygen-containing monoterpenes, e.g., 3-carene-10-ol, myrtenol, *trans*-verbenol, and verbenone, were identified from hindgut extracts of both sexes of both species. Compared to *T. minor*, *T. piniperda* contained additional compounds and in larger amounts. The amounts were highest in both species at the time when the beetles had bored into contact with the resin-producing xylem-phloem tissue. The synthesis of (1*S*,6*R*)-3-carene-10-ol by photooxidation of (+)-(1*S*,6*R*)-3-carene is described. In comparative electroantennogram (EAG) measurements on males and females of both species, the most active of the tested compounds was *trans*-verbenol. Laboratory bioassays of walking beetles showed that *T. piniperda* was attracted to uninfested pine logs. *T. minor* was more strongly attracted to pine logs infested with females than to uninfested pine logs, indicating a female-produced aggregation pheromone. Field tests confirmed that *T. piniperda* was strongly attracted to pine logs. The attraction of *T. minor* to logs was significant only when logs were combined with racemic *trans*-verbenol and (1*S*,6*R*)-3-carene-10-ol. *T. minor* was also

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attracted to a combination of these monoterpene alcohols alone. We suggest that host and mate location in *T. piniperda* is achieved by means of a kairomone composed of host monoterpenes, while *T. minor* utilizes a primitive pheromone synergized by host odors. Evolution of host colonization strategies of the two beetles are discussed.

Key Words—Host attraction, pheromone, *Pinus sylvestris*, *trans*-verbenol, 3-carene-10-ol, myrtenol, verbenone, EAG, kairomone, *Blastophagus*, *Tomicus minor*, *Tomicus piniperda*, Coleoptera, Scolytidae, pine shoot beetles.

INTRODUCTION

The lesser pine shoot beetle *Tomicus minor* (Hart.) and the larger pine shoot beetle *Tomicus piniperda* (L.) (synon. *Blastophagus* Eich., synon. *Myelophilus* Eich.) (Coleoptera, Scolytidae) are both present in the palearctic region from Europe to Siberia and Japan. The two species are sympatric and mostly colonize Scots pine, *Pinus sylvestris* (L.), in western Europe but occasionally other trees. Both species hibernate as adults and disperse in the spring as soon as the daily temperature exceeds about 12°C. They usually establish galleries in the phloem of windthrown or otherwise weakened trees. *T. minor* is usually found in the smoother bark on the branches or higher trunk while *T. piniperda* prefers the thicker bark. Females in both species initiate galleries in the bark and are followed by a male. After two to three months, the brood emerges and flies to shoots of the current year's growth where they feed and mature. As a result of this feeding, both species cause growth losses and pruning of shoots. In addition to these effects, *T. minor* carries fungi that cause blue staining of the wood (cf. Francke-Grosmann, 1952). The bionomics of these species and their silvicultural importance have been reported by Richtie (1917), Bakke (1968), Postner (1974), and Långström (1983).

Kangas et al. (1970b) found that a volatile pine fraction, containing α-terpineol, and *cis*- and *trans*-carveol, was attractive to *T. minor* beetles in laboratory bioassays. Bakke and Jordal (personal communication) found *trans*-verbenol in hindguts of *T. minor*, and their field trapping experiments indicated that this compound may act as a pheromone component.

Earlier studies on the semiochemical system of *T. piniperda* have searched for aggregation pheromones (Schönherr, 1972; Carlé, 1978; Carlé et al., 1978; Francke and Heemann, 1976; Byers et al., 1985) with no apparent success, while evidence for a strong host attraction has accumulated (Kangas et al., 1965; Löyttyniemi and Hiltunen, 1976; Byers et al., 1985; Schroeder and Eidmann, 1986).

The aim of this study is to investigate the role of host- and beetle-produced odors in the host- and mate-finding of *T. minor* and *T. piniperda*.

METHODS AND MATERIALS

Collection of Beetles and GC-MS Analyses of Gut Volatiles. Beetles of both species were collected at Blentarp, 20 km southeast of Lund in the southernmost province of Sweden, from March 26 to April 15, 1982, while the beetles were dispersing and constructing galleries on Scots pine. *T. minor* were collected from several windthrown trees and large branches, while *T. piniperda* were taken from four windthrown trees (A–D). The diameter at breast height (DBH) of trees A, B, and C was approximately 30 cm and tree D was 15 cm. Tree C was cut into three sections, and the beetles were taken from the middle section. The beetles were treated and stored in liquid nitrogen as described by Birgersson et al. (1984).

Both *T. minor* and *T. piniperda* were separated according to the attack phase during their gallery construction in host trees (see Table 1). Attack phases were chosen in such a way that comparisons of the two species would be facilitated. However, an exact correspondence between phases in the two species was not possible to achieve because of their different behaviors (Table 1). Therefore, we divided the phase "pairs boring" into three subphases for *T. piniperda* according to the length of the tunnel made. Under favorable weather conditions, the time span between finding a suitable host (phase "walking") to egg-laying is three to six days.

Hindguts were dissected and extracted essentially as described by Birgersson et al. (1984). Females were also examined for presence of sperm (filled spermatheca) or eggs. Pentane extracts obtained from 8 to 25 guts were transferred to small glass ampoules which were sealed under nitrogen and stored at −20°C until analysis with a Finnigan 4021 gas chromatograph–mass spectrometer. GC capillary columns used were 25 m × 0.2 mm ID or 50 m × 0.35 mm ID of fused silica coated with OV-351 (Alltec Assoc.), film thickness 0.57 μm, or with Superox FA (Supelco Inc.), film thickness 0.37 μm, respectively. The terpenoid compounds analyzed in this study elute in the same order from both stationary phases. Helium was used as carrier gas at a velocity of 0.3 m/sec. The injector temperature was 210°C. After injection, the GC oven temperature remained at 50°C for 4 min and then was increased to 230°C at 8°C/min.

Heptyl acetate (30 ng/μl pentane) was added as an internal standard to the extraction solvent for quantitative calculations and comparisons of retention characteristics. Heptyl acetate was chosen as its retention time neither interferes with monoterpene hydrocarbons nor their oxygenated derivatives on the stationary phases used in this study. In our material, however, the heptyl acetate was to some extent (less than 15%) transformed to 1-heptanol and very small amounts of heptanal. For the quantitative determinations the sum of heptyl acetate, heptanol and heptanal were assumed to equal the amount of heptyl acetate originally added. A response factor of 1 was used for the quantification of the

TABLE 1. DESCRIPTION OF ATTACK PHASES, MATING, AND FEMALE EGG NUMBERS

Attack phases	No of beetles collected[a]				Females mated (%)		Eggs in ovaries ($\bar{X}$)[b]	
	T. minor		*T. piniperda*					
	♂	♀	♂	♀	*T. minor*	*T. piniperda*	*T. minor*	*T. piniperda*
Hibernating	0	0	49	32	—	28	—	0
Walking on host bark	10	8	14	26	25	46	0	0
Single beetles in tunnel, boring just started	0	69	22	26	49	81	0.1	0
Pairs of beetles in tunnels[c]	93	106	—	—	79	—	0.2	—
1. male waiting at entrance	—	—	79	55	—	87	—	0
2. male inside tunnel	—	—	40	47	—	81	—	0
3. as 2. and resin contacted	—	—	33	34	—	85	—	0
Nuptial chamber formed	42	46	166	138	93	97	1	0.7
Egg laying								
Galleries <2 cm	17	17	0	0	100	—	1.3	—
Galleries >2 cm	23	21	55	56	100	100	1.3	0.8
	185	267	458	414				
	452		872					

[a] All insects collected were used both for studies of mating and egg content and for analyses of volatiles in guts.
[b] 0–3 eggs in *T. minor*, 0–2 eggs in *T. piniperda*.
[c] Attack phase "Pairs" in *T. piniperda* was separated into three subphases according to the length of the tunnel and the position of the male, as this species was sampled from thicker bark than *T. minor*. In the smaller species, formation of pairs proceeds more rapidly due to the thinner bark.

oxygenated monoterpenes relative to the internal standard. At least 0.05 ng/ extract was required for a reliable quantification of identified compounds.

Chemicals. For all identified compounds, synthetic references were available so that relative retention times and mass spectra could be compared with the compounds in the insect extracts. Myrtenal was obtained from myrtenol by treatment with chromium trioxide dipyridine complex in acetic acid (Baeckström, 1978) and analyzed immediately after synthesis. (1*S*,6*R*)-3-Carene-10-ol was obtained for reference by lithium aluminium hydride reduction of chaminic acid from the cypress *Chamaecyparis nootkatensis* (Lamb.).

The chemicals used for electroantennography, laboratory bioassay, and field trapping were 2-methyl-3-buten-2-ol, 97%; (*R*/*S*)-ipsenol, 85%; (+)-(1*R*,4*S*,5*R*)-*trans*-verbenol here called (*R*)-*trans*-verbenol, containing 12% *cis*-verbenol and <0.1% verbenone; (−)-(1*S*,4*R*,5*S*)-*trans*-verbenol here called (*S*)-*trans*-verbenol, containing <5% (*R*)-isomer and 12% *cis*-verbenol and 1% verbenone; (*R*/*S*)-*cis*-verbenol, containing 12% *trans*-verbenol; (*R*/*S*)-verbenone, 80%; (−)-*cis*-myrtanol, 95%; 2-phenylethanol, 99%; (*R*/*S*)-frontalin, >95%; (*R*/*S*)-*exo*-brevicomin, >95%; (−)-(1*S*,5*S*)-α-pinene and (+)-(1*R*,5*R*)-α-pinene here referred to as (*S*)-α-pinene and (*R*)-α-pinene, 99%; (1*S*,6*R*)-3-carene, $[\alpha]_D^{20} = +21°$; terpinolene, >99%; and myrcene, >99.8%. The chemical standards were obtained from Aldrich Chem. Comp., Steinheim, FRG, Borregaard Ind. Ltd, Sarpsborg, Norway, Chem. Samples Co., Ohio, US, or Fluka AG, Buchs, Switzerland.

Synthesis of (1S,6R)-3-Carene-10-ol for Biological Tests. Gollnick et al. (1965) and Gollnick and Schade (1966) have described a synthesis of (1*S*,6*R*)-3-carene-10-ol (7) from (1*S*,6*R*)-3-carene and their scheme was followed, although each step in the sequence was modified (see Figure 1). The photooxidation of 3-carene (1) was carried out in the presence of $TBABH_4$ according to Baeckström et al. (1982), and the crude reaction mixture was subjected to medium-pressure liquid chromatography (MPLC) on silica gel. The fractions containing monooxygenated products were combined and selectively acetylated by treatment with equal amounts of acetic anhydride and pyridine at room temperature. The acetate (5) of alcohol (4) was isolated by MPLC on silica gel and hydrolyzed with KOH in methanol. This gave the crystalline alcohol (4) which was transformed to the aldehyde (6) by an oxidative rearrangement using PCC and pTSOH in dichloromethane (Baeckström et al., 1982). Reduction of the aldehyde (6) with LAH gave the desired alcohol (7). The absolute configuration of the enantiomers of acid (8) have been determined. The (+)-(1*S*,6*R*)-enantiomer is called chaminic acid and the (−)-(1*R*,6*S*)-enantiomer is called isochamic acid (Norin, 1964). The aldehyde (6) was oxidized to the corresponding acid with sodium chlorite (Lindgren and Nilsson, 1973), and its specific rotation, [^{1}H]NMR spectrum, and melting point were found to be the same as those of chaminic acid (8) [m_p = 104.5–105.5°C, Lit. = 103–106°C, $[\alpha]_{589} = +7.2°$ (c = 3.4, MeOH), Lit. = +6 (c = 3.9, MeOH)].

FIG. 1. Reaction scheme for synthesis of 3-carene-10-ol (7) from 3-carene (1).

Electroantennography (EAG). Antennae were prepared according to Van Der Pers et al. (1984). The EAG responses were monitored on an oscilloscope, and maximum amplitudes recorded by a digital voltmeter as described by Bjostad and Roelofs (1980). The stimulation technique was essentially the same as that described by Van Der Pers (1981). A piece of filter paper (2 cm^2), with the test compound was put into a disposable 5-ml plastic syringe. No correction was made for differences in volatility among the test compounds. For each test run on an antenna the "mean response" was defined by subtracting the blank response from all measurements and then calculating the mean of the resulting amplitudes from 12 straight-chain alcohols (methanol to dodecanol), 2-methyl-3-buten-2-ol, ipsenol, *cis*- and *trans*-verbenol, verbenone, myrtenol, *cis*-myrtanol, phenylethanol, frontalin, and *exo*-brevicomin. All EAG responses are expressed as percent of this "mean response."

Laboratory Bioassay of Walking Beetles. T. minor were excise from windthrown pine trees two to three days after the first dispersal period. *T. piniperda* were picked during dispersal days while they were walking on pine trunks in a forest 30 km east of Lund, in southernmost Sweden. Beetles were

kept in the dark, 100% relative humidity, 4°C until used for testing. The open arena bioassay was as described by Byers and Wood (1981). Ten beetles at a time were released 20 ± 2 cm from the odor source on an open arena with a laminar air flow, approximately 0.6 m/sec. A beetle was recorded as attracted if it had approached closer than 1 cm from the odor source within 2 min after release. Unresponsive beetles were given a second try. Odors from pine logs for bioassay were obtained either directly by pumping air through a glass jar with a log, or indirectly after adsorption on Porapak Q and diethyl ether solvent extraction. In the latter case the adsorbed components were fractionated with capillary GC as described by Byers et al. (1985). The fractionated host compounds and the synthetic compounds were released from 5-μl glass capillaries placed in the air stream.

Field Trapping. The attractivities of a mixture of the racemic *trans*-verbenol and (1*S*,6*R*)-3-carene-10-ol as well as host odors were tested in three field-trapping experiments. The monoterpene alcohols were used in three different baits: alone (MT-OH), together with fresh pine logs (Log + MT-OH), and together with purified host monoterpenes (MT-OH + MT). The host monoterpenes used, α-pinene, terpinolene, and 3-carene, had earlier been found to be attractive to *T. piniperda* (Byers et al., 1985).

Trapping was done in a homogeneous, evenly aged (about 70 years) pine stand 30 km east of Lund, Sweden, in April–May in 1983 and 1984. The trap logs were covered with brass nets (No. 60) to prevent arriving beetles from boring. Logs and vials with synthetic terpenes were surrounded by sticky trap, hardware cloth (No. 4), and coated with Stickem Special® (Byers and Wood, 1980). The monoterpene alcohols were each released from a 1-ml polyethylene vial (Kartell, Italy), loaded with 50 μl of the compound. The release rate of (*R*)-*trans*-verbenol from such vials was estimated by weight loss over 21 days in the laboratory to be 0.20 mg/day ($N = 8$, $r = 0.999$, hole size 4.2 mm, 20°C). The release rates of α-pinene, 3-carene, and terpinolene were each about 30 mg/day at 18°C. Treatments were assigned at random to traps placed on steel poles 1.5 m above ground and 10 m apart in a line. Rearrangement of treatment traps and collection of beetles were performed after at least 15 beetles were caught on the most attractive trap. The catches from 1984, 12 replicates, were transformed according to log (catch + 0.25) which gave homogeneous variances before ANOVA followed by Duncan's multiple-range test.

RESULTS

Attack Phases, Mating, and Egg-Laying. When walking on the host tree before entering the bark, 25% of *T. minor* and 46% of *T. piniperda* females were mated, and in both species at least 90% were mated when the nuptial chambers had been constructed (Table 1). In *T. piniperda* no fully developed

eggs could be found in the ovaries until after the nuptial chambers had been completed, while in *T. minor* some beetles contained eggs just after they had started boring ($\overline{X}$ = 0.1 eggs per female). When construction of egg galleries had begun, the mean number of eggs per female was 1.3 for *T. minor* and 0.8 for *T. piniperda*.

Volatile Compounds in Hindgut. The major volatiles found in the hindguts of the two pine shoot beetles were oxygenated monoterpenes (Table 2). In addition, smaller amounts of monoterpene hydrocarbons and sesquiterpenes were found. In *T. minor*, both the number of compounds and the amounts of detectable oxygenated monoterpenes were lower than in *T. piniperda*. Three compounds, 3-carene-10-ol, *trans*-verbenol, and myrtenol, dominated in both sexes of the two species (Table 2). Less than 2 ng per beetles of *cis*-verbenol, verbenone, and myrtenol were present in *T. minor* and less than 4 ng per beetle of verbenone, 3-carene-10-al, myrtenal, and borneol were found in *T. piniperda*. Small amounts (< 5 ng/beetle) of a few unidentified oxygen-containing monoterpenes were sometimes found, and none of these compounds were detected in both species.

In general the amounts of the oxygenated monoterpenes reached a maximum in both species when the beetles had bored through the outer bark and contacted the resin-containing phloem and xylem. In the phases following construction of the nuptial chamber, the amount of oxygenated terpenes decreased and continued to decline during egg-laying. This pattern is illustrated in Figure 2A–C for the three major oxygenated terpenes, *trans*-verbenol, 3-carene-10-ol, myrtenol, and for verbenone (Figure 2D).

TABLE 2. OXYGENATED MONOTERPENES IN GUT OF PINE SHOOT BETTLES WHEN BORING IN RESIN-CARRYING TISSUE (ATTACK PHASE: "PAIRS")

	Monoterpene, ng/beetle (mean value ± standard deviation)			
	Tomicus minor		*Tomicus piniperda*	
Compound	93 ♂ $N = 5^a$	100 ♀ $N = 6^a$	73 ♂ $N = 4^a$	81 ♀ $N = 5^a$
trans-Verbenol	2.8 ± 2.0	6.8 ± 5.8	6.8 ± 0.9	6.4 ± 1.2
cis-Verbenol	0	0.3 ± 0.3	0	0
Verbenone	1.0 ± 0.4	0.4 ± 0.3	3.5 ± 0.6	2.3 ± 0.5
3-Carene-10-ol	11.6 ± 5.9	7.2 ± 5.9	27.9 ± 10	10.9 ± 3.5
3-Carene-10-al	0	0	0.3 ± 0.3	0.4 ± 0.1
Myrtenol	1.6 ± 1.0	1.4 ± 1.4	15.6 ± 4.4	9.4 ± 2.2
Myrtenal	0	0	0.6 ± 0.2	0.5 ± 0.2
Borneol	0	0	1.6 ± 0.8	1.0 ± 0.3

[a] N = number of extracts into which the beetles were divided at analysis.

The guts of *T. minor* females on the average contained more *trans*-verbenol than their conspecific males (Figure 2A). *T. piniperda* males, compared to the females, generally seemed to contain somewhat larger amounts of all the terpenes. However, it should be noted that the range of variation of samples prepared from the same sex and attack phase exceeds the differences between the sexes.

To determine the influence of various host trees on the content of oxygenated monoterpenes in *T. piniperda*, beetles were separated and extracted according to the specific tree they attacked. Table 3 shows the chemical analyses of beetles from the phase in which nuptial chambers have been formed. The insects collected from trees A and D contained significantly larger amounts of oxygenated monoterpenes than those collected from tree B and log C ($P < 5\%$, Wilcoxon signed-ranks).

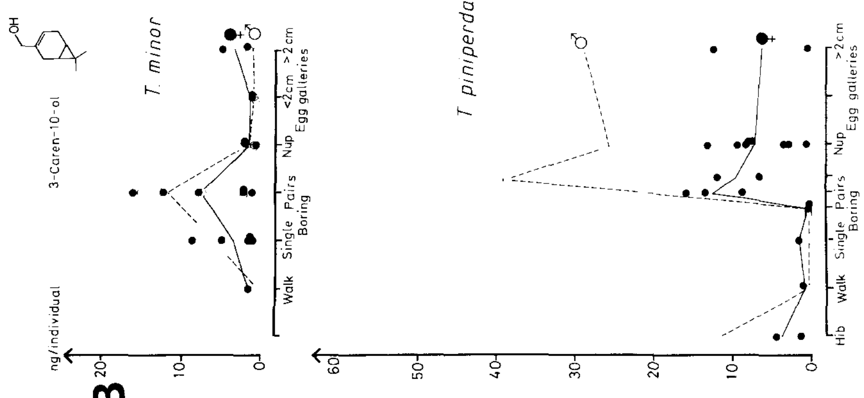
Electroantennography. As a first step in determining the biological activities of the compounds identified in beetles, we recorded EAG responses in both species (Figure 3). In addition, we tested the response to some compounds attractive to other Scolytidae, as well as some host pine monoterpenes (Figure 3), and a homologous series of 12 primary straight-chain alcohols (methanol to dodecanol, not shown in the figure) was included. The straight-chain alcohols evoked a similar response pattern in both species and sexes. The responses increased with chain length to a maximum between pentanol to heptanol and then decreased with further increase in chain length. The amplitude of these responses to the alcohols were of the same order of magnitude as the other compounds tested.

For *T. minor* the strongest response was elicited by *trans*-verbenol followed by *cis*-verbenol. In *T. piniperda*, on the other hand, verbenone evoked the strongest response followed by *cis*-verbenol for the females and *trans*-verbenol for the males. However, the differences in response to *trans*-verbenol and verbenone were not significant either between the species or the sexes ($P < 5\%$, Student's t test).

Of the three major monoterpene alcohols in the beetles guts, *trans*-verbenol evoked a significantly higher response in both species and sexes compared to 3-carene-10-ol ($P < 5\%$, Wilcoxon signed rank), while myrtenol gave an intermediate response. No significant difference was observed for either species when comparing the two enantiomers of *trans*-verbenol at the same dose.

When *T. minor* antennae were exposed to different doses of (1*S*,6*R*)-3-carene-10-ol and the two enantiomers of *trans*-verbenol, both *trans*-verbenols caused signals at least twice as strong as those from 3-carene-10-ol, and they also had lower thresholds (Figure 4). The (*S*)-*trans*-verbenol was slightly more active in the tests than the (*R*)-enantiomer, but the difference was not significant.

The antennae of both species were also exposed to some major host tree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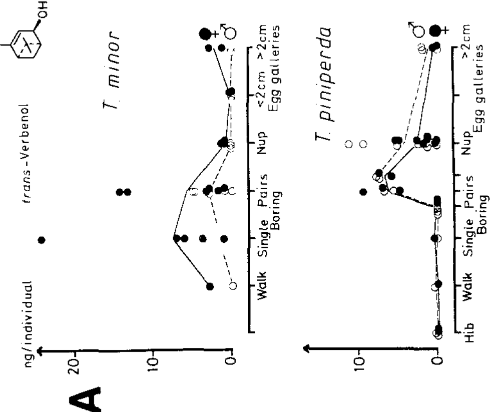
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Single Pairs
Boring
Nup
>2cm
Egg galleries

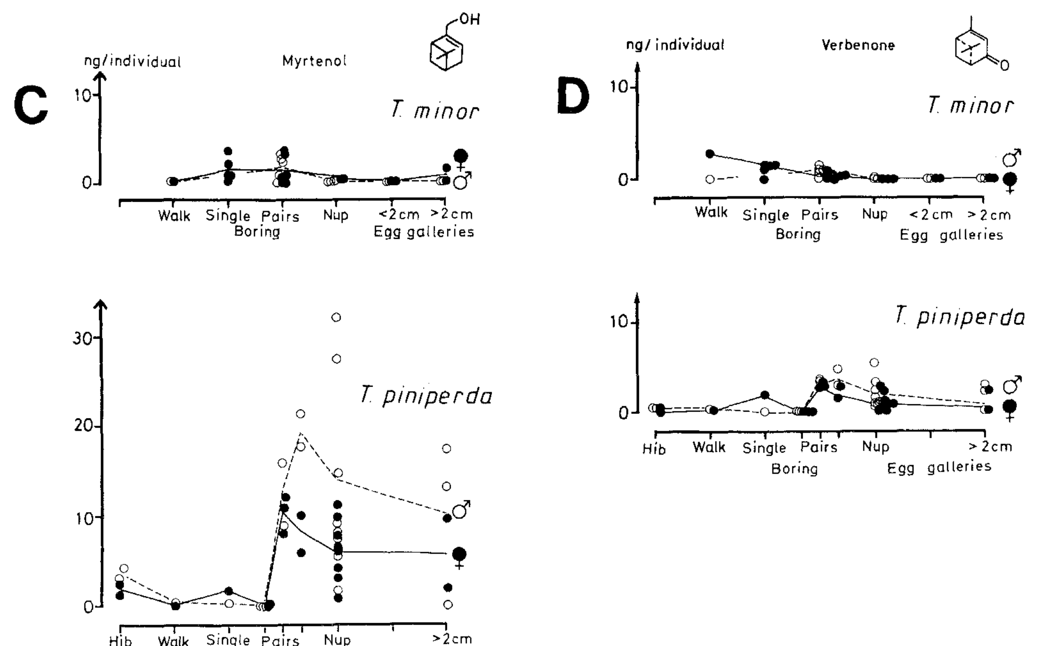

FIG. 2. Amount of (A) *trans*-verbenol, (B) 3-carene-10-ol, (C) myrtenol, and (D) verbenone in guts of *T. minor* and *T. piniperda* in different attack phases. Each point gives the result of an analysis of 8–25 beetles, expressed as nanograms compound per beetles. The broken lines for males and solid lines for females connect the mean amounts for all beetles of each phase and sex. See Table 1 for description of attack phases.

TABLE 3. OXYGENATED MONOTERPENES IN GUTS OF *T. piniperda* ATTACKING FOUR DIFFERENT TREES (ATTACK PHASE: NUPTIAL CHAMBER FORMED)

Compounds		Monoterpenes (ng compound/beetle)			
		Tree A ♂ N = 50 ♀ N = 47	Tree B ♂ N = 43 ♀ N = 39	Tree C ♂ N = 55 ♀ N = 33	Tree D ♂ N = 15 ♀ N = 19
trans-Verbenol	♂	7.59	2.81	0.80	9.33
	♀	3.06	1.80	0.47	4.95
Verbenone	♂	3.77	1.57	0.68	3.29
	♀	1.24	0.66	0.14	2.12
3-Carene-10-ol	♂	45.8	22.8	7.18	32.8
	♀	6.50	10.3	2.50	7.78
3-Carene-10-al	♂	0.48	0.36	0.13	1.41
	♀	0.25	0.21	0.00	0.27
Myrtenol	♂	22.8	11.1	4.94	27.4
	♀	5.80	6.49	3.33	9.9
Myrtenal	♂	0.37	0.28	0.20	0.43
	♀	0.33	0.18	0.00	0.35
Borneol	♂	1.24	0.76	0.22	1.03
	♀	0.58	0.84	0.21	0.58
Unidentified	♂	1.19	0.61	0.16	1.46
	♀	0.38	0.59	0.00	0.48
Unidentified	♂	0.82	0.55	0.44	1.53
	♀	0.37	0.31	0.17	0.72
Unidentified	♂	2.90	2.15	0.78	3.64
	♀	0.74	1.34	0.30	0.98
Σ (ng/beetle)	♂	87	43	15.5	82
	♀	19	23	7.1	28

monoterpenes: (*R*)- and (*S*)-α-pinene, (1*S*,6*R*)-3-carene, terpinolene, and myrcene. The responses to these compounds were generally lower than to the oxygenated monoterpenes. No differences between sexes were found, while *T. piniperda* seemed to respond more strongly than *T. minor* to these host compounds.

Laboratory Bioassay with Walking Beetles. Behavioral tests were carried out under controlled conditions with beetle- and host-produced compounds. In the open-arena olfactometer both sexes of *T. minor* were attracted to odors from a pine log, while introduction of females into the log further increased the attraction of both sexes (Table 4, experiment 1). In contrast, *T. piniperda* beetles

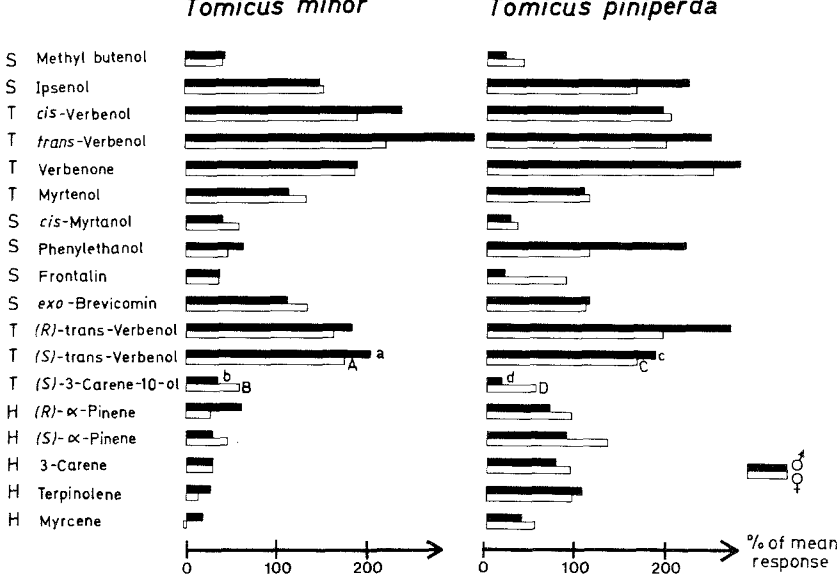

FIG. 3. EAG response of *T. minor* and *T. piniperda*, to a series of oxygenated monoterpenes produced by *Tomicus* (T), other scolytid pheromone substances (S), and host monoterpenes (H). Each insect antenna was exposed to the chemicals in the order they appear in the figure. Each compound (500 μg) was added on filter paper. For the comparison between (*S*)-*trans*-verbenol and (*S*)-3-carene-10-ol, within each species and sex, bars marked with different letters denote significantly different responses ($P < 5\%$, Wilcoxon signed-rank). Sample size was 20/16 (males/females) in *T. minor* (host compound 6/6) and for *T. piniperda* 8/15.

were strongly attracted to the pine log alone with no apparent increase in attractivity when conspecific females were present in the log. In experiment 2, Table 4, the attractiveness of the synthetic compounds, (*R*)- and (*S*)-*trans*-verbenol and (1*S*,6*R*)-3-carene-10-ol, to *T. minor* males was compared and 3-carene-10-ol was not found to be attractive. In experiment 3 the same compounds were tested together with the monoterpene hydrocarbon fraction from pine. Again, the response to (1*S*,6*R*)-3-carene-10-ol was not significantly different from the solvent alone, but the combination of host terpenes and (*S*)-*trans*-verbenol was active. The strongest response (97%) of *T. minor* males was to a combination of a pine log and a low release of synthetic (*S*)-*trans*-verbenol (experiment 4).

Field Trapping. In the first test (Table 5, A1), performed towards the end of the flight period in 1983, indications were found that *T. minor* was attracted by the three alcohols (*R*)- and (*S*)-*trans*-verbenol and (1*S*,6*R*)-3-carene-10-ol alone (MT-OH) and in combination with pine logs. Similarly, a subtractive

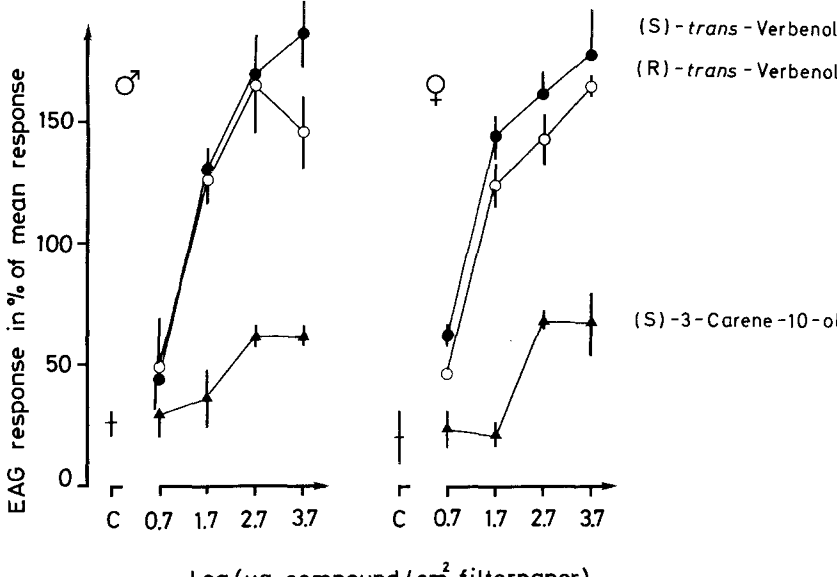

FIG. 4. Mean EAG response of *T. minor* to different doses of (*S*)- and (*R*)-*trans*-verbenol and (1*S*,6*R*)-3-carene-10-ol applied as hexane solutions on a filter paper. Control was hexane alone. Bars represent the standard deviation.

assay of the three alcohols at the end of the 1983 flight period gave no significant differences in attraction of either species between logs baited with (1): MT-OH, (2): (*R*)+(*S*)-*trans*-verbenol, (3): (*S*)-*trans*-verbenol + (1*S*,6*R*)-3-carene-10-ol, or (4): (*R*)-*trans*-verbenol + 3-carene-10-ol. The pooled catch of *T. minor* had 68.1% males (N = 91, 95% CI-58–77), while *T. piniperda* catch was 51.2% males (N = 374).

The second test (A2) in 1984, started in the middle of the flight period, showed that traps containing *trans*-verbenols, 3-carene-10-ol, and a fresh pine log caught more *T. minor* than any of the other four treatments and was significantly different from a log alone or the blank (Figure 5). The difference in total catch between a log alone (31 beetles) and a log with the monoterpene alcohols (275 beetles) was especially prominent. For *T. minor* the baits containing the monoterpene alcohols alone differed significantly from the blank, while the log alone was not significantly different from the blank.

For *T. piniperda*, all three baits containing either pine log or the host monoterpene hydrocarbons caught significantly more beetles than the blank. The bait with the monoterpene alcohols alone did not catch more than the blank. An increase in catch was obtained when the monoterpene alcohols were added to the log, but this increase was not statistically significant.

TABLE 4. ATTRACTIVITY OF NATURAL AND SYNTHETIC VOLATILES IN LABORATORY BIOASSAY

	Beetles responding, % (N = 30)			
	Tomicus minor		*Tomicus piniperda*	
Stimulus	Male	Female	Male	Female
Experiment 1				
Pine log	20 ″ Ψ[c]	17 ″ Ψ	73 ″ Ψ	67 ″ Ψ
Pine log infested with 30 conspecific females	90 § Ψ	87 §	63 ″ Ψ	63 ″
Experiment 2				
(*S*)-*trans*-verbenol + (*R*)-*trans*-verbenol[a]	47 §			
(*S*)-3-carene-10-ol	7 ″			
(*S*)-*trans*-verbenol	27 ″ §			
(*R*)-*trans*-verbenol	23 ″ §			
Experiment 3				
Pine fraction[b]	20 ″			
Pine fraction + (*S*)-*trans*-verbenol	57 §			
Pine fraction + (*R*)-*trans*-verbenol	33 ″ §			
Pine fraction + (*S*)-3-carene-10-ol	7 ″			
Experiment 4				
Pine log	67 ″	40[d] ″		
Pine log + (*S*)-*trans*-verbenol	97 §	60 ″		

[a] 220 ng/min of monoterpene alcohols in diethylether were released from capillaries in exp. 2 and 3; and 70 ng/min released from a closed 1-ml PE vial in exp. 4.
[b] Monoterpene hydrocarbon fraction in diethyl ether after entrainment and GC fractionation.
[c] ″, §, Values followed by the same symbol are not significantly different within a species and sex by chi-square corrected for continuity at $P < 5\%$ (significance level adjusted for number of comparisons) in each experiment. Ψ, Response different between species within sex by chi-square corrected for continuity at $P < 5\%$.
[d] 40 females tested.

T. minor males were caught in a significantly higher proportion than females in the traps containing both a log and the monoterpene alcohols in all three tests (Table 4). For *T. piniperda*, on the other hand, no sex ratios significantly differed from unity.

DISCUSSION

Pheromone Activity in Tomicus. In *T. piniperda* (including *T. destruens* Woll.), Schönherr (1972) and Carlé (1974, 1978) report indications of pheromonal attraction to beetle infested logs. However, appropriate statistical tests and controls are lacking in these three studies. Kangas et al. (1970a) and Carlé

TABLE 5. SEX RATIO OF *T. minor* AND *T. piniperda* CAUGHT AT STICKY TRAPS SURROUNDING LOGS OR SYNTHETIC BAITS, SPRING 1983 AND 1984, SKÅNE, SWEDEN.

	Sex-Ratio in Catch					
	T. minor			*T. piniperda*		
Baits[a]	Sample size[b]	Males (%)	95% CI[c]	Sample size[b]	Males (%)	95% CI[c]
Test A1, activity of pheromone, April 29–May, 19, 1983, 6 replicates						
Log +MT-OH	96	62.5[d]	53–72	124	48.7	38–56
MT-OH	50	76.0[d]	63–86	26	42.3	26–61
Log	12	(50)[e]	25–75	76	47.4	37–58
Blank	4	(50)[e]	15–85	22	59.1	39–77
Test A2, activity of pheromone, April 14–27, 1984, 12 replicates						
Log +MT-OH	275	60.1[d]	54–66	473	50.6	46–55
MT-OH	86	48.3	38–59	32	61.3	44–76
MT +MT-OH	75	58.7	47–69	166	56.8	50–65
Log	31	56.2	39–72	294	54.0	49–60
Blank	4	(50)[e]	15–85	21	60.0	37–76

[a]Bait designations: MT-OH = (*S*)-*trans*-verbenol + (*R*)-*trans*-verbenol + 3-caren-10-ol, oxygenated monoterpenes released from separate vials at approx. 0.2 mg/day, see Methods. MT = (*S*)-α-pinene + (*R*)-α-pinene + 3-carene + terpinolene, monoterpene hydrocarbons released from separate vials at approx. 30 mg/day (Byers et al., 1985), see Methods.
[b]Sample size equal to or less than total catch, as maximum 100 individuals were sexed from each replicate and some individuals were too damaged to sex.
[c]Binomial 95% confidence interval (Byers & Wood 1980).
[d]Value significantly different from an equal sex ratio.
[e]Value calculated on less than 20 individuals.

(1978) in laboratory bioassays claimed *trans*-verbenol to be attractive, but no statistics were presented. On the other hand, field-trapping experiments by Perttunen et al. (1970) did not find that the presence of boring females (assumed to be the pheromone-producing sex) enhanced the attractiveness of logs. The results of the present study complement these results and those of Byers et al. (1985), who developed a systematic method for isolating semiochemicals from pine logs infested with *T. piniperda* (sexes together or each alone) and found no evidence for a long-range pheromone for mate and host location. Instead, pine volatiles, (*R*)- and (*S*)-α-pinene, 3-carene, and terpinolene, were found in field and laboratory experiments to be attractants (Byers et al., 1985). These three compounds were later shown to induce attacks by *T. piniperda* on vigorous trees, unsuitable for brood development (Schroeder and Eidmann, 1987). Kangas et al. (1965) and Oksanen et al. (1968) concluded from laboratory bioassays (without any statistical analysis of data) that *cis*- and *trans*-carveol

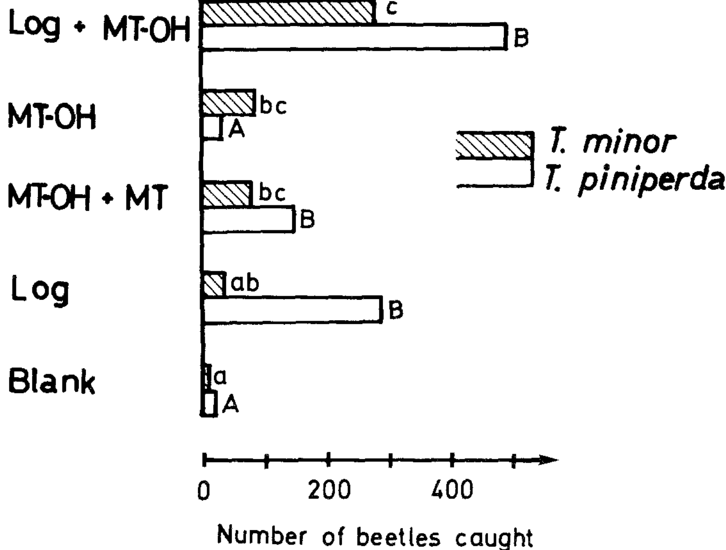

FIG. 5. Catch of *T. minor* and *T. piniperda* on sticky traps with five different baits. The lengths of the bars correspond to the total catch in 12 replicates in 1984. Bars followed by the same lower or upper case letters form homogeneous subsets [$P < 5\%$, Duncan's multiple-range test after ANOVA on log (catch + 1/4)]. Baits marked with MT-OH contain (*S*)- and (*R*)-*trans*-verbenol and (1*S*,6*R*)-3-carene-10-ol and those marked with MT contain (*S*)- and (*R*)-α-pinene, terpinolene, and (*S*)-3-carene.

together with α-terpineol were the most active host compounds and thus responsible for "primary attraction." However, their bioassays were performed in closed-choice chambers with no odor gradient at equilibrium. In our open-arena walking bioassay the odor is mixed with a stream of air. This allows both an odor gradient and a wind direction in which the beetles can orient and thus simulates the natural condition more closely.

The existence of a pheromone in *T. minor* produced by the female is indicated by the female initiated galleries, the strong bioassay response of both sexes to female infested logs, and the >50% males caught at traps baited with (*S*)- and (*R*)-*trans*-verbenol and 3-carene-10-ol. It is, however, possible that males also may contribute to the pheromonal attraction as their production of the three alcohols is almost as large as that of the females (Table 2). In a large sample of pine shoot beetles attracted to pine logs or feeding in pine shoots, Långström (1980, 1983) found an equal or slightly female biased sex ratio for both species in middle Sweden. Thus the higher percentage of males of *T. minor* at pheromone traps probably does not reflect the mean sex ratio of the population. In contrast, *T. piniperda* did not respond more to female infested logs than

to uninfested logs, and the sex ratio at traps baited with the alcohols was not different from unity.

The fact that a long-range pheromone has not been found for *T. piniperda* in this or other studies is notable, as pheromone communication in scolytid biology has almost become a paradigm (Borden, 1982; Wood, 1982). However, in at least two other cases research has failed to prove a pheromonal attraction but instead an attraction to host odors (*Pseudohylesinus nebulosus*; Ryker and Oester, 1982) or to odors of a decaying host (*Hylurgopinus rufipes*; Gardiner, 1979; Lanier, 1983). *P. nebulosus* is similar to *T. piniperda* in several respects: not only does it disperse early in spring and attacks only felled or weakened trees, but logs with male and female *P. nebulosus* were also somewhat more attractive than logs alone, parallel to the findings of Byers et al. (1985). In each of these studies the increase in attraction was attributed to a large release of host volatiles from logs with excavating beetles rather than to a weak pheromonal attraction.

Semiochemical Production and EAG Responses. Both the (*R*)- and (*S*)-isomers of *trans*-verbenol were tested in the biological experiments because the absolute configuration of the beetle produced compound was not determined. In the EAG tests with *T. minor*, *trans*-verbenol induced the largest total response and the (*S*)-enantiomer the highest dose–response. Bakke and Jordal (personal communication) also found indications that (−)-(*S*)-*trans*-verbenol acts as an attractant for *T. minor* in field-trapping experiments. When considering the activity of *trans*-verbenol, the relatively large impurity of *cis*-verbenol could be important. In fact, the amount of *cis*-isomer (about 10%) in the synthetic *trans*-verbenol used in our field tests is about as large as the ratio of *cis* to *trans* naturally found in hindguts of *T. minor*. Thus, the studies reported here do not uniequivocally support *trans*-verbenol as the only active component in the *T. minor* pheromone.

Only the (1*S*,6*R*)-enantiomer of 3-carene-10-ol was used in laboratory and field experiments since its precursor, 3-carene of the wood oil in Scots pine, is known to possess the (1*S*,6*R*)-configuration (Semmler and von Schiller, 1927; Aschan, 1928; Norin, 1964). 3-Carene-10-ol has earlier been identified in the mountain pine beetle, *Dendroctonus ponderosae* (Conn et al., 1983), but the absolute configuration was not determined and the compound was not found to have any effect on field trap catches. Our results with the laboratory olfactometer and EAG also indicate that 3-carene-10-ol is not an essential attractive pheromone component in *T. minor*.

The demonstration of the presence of *trans*-verbenol, myrtenol, and verbenone in *T. piniperda* hindguts is in agreement with earlier studies (Francke and Heeman, 1976; Carlé et al., 1978). However, Francke and Heeman's observation that virgin females and males have relatively high amounts of verbenone was not confirmed in our study. The amounts of verbenone were highest

in both sexes just before the construction of egg niches and at that time most females were mated.

The value of electrophysiology as a screening method for attractive pheromone candidates in bark beetles is probably limited. For example, it is known that bark beetles can have receptors highly sensitive to pheromone components of other species which act as allomones (Light and Birch, 1982). In fact, we found significant EAG activity in both *Tomicus* species to several bark beetle pheromone compounds that were not detected in either *Tomicus* species. Our study also showed that several monoterpenes attractive to *T. piniperda* gave smaller EAG responses than *trans*-verbenol and other oxygenated monoterpenes. These oxygenated compounds could, however, elicit other, as yet undiscovered, pheromonal activities at close range.

In the hindgut extracts, large amounts of 1-heptanol were found. When 1-heptanol, a pheromone component of *Dendroctonus vitei* (Renwick et al., 1975), and some homologs were at first included in the EAG tests, both species were almost as sensitive to these straight chain alcohols as to the oxygenated monoterpenes. However, it was later found, by addition of hexyl and octyl acetate to the gut extraction solvent, that the heptanol originated from transformations of the heptyl acetate that was used as an internal standard. Short-chain alcohols are also known to evoke EAG response in the Colorado beetle (Visser, 1979) and some lepidopteran species (Van Der Pers, 1981).

Evolutionary Considerations. How can the two *Tomicus* species be placed in a larger evolutionary framework of olfactory orientation during host colonization in bark beetles? The first "primitive" system to evolve may have relied only on host plant compounds for host location and acceptance, as in many phytophagous insects. As the use of long-range aggregation pheromones evolved, the need to utilize host attractants could have decreased in significance. In most insects, the egg-laying potential of the female is the most critical resource for reproduction, and females produce a sex pheromone attracting only males from a distance. In some insect groups, males produce pheromone and provide essential resources for reproduction like nuptial gifts in scorpionflies (Bornemissza, 1964) or announce clumped resources for reproduction, as in some stored-product beetles (Burkholder and Ma, 1985) and in polygynous bark beetles (Kirkendall, 1983). Female-produced aggregation pheromones, however, seem to be restricted to bark beetles and function in host-killing cooperation to establish a food resource after successful colonization of a tree.

Possibly, an early step in pheromone system evolution was represented by groups that modified host compounds for use as pheromone components (many *Ips*; Wood, 1982). More advanced systems have complex bicyclic ketals (*Dendroctonus*, *Pityogenes*) or tricyclic ketals (*Trypodendron*, *Scolytus*) of which the carbon skeletons are not found in the host. These compounds are, however, usually still synergized by host compounds (Borden, 1982; Wood, 1982). Other

advanced systems include loss of host synergism but with monoterpene alcohols in conjunction with short-chain alcohols (*I. typographus*; Bakke et al., 1977; *I. cembrae*; Stoakley et al., 1978). The small difference between the sexes of *T. minor* in the production of the major component *trans*-verbenol indicates a primitive system not far evolved from a simple detoxification of host terpenes.

Both *Tomicus* species apparently have a similar production and sensory perception of oxygenated compounds suitable as chemical messengers but *T. piniperda* has not acquired (or maintained) a pheromone system. The life history of the two species differs radically in the mode of feeding by the larvae. In the genus *Tomicus*, three or four palearctic species exist (Schedl, 1946), but of these *T. minor* is the only species which has evolved the fungus-feeding habit (xylomycetophagy; Kirkendall, 1983). This fungus feeding may have allowed an expansion or shift of the spatial niche to the thin-barked sections of the pine, where *T. piniperda* cannot reproduce readily. The xylomycetophagus population may have avoided gene flow from the phloem-feeding population by beginning to use a long-range pheromone. The fact that *T. minor* attacks thin bark (often <0.5 mm) and thus contacts resinous tissue more rapidly would make it easier for this species to develop a "frass pheromone" (Vité et al., 1972), which could be released comparatively quickly (within a day). In contrast, *T. piniperda* may take from one to several days during the early spring conditions (low day-time temperature) to penetrate the thicker cortex bark, and by this time most beetles have landed on the tree in response to host monoterpenes. On the other hand, *T. minor* is reputed to colonize standing, weakly stressed trees more often (Postner, 1974), presumably with the aid of its aggregation pheromone. A further evolution towards a more rapid acting pheromone system might allow *T. minor* to attack viable host trees and expand its niche considerably.

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PATTERNS AND SOURCES OF LEAF TANNIN VARIATION IN YELLOW BIRCH (*Betula allegheniensis*) AND SUGAR MAPLE (*Acer saccharum*)

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Abstract—Leaves from forest-grown sugar maple (*Acer saccharum* Marsh) and yellow birch (*Betula allegheniensis* Britt.) trees were analyzed for four tannin measures (hydrolyzable and condensed tannins, total phenolics, and protein binding) at three times during the growing season. Fifteen-year-old half-sib sugar maples from four provenances, representing the geographical extremes of the sugar maple range and growing in a common garden, were examined for the same traits. We found no significant geographic or seed source component to variation in three of the four tannin measures. We found significant seasonal changes in both birch and maple leaf tannins. Within-canopy leaf tannin variation tended to obscure differences between trees in maple, but in birches between-tree differences in leaf tannin content were more readily found. We also found a significant negative correlation between leaf protein binding capacity and leaf wet weight.

Key Words—*Acer saccharum*, Aceraceae, *Betula allegheniensis*, Betulaceae, tannins, phenolics, variability.

INTRODUCTION

Tannins are polyphenolic secondary compounds that have antifeedant and toxic effects on a variety of herbivores (Feeny, 1970; Chan et al., 1978; Reese et al., 1982) as well as antipathogenic properties (Swain, 1979). Susceptibility/resistance of chestnut trees to chestnut blight has been attributed to variable tannin production (McCarroll and Thor, 1985). Tannins in sugar maple (*Acer saccharum* Marsh) bark and roots may determine tree resistance to fungal attack

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(Wargo, 1972). Although their impact on defoliators is uncertain (Bernays, 1981; Martin and Martin, 1982, Lawson et al., 1984), these compounds may constitute 20% of sugar maple leaf dry weight and 10% of yellow birch (*Betula allegheniensis* Britt.) dry weight (Schultz et al., 1982) and are likely to represent a substantial metabolic investment by these trees (Mooney, 1983).

Birch and maple leaf tannins can vary substantially in space and time (Schultz et al., 1982). There are many possible causes of this variation, including increased synthesis in response of leaf damage (Baldwin and Schultz, 1983), seasonal change (Schultz et al., 1982), leaf age (Schultz et al., 1982), and individual variation arising from genotypic and/or site differences.

The last two sources of variation of other secondary compounds, e.g., monoterpenes in conifers are known to be heritable (Bridgen and Hanover, 1982; Strauss and Critchfield, 1982) and geographic variation are readily identified (Zavarin et al., 1979; Lester, 1974). Although the heritability of tannin production is well established for sorghum, where it has been employed in the development of pest-resistant cultivars (Woodruff et al., 1982), it is unstudied in trees. Understanding patterns and causes of leaf tannin variation might also help promote pest-resistance in trees.

We report here analyses of tannin and phenolic variation in two northern hardwood tree species, including maple trees grown in a common plantation from known seed and geographic sources. We find that within-tree and individual variation (related in part to leaf size) and seasonal changes are too great to permit us to identify variation arising from family, population, or even geographic sources.

METHODS AND MATERIALS

Tannin Analysis. Birch and maple leaves were collected between 8 and 9 AM and immediately placed on ice. Weighed leaves (to 0.2 mg) were flash-frozen and ground to a powder in liquid N_2 within 20 min of collection and extracted individually in aqueous MeOH (1 : 1 v/v) under N_2 for an hour at 70°C (Schultz et al., 1981). Leaf dry weights were determined gravimetrically. Extracts were filtered through Whatman No. 1 and the phenolic content was estimated by the Folin-Denis technique (Swain and Hillis, 1959). The tanning capacity of the extracts was estimated by protein precipitation; fresh hemoglobin was used as the substrate (Schultz et al., 1981). Hydrolyzable tannins were estimated with an iodate technique (Swain, 1979). These tannin values are expressed as percent tannic acid equivalents per gram dry leaf weight (%TAE) from tannic acid standard curves. Condensed tannins were measured as proanthocyanidins (Bate-Smith, 1975) and expressed as percent purified red oak condensed tannin (%ROT) per gram dry weight leaf (Schultz and Baldwin, 1982). These techniques are highly replicable; tannin and phenolic measurements made

on 20 homogeneous maple leaf samples of varying weight (75–2500 mg dry wt) had coefficients of variation below 7%.

Analysis of Geographic Variation. Fifteen-year-old half-sib sugar maples grown from seed in a provenance plantation established and maintained by the USDA-FS Aiken Sugar Maple Lab located in Millbrook, New York, were studied. Four seed sources from the approximate geographical extremes of the sugar maple range were chosen: Mille Lacs, Minnesota; Harlem County, Kentucky; Tucker, West Virginia; and Quebec City, Canada. Four trees from each seed source—one tree from each of four randomized blocks within the plantation—were sampled. Five leaves were collected 2.5 m above ground from shaded stems in the southern canopy of each tree on July 8, 1981. Selecting widely spaced individuals should have minimized intertree "pheromonal" interactions (Rhoades, 1983; Baldwin and Schultz, 1983).

Analysis of Seasonal Variation. Five 40- to 50-year-old forest sugar maple and yellow birch trees of unknown seed origin, with diameters at breast height (DBHs) of 15–22 cm, were sampled near Norwich, Vermont, on three dates during the growing season: May 19, June 10, and July 20. Five sugar maple leaves from one whorl from each tree were collected at each time period. All maple leaves were taken from the the west canopy, 2.5 m above the ground, from shaded stems, and were derived from primordia established during the previous year, thus minimizing chemical differences which might be due to leaf age or position. The first five fully expanded leaf pairs on the birch trees, at the same height and aspect as the maples, were collected. One randomly chosen leaf from each pair was selected for tannin analysis, the other was used for percent dry weight determination. The five birch leaves thus represent an age gradient. No branch was sampled twice, to avoid wound-induced changes in tannin chemistry (Baldwin and Schultz, 1983; Schultz and Baldwin, 1982), and widely spaced individuals were selected in an attempt to minimize "pheromonal" interactions (Rhoades, 1983; Baldwin and Schultz, 1983).

Statistical Methods. Many of the chemical measures were heteroscedastic between sampling dates or provenances which invalidates the use of two-way ANOVAs; thus Kruskal-Wallis one-way ANOVAs were used to compare groups (Sokal and Rohlf, 1969). A Pearson product-moment correlation matrix was calculated after the chemical data were transformed to z scores (Cooley and Lohnes, 1971). Leaf chemical values were regressed against leaf weight and dry weights and the significance of the slopes was determined with an F test (Sokal and Rohlf, 1969).

RESULTS AND DISCUSSION

Sugar maple trees grown from seeds collected at the geographical extremes of the sugar maple range showed no significant differences in leaf total phenolic

TABLE 1. TOTAL PHENOLIC CONTENTS, PROTEIN BINDING COEFFICIENTS, AND HYDROLYZABLE TANNIN CONTENTS AS PERCENT DRY WEIGHT TANNIC ACID EQUIVALENTS; PROANTHOCYANIDIN CONTENTS AS (PERCENT) DRY WEIGHT PURIFIED RED OAK TANNIN, OF SUGAR MAPLE LEAVES FROM 4 PROVENANCES IN COMMON GARDEN[a]

Provenance	Total phenolics	Protein binding	Hydrolyzable tannins	Proanthocyanidins
Kentucky	16.6 (2.3)	43.7 (8.0)	43.5 (7.7)	0.62 (0.31)
Minnesota	18.1 (2.8)	43.6 (7.7)	50.5 (11.3)	0.42 (0.12)
Quebec	17.7 (1.7)	43.1 (11.0)	55.8 (13.2)	0.53 (0.23)
West Virginia	18.5 (4.8)	43.3 (9.4)	44.5 (5.8)	1.36 (0.94)
H[b]	1.86 (ns)	1.36 (ns)	7.73 (ns)	13.35 ($P < .005$)

[a] Means (standard deviation).
[b] H = Kruskal-Wallis one-way ANOVA statistic. (ns = $P > 0.05$).

content, protein binding capacity, or hydrolyzable tannin content (Table 1). However, trees grown from the West Virginia seed source had higher condensed tannin contents than those from the other provenances.

Tannin measurements varied substantially among individual trees. Hydrolyzable tannin contents had between-tree coefficients of variation (CV) of 23.0%, protein binding coefficient CV of 21.1%, total phenolic content CV of 18.3%, and condensed tannin content CV of 87.5%. However, little of this variation was associated with seed source; for example, trees with both the highest and lowest mean total phenolic contents were grown from seed collected in Mille Lacs, Minnesota.

Seasonal changes were a major source of variation. Sugar maple leaf total phenolic contents, protein binding coefficients, and hydrolyzable tannin contents tended to decline from May 19 to June 23, with very little further decline by July 20 (Table 2). Together with the yellow birch results below, this is the first report of seasonal changes in hydrolyzable tannins of forest trees. Maple condensed tannin contents could not be detected in the May sampling and had reached a plateau by the June and July sampling dates (Table 2). This pattern differs from that found by Feeny and Bostock (1968) for *Quercus robur*, wherein an almost continuous seasonal increase in condensed tannins was found.

The phenology of maple condensed tannin synthesis suggests that the high condensed tannin content found in leaves from the West Virginia seed source is not a result of altered phenology in these trees; their condensed tannin content is substantially above both forest and plantation trees sampled at approximately the same time. However, only three of the four West Virginia trees displayed this high tannin content. Several nongenetic factors could have generated elevated tannin contents in some of the West Virginia trees, including previous

TABLE 2. TOTAL PHENOLIC CONTENTS, PROTEIN BINDING COEFFICIENTS, AND HYDROLYZABLE TANNIN CONTENTS AS PERCENT DRY WEIGHT TANNIC ACID EQUIVALENTS; PROANTHOCYANIDIN CONTENTS AS PERCENT DRY WEIGHT PURIFIED RED OAK TANNIN OF SUGAR MAPLE LEAVES COLLECTED FROM 5 TREES NEAR NORWICH, VERMONT ON 3 DIFFERENT DATES[a]

Sampling date	Total phenolics	Protein binding	Hydrolyzable tannins	Proanthocyanidins
May 19	28.4 (9.1)	40.3 (10.0)	87.1 (48.4)	ND
June 23	18.2 (2.7)	27.7 (12.6)	48.6 (11.8)	0.58 (0.25)
July 20	16.1 (1.9)	31.0 (8.5)	37.9 (26.3)	0.45 (0.18)

[a] Means (standard deviation) ND = not detected.

damage or infection. In laboratory grown maples, as little as 10% leaf area removal results in significantly increased tannin production (Baldwin and Schultz, 1983).

All plantation trees sampled at the same time as forest trees had significantly greater protein binding coefficients. Differences between the two sets of trees could derive from superior growth conditions on the plantation.

These results indicate that the chemical assays we used (with the possible exception of condensed tannin measurements) are likely to be poor markers for identifying geographic origin or seed source of sugar maples, because within-tree and seasonal variation obscure patterns in static chemical traits. However, other tree characteristics (e.g., ability to respond chemically to pest attack), could exhibit stronger geographic or family variation.

Yellow birch leaves exhibited a more complicated seasonal pattern (Table 3) than maple. Hydrolyzable tannin content decreased seasonally to a plateau

TABLE 3. TOTAL PHENOLIC CONTENTS, PROTEIN BINDING COEFFICIENTS, AND HYDROLYZABLE TANNIN CONTENTS AS PERCENT DRY WEIGHT TANNIC ACID EQUIVALENTS; PROANTHOCYANIDIN CONTENTS AS PERCENT DRY WEIGHT PURIFIED RED OAK TANNIN OF YELLOW BIRCH LEAVES COLLECTED FROM 5 TREES NEAR NORWICH, VERMONT ON 3 DIFFERENT DATES[a]

Sampling date	Total phenolics	Protein binding	Hydrolyzable tannins	Proanthocyanidins
May 19	12.2 (3.8)	15.7 (5.8)	68.9 (25.6)	1.15 (1.30)
June 23	6.8 (2.7)	46.2 (15.3)	33.1 (10.2)	1.42 (0.85)
July 20	33.1 (6.7)	18.8 (5.4)	41.1 (11.3)	1.16 (0.52)

[a] Means (standard deviation).

as it did in maple. Condensed tannin and protein binding coefficients increased in the June sampling date and decreased in July. Birch total phenolic contents displayed the reverse pattern, decreasing in June and increasing in the July sampling.

Leaf chemistries of individual birch and maple trees, however, did not change in an orderly, parallel fashion. No forest maple tree maintained the same rank among the five individuals sampled through all time periods for any chemical measure. With a few exceptions, it is difficult to discern statistical differences between leaf tannin contents in maple trees at any particular sampling period (Table 4). Yellow birch exhibits less within-canopy variation, and statistical differences between trees are more frequently discerned (Table 4). These general seasonal patterns were similar to those found previously for both maple and birch (Schultz et al., 1982), but were temporally shifted so that plateaus were reached about three weeks earlier.

We found no consistent relationship between leaf tannin values and position in the whorl for forest maple trees or position on the branch for the forest birch trees. This was not surprising for maple because the leaves in a whorl do not differ greatly in age (Gregory, 1980). Previous studies (Schultz et al., 1982) had led us to expect higher phenolic contents in the terminal, younger leaves of yellow birch, which flushes new leaves throughout a long growing season (Sharik and Barnes, 1976). We regressed the leaf tannin and phenolic contents

TABLE 4. RESULTS OF KRUSKAL-WALLIS ONE-WAY ANALYSIS OF VARIANCE FOR TOTAL PHENOLICS, HYDROLYZABLE AND CONDENSED TANNIN, AND PROTEIN BINDING DIFFERENCES FROM 5 YELLOW BIRCH AND 5 SUGAR MAPLE TREES SAMPLED THREE TIMES DURING SEASON[a]

	Total phenolics	Protein binding	Hydrolyzable tannins	Proanthocyanidins
Sugar Maple				
Between dates	$P < 0.005$	$P < 0.005$	$P < 0.005$	NS
Between trees				
May 19	NS	$P < 0.05$	NS	—
June 23	NS	NS	NS	$P < 0.01$
July 20	$P < 0.005$	NS	NS	NS
Yellow Birch				
Between dates	$P < 0.005$	$P < 0.005$	$P < 0.005$	NS
Between trees				
May 19	$P < 0.01$	NS	NS	$P < 0.005$
June 23	$P < 0.005$	$P < 0.01$	$P < 0.01$	$P < 0.005$
July 20	$P < 0.01$	NS	$P < 0.025$	$P < 0.005$

[a]NS = $P > 0.05$.

on leaf wet weights; results were the same when leaf dry weights were used. There were no significant differences in leaf dry weights between trees or time periods. We identified a significant negative relationship between protein binding capacity and sugar maple leaf weight for the June ($r = -0.51$, $DF = 24$, $F = 8.0$, $P < 0.01$) and July ($r = -0.61$, $DF = 24$, $F = 13.7$, $P < 0.005$) sampling dates; no other slopes differed significantly from zero. However, leaf size was found to predict protein binding in sugar maple.

In yellow birch we found similar negative correlations between leaf weight and protein binding capacity for all time periods: May ($r = -0.41$, $DF = 24$, $F = 4.66$, $P < 0.05$), June ($r = -0.51$, $DF = 24$, $F = 7.73$, $P < 0.025$), and July ($r = -0.76$, $DF = 24$, $F = 30.98$, $P < 0.001$). We also found a positive correlation between condensed tannin content and leaf weight for the July sample ($r - 0.54$, $DF = 24$, $F = 10.68$, $P < 0.001$). No other regressions for birch were significant.

The observation that smaller birch and maple leaves, regardless of their age, have higher protein binding coefficients than larger leaves suggests that tannin synthesis may be influenced by leaf expansion and growth. A similar situation was reported by Zucker (1982), who showed that the largest *Populus angustifolia* leaves had the lowest total phenolic contents. If a leaf is limited in the amount of phenolic compounds it can produce because of biosynthetic competition with growth processes, then smaller leaves should have higher tannin contents. Competition for precursors could occur at several points, including the deamination of phenylalanine (or tyrosine) at entry to the phenylpropanoid biosynthetic pathway, or between lignin synthesis and the synthesis of other phenolics. The first case would represent a direct trade-off between protein synthesis (growth) and phenolic synthesis. In the latter case, larger leaf size may result from shunting more precursors into structural lignins than into condensed tannins. However, if hydrolyzable tannins make a large contribution to the protein binding capacity of a leaf, the biosynthetic trade-offs become less clear; gallic acid, the precursor of hydrolyzable tannins, can originate from the shikimate acid pathway, as well as the phenylpropanoid pathway. The observations that many of the tannin measures decrease seasonally and that in both maple and birch the strength of the correlation with leaf weight increases seasonally are consistent with the hypothesis that lignification and tannin synthesis are competitive processes.

In their classic study of *Quercus robur* L. tannins, Feeny and Bostock (1968) reported seasonal and leaf age increases in tannin concentrations. Except for condensed tannins in maple, our results differ substantially. Although these differences may reflect differences among the species involved, several investigators have found higher tannin or phenolic concentrations in younger leaves (e.g., Coley, 1983), including oak (Faeth, 1986; Schultz and Baldwin, unpublished). Each of these studies also employed different analytical methods. We

suspect that the relationship between growth and phenolic synthesis is highly dynamic and complex in young, rapidly expanding tree leaves. Only very detailed (e.g., on a daily basis) studies of phenolic biosynthesis in expanding leaves will elucidate this interaction.

Regardless of the mechanism, the correlation between leaf size and protein binding capacity could have important ecological and evolutionary implications for maple and birch feeders. If herbivores can distinguish between leaves of different weights (or sizes), then they may be able to increase their foraging efficiency for leaves of high food quality (e.g., Zucker, 1982).

In previous work (Baldwin and Schultz, 1984), we found a significant negative relationship between individual tree leaf weight and Folin-Denis reactive phenolics for unstressed laboratory-grown yellow birch trees. This correlation was lost if the trees were damaged or shared the atmosphere with trees that had been damaged. The observation that forest-grown birches do not, as a group, exhibit the negative total phenolic content–leaf weight relationship could be due to nearby leaf damage, either on the same tree or on nearby conspecifics. Moreover, the observation that undamaged birch and late-season maple leaves exhibit a negative protein binding capacity–leaf weight relationship indicates that tannin synthesis, unlike phenolic synthesis, may be less responsive to nearby leaf damage. These results were consistent with previous work (Baldwin and Schultz, 1983) on laboratory-grown maple trees.

Calculation of a Pearson's product–moment correlation matrix allowed us to determine correlation coefficients between pairs of chemical traits of the 80 leaves analyzed from the maple plantation. Protein binding coefficients, which measure the in vitro protein binding capacity of tannins, were significantly correlated with total phenolic contents ($r = 0.65$, $P < 0.05$). Hydrolyzable and condensed tannin contents were not significantly correlated with tanning coefficients, and the three chemical measures were not significantly correlated with each other.

Correlation coefficients calculated from the forest maples differed dramatically from those in the plantation grown maples. Protein binding coefficients were not significantly correlated with any of the chemical measures. Total phenolic content was positively correlated with condensed tannin content ($r = 0.62$, $P < 0.05$) but negatively correlated with hydrolyzable tannin content ($r = -0.70$, $P < 0.01$) and condensed and hydrolyzable tannin contents were negatively correlated ($r = -0.78$, $P < 0.01$).

The relationships among the tannin measurements appear to vary from site to site and/or seasonally. These results could be caused by several factors. First, synthesis of various tannin types might not be closely coordinated in a tree; there is no reason, for example, to expect any particular relationship between hydrolyzable and condensed tannin synthesis. Second, the individual experiences of each tree could alter tannin chemistry and synthesis as the season pro-

gresses. Finally, the nature of the analyses employed here is such that the functional and synthetic relationships of the product measured are uncertain. Most of these analyses are influenced by qualitative composition of plant phenolics as well as concentration of the "target" molecules (Hagerman and Butler, 1978). Martin and Martin (1982) found a similar lack of correlation between chemical measures of tannins and functional measures such as protein binding.

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FORMIC ACID IN CAUSTIC CEPHALIC SECRETIONS OF STINGLESS BEE, *Oxytrigona* (HYMENOPTERA: APIDAE)

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Abstract—The cephalic extracts of two species in the stingless bee genus *Oxytrigona* were analyzed. Extracts made in diethyl ether, and then derivatized with diphenyldiazomethane, revealed large quantities of formic acid, potentially a major defensive secretion of this stingless bee group. We additionally identify several hydrocarbons, aldehydes, ketones, and acetates. Novel diketones identified by others were detected in both species.

Key Words—Defensive secretions, formic acid, *Oxytrigona*, Hymenoptera, Apidae, stingless bees, mandibular glands, Meliponinae.

INTRODUCTION

Stingless bees (Meliponinae) may react to colony disturbance by rapid and persistent attack of intruders, as do other eusocial apids, the honeybees and bumblebees (Plowright and Laverty, 1984; Sakagami, 1982; Michener, 1974). However, their primary lines of defense are biting or, in unaggressive species, concealment within the nest (Roubik, 1983). Chemical secretions play a role in eliciting defensive behavior by apids in general, often arising from mandibular gland compounds released during biting (Duffield et al., 1984; Smith and Rou-

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bik, 1983; Johnson et al., 1985). In addition to an atrophied sting apparatus, meliponine bees display further unusual traits, noteworthy of which is a secretion produced by "firebees," several species of neotropical *Oxytrigona* (Camargo, 1984). The two mandibular glands of *Oxytrigona* are unusually enlarged and occupy most of the head capsule (Kerr and da Costa Cruz, 1961). Symptoms of attacks on humans range from eventual appearance of itching blisters to an immediate burning sensation if the skin is broken by a biting bee; each leaves a lasting scar (Michener, 1974; personal observation). One species of *Oxytrigona* produces chemically novel diketones (Bian et al., 1984). We examined this and an additional species of *Oxytrigona* from Panama, and applied analytic methods necessary to detect formic acid, which resembles the odor of live bees (Schwarz, 1948, p. 469). Its presence in both species suggests that it is a major defensive secretion of *Oxytrigona*.

METHODS AND MATERIALS

Live worker bees of *Oxytrigona daemoniaca* (Camargo, 1984; J.M.F. Camargo, personal communication) were collected in January 1982 from a nest found within the Atlantic coastal forest of eastern Panama. The heads of four live bees were immediately placed in diethyl ether. Foraging workers of *Oxytrigona mellicolor* (=*O. tataira mellicolor*) were collected and the heads extracted in diethyl ether on Barro Colorado Island in central Panama during April 1984. All extracts were sealed in glass vials prior to shipment; after arrival, they were stored under nitrogen gas at 10°C. Prior to analysis, the ether extracts were each divided between two vials, of which one was later treated with diphenyldiazomethane to yield the diphenyl methyl ester of the carboxylic acids in the extract (Smith and Howard, 1955). The underivatized vial of each pair was then used for comparison in the chemical analyses.

Because of odor and irritating quality, we considered the possibility that formic acid might be a component of the secretion. In order to estimate the loss of formic acid in the extracts due to its low solubility in some solvents, mixtures of synthetic formic acid in different solvents were kept at room temperature for 48–72 hr prior to analysis, as extracts would be during shipment. Using the esterification techniques outlined above, the recovery of formic acid was low from both dichloromethane and hexane (15% and 5%, respectively) but excellent from diethyl ether (95%).

For the first series of extracts in 1982, extracts of *O. daemoniaca* and mixtures of formic, acetic, propanoic, and butanoic acids in solvent were both separately and concurrently injected splitless before and after treatment with the esterification reagent onto a Hewlett-Packard HP 5092 gas chromatograph coupled to an HP 3390 data system. Final identifications of these mixtures were

made with a Girdel capillary gas chromatograph with split Ros injection coupled to a Ribermag R-10-10 quadrupole mass spectrometer and a PDP 8/A computer system in the Department of Chemistry, University of Kansas. Both instruments were fitted with 25-m vitreous silica capillary columns coated with SE-30 stationary phase. The temperature program on both instruments was from 100 to 200°C at a ramp rate of 10°C/min after a 5-min isothermal time.

For the second series in 1984, derivatized and underivatized extracts were injected splitless onto a Carlo-Erba GC and then also onto a Varian GC-MS instrument in the Department of Chemistry of the University of Tübingen, West Germany. Both instruments were equipped with a 25-m SE-54 fused silica capillary column. Temperature programming was from 70 to 200°C at 8°C/min after a 5-min isothermal time.

RESULTS

After treatment with diphenyldiazomethane, ether extracts of *O. daemoniaca* and *O. mellicolor* yielded a component which had an identical mass spectrum and retention time to those of synthetic diphenyl methyl formate formed in the ether–synthetic formic acid mixtures after similar treatment (Figures 1 and 2). In no case did any of the remaining eluted material match the retention time and/or the mass spectra of the diphenyl methyl esters of acetic, propanoic, or butyric acids. Quantification of the formate ester peak in *O. daemoniaca* by coinjection of the animal extracts on the GC together with known amounts of an internal standard demonstrated that formic acid was present in the extracts in excess of 10–20 μg per individual worker.

Other components identified from *O. mellicolor* (Figure 3) have identical mass spectra to the compounds identified in this species by Bian et al. (1984). Mass spectra and retention times of the eluted materials from our extracts were compared with those from Bian et al., with synthetic analogs when they were available, and with published mass spectra.

Two of the eluted compounds had identical mass spectra to the diketones which have been identified only in this subgenus of stingless bee (Bian et al., 1984). Peaks 2 and 4 (Figure 3) correspond to (*E*)-3-hepten-2,5-dione and (*E*)-3-nonen-2,5-dione, respectively. The three peaks under four represent different isomers and the saturated analog to nonen-2,5-dione, all of which were present in the analyses of Bian et al. We therefore provide independent confirmation of the presence of these novel compounds in the mandibular glands of a second species of *Oxytrigona*.

Additional components, most of which were identified by Bian et al. (1984) from *O. mellicolor*, are as follows with their respective peak numbers from Figure 3 in parentheses: 2-Heptanone (1) was present in large quantities and

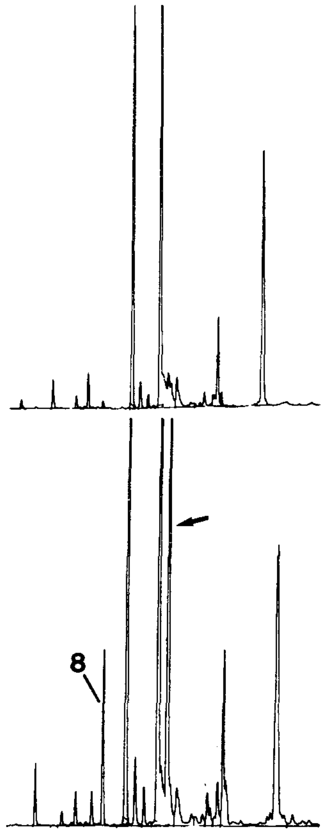

FIG. 1. Capillary gas chromatogram from an ether extract of *O. mellicolor* after treatment with diphenyldiazomethane (bottom). Chromatograms from derivatized ether extracts of *O. daemoniaca* are identical. The peak indicated (arrow) corresponds to diphenyl methyl formate, which appeared after treatment with the reagent. The other peaks have identical mass spectra and retention times to compounds found either in underivatized extracts or in the reagent (top). For reference purposes, the peak numbered eight refers to the peak of the same number in Figure 3.

may be metabolically related to the diketones. The even and odd carbon numbered saturated hydrocarbons undecane through heptadecane (3, 5, 6, 8, 11), as well as the monounsaturated pentadecene and heptadecene (7, 10), were present. Pentadecene and heptadecane were not found in *O. mellicolor* studied by Bian et al. (1984). Dodecyl-, tetradecyl-, and hexadecyl acetates (9, 12, 13) were present in the bees from Venezuela and from Panama. Additionally, hexadecanal was present in extracts of *O. daemoniaca*.

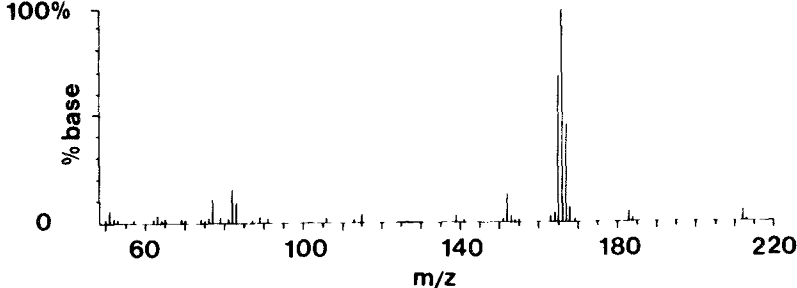

FIG. 2. Mass spectra of diphenyl methyl formate in the ether extracts of *O. mellicolor*.

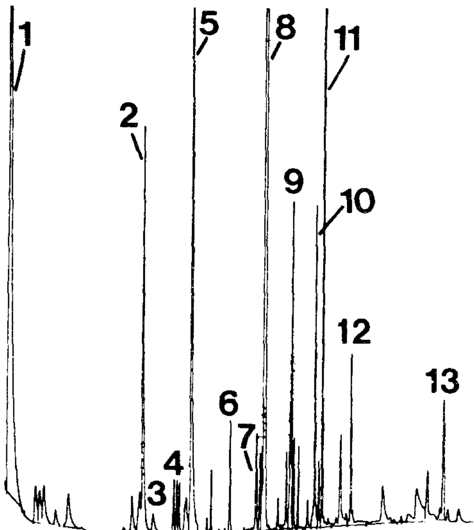

FIG. 3. Capillary gas chromatogram from head extracts of worker *O. mellicolor*. Numbers above the peaks correspond to the following substances (see text): 1, 2-heptanone; 2, (*E*)-3-hepten-2,5-dione; 3, undecane; 4, (*E*)-3-nonen-2,5-dione; 5, tridecane; 6, pentadecane; 7, pentadecene; 8, hexadecane; 9, dodecylacetate; 10, heptadecene; 11, heptadecane; 12, tetradecylacetate; 13, hexadecylacetate.

DISCUSSION

The repellent properties of formic acid, and the pain and itching it produces in humans (Eisner, 1970; Blum, 1981), demonstrate its value to *Oxytrigona* in defense of nests from vertebrate predators. Our findings provide the first evidence of employment of formic acid as a defensive substance in the Apoidea

and confirm the two observations reported in Schwarz (1948). Cederberg (1977) identified butyric acid as a major component in the mandibular gland secretion of the bumblebee *Bombus lapidarius*; low-molecular-weight carboxylic acids are commonly utilized in the Arthropoda as defensive substances (Blum, 1981). Moreover, formic acid was the first insect venom to be described (review by Schmidt, 1982) and is synthesized in vivo by deamination of serine (Hefetz and Blum, 1978). It can easily escape detection in chemical analyses because of its high volatility and low solubility in commonly used solvents. As extracts of *O. daemoniaca* were of heads, we do not show conclusively that the formic acid originates in the mandibular glands; it is conceivable that other cephalic exocrine glands produce the acid.

The combination of a highly complex exocrine secretion and persistent biting behavior must greatly enhance the defensive efficacy of the formic acid in the firebees. In many cases, acids occur in biting and/or stinging arthropods such as, for example, the formicine ants (Schmidt, 1982; Hermann and Blum, 1981). The integument of the attacked individual acts as a barrier to the penetration of the acid and must first be broken through mechanical damage; other substances in the secretion enhance the penetration properties of the acid mixture (Rembold, 1962; Eisner et al., 1968) and may account for the complexity of the cephalic secretion of *Oxytrigona* in comparison with some stingless bee species (Blum, 1981).

Occasional food robbing of apiary colonies of the honeybee *Apis mellifera* has been reported (Bian et al., 1984) and is known to us. However, nests of *Oxytrigona* can be kept together in an apiary with nests of other stingless bee species without similar robbing behavior on the part of the former. Additionally, unlike *Oxytrigona*, the four obligate cleptobiotic stingless bee species lack a pollen-carrying apparatus, have an extremely enlarged gut, and do not visit flowers (Cruz Landim and Rodrigues, 1967; Nogueira-Neto, 1970; Roubik, 1980, 1983). Therefore, food robbing by *Oxytrigona* may be a behavioral phenomenon unique to its relationship with *Apis* and not to its relationship with other stingless bee species.

Formic acid is undoubtedly responsible for some of the symptoms resulting from a firebee attack. However, other symptoms, such as blistering and other long-term effects (Michener, 1974), are not characteristically caused by formic acid. It is necessary to examine other components in the cephalic secretion of the firebees, including possible proteinaceous venoms, to determine how they may enhance the defensive efficacy of the secretion.

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FEEDING DETERRENT COMPOUNDS TO THE BOLL WEEVIL, *Anthonomus grandis* BOHEMAN[1] IN ROSE-OF-SHARON, *Hibiscus syriacus* L.[2,3]

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Abstract—The Rose-of-Sharon, *Hibiscus syriacus* (L.), can be a significant alternate host plant for the boll weevil, *Anthonomus gradis* (Boh.). Boll weevils are known to be deterred from feeding and ovipositing in the buds unless the calyx is removed. This investigation was initiated to identify calyx allelochemicals that deter feeding with the eventual strategy of breeding for cotton lines high in these allelochemicals in the appropriate tissues. The feeding deterrency of calyx tissue from the buds of Rose-of-Sharon for the boll weevil was confirmed. The most active deterrent fraction was found to contain mostly fatty acids and their methyl esters. Saturated fatty acids and their methyl esters were generally found to be stimulatory, while the unsaturated species were found to be deterrent. Higher quantities of the fatty acids, particularly the unsaturated species, were found in Rose-of-Sharon calyx tissue than in the buds without calyx. This supports the hypothesis developed through the isolational work and testing of standards that the unsaturated fatty acids are significant deterrents of boll weevil feeding.

Key Words—Boll weevil, *Anthonomus grandis* Boh., Coleoptera, Curculionidae, Rose-of-Sharon, *Hibiscus syriacus* L., feeding deterrent, unsaturated fatty acids.

[1] Coleoptera: Curculionidae.
[2] Malvales: Malvaceae.
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INTRODUCTION

The boll weevil, *Anthonomus grandis* Boh., is usually considered host-specific to cotton, but Coad (1914) was first to report its infestation of, and emergence from Rose-of-Sharon (ROS), *Hibiscus syriacus* L. Maxwell et al. (1965) and Parrott et al. (1966) reported that the calyx, which encloses the developing ROS bud, deterred feeding and oviposition by the boll weevil. When the calyx was removed, weevils fed on, and oviposited in buds at about the same rate as on cotton flower buds. Feeding and oviposition on the cotton buds were deterred when a water extract of the ROS calyx was applied to its surface. In subsequent chemical investigations at this laboratory, Hanny et al. (1972) identified 10 carotenoids in the bud, leaf, and flower of ROS, three of which (cryptoxanthin, chrysanthemaxanthin, and antheraxanthin) were present in ROS but not in cotton, *Gossypium hirsutum* L., while two (flavoxanthin and violaxanthin) were found in cotton but not in ROS. Additionally, the essential oils of ROS buds and flowers were found to contain at least 65 components including hydrocarbons, carbonyls, alcohols, and fatty acid methyl and ethyl esters (Hanny et al., 1973). However, no biological evaluations were conducted as part of either investigation, and, to our knowledge, no substantive information about the chemical nature of the deterrent(s) has been obtained since. The general interest in natural antifeedants, with the potential that their concentration in the host (cotton) could be enhanced by breeding selections, or eventually by gene insertions, has led to a resumption of efforts to chemically define the feeding (and oviposition) deterrents in this alternate host of the boll weevil.

METHODS AND MATERIALS

Insects. Insects used in this study were from a colony of "wild" strain boll weevils (red coloration) as defined by Bartlett (1967). The boll weevils were reared from egg to adult on a Pharmamedia®-based (Trader's Oil Mill Co.; Ft. Worth, Texas) diet prepared after the method of Lindig (1979). Male boll weevils are field collected and mated to laboratory-reared females on a yearly basis to maintain the genetic diversity present in the colony. Holding conditions are 28.3°C, 50 ± 5% relative humidity, and 14:10 light–dark photoperiod.

Four- to seven-day-old boll weevils from the rearing colony were taken three days prior to the bioassay and fed either cotton squares or artificial diet as applicable (see below). All food was removed 24 hr prior to testing to starve the males and females that had been separated from one another.

Cotton Bud Bioassays. Fresh, debracted cotton squares were rinsed in running tap water and air dried prior to bioassay. Test compounds were solubilized in an appropriate solvent, and the buds, marked with paper tags pinned in line with the stem, were dipped into the solutions. Approximately 25 μl of solution

remained on each bud. Therefore, solute concentration was adjusted to allow 1.5 mg of test material to remain on each bud after application and air drying for 1 hr. Two to six buds, depending upon the number of samples of test compounds, were placed into 10-cm Petri dishes and either 10 male or 10 female insects added. A control bud was always present in each of the dishes. The bioassays were conducted in the dark at 28°C and 50 ± 5% relative humidity. Feeding activity was determined as the number of punctures found in the cotton squares using a dissecting microscope at the end of 3 and again at 6 hr.

Plate Bioassay. The plate bioassay has been described elsewhere in detail (Bird and Hedin, 1986). Briefly, a reusable beeswax–paraffin chamber was prepared from a standard 10-cm Petri dish. Six 13-mm wells were cut into the wax layer equidistant from one another, 2 cm from the center of the dish, with a No. 9 cork borer and filled with a cooling Pharmamedia (3.0%)–agar (2.5%) solution which was allowed to solidify with a 1- to 2-mm high convex surface. Samples (100 μg for known compounds; 200 μg for extracts and chromatographic fractions) in appropriate solvents were randomly applied as 20-μl aliquots to 15-mm lens paper disks covering each feeding site. After application of the samples to the disks, the chambers were left uncovered 30 min to allow solvent evaporation. Feeding activity was determined as the number of punctures per paper disk after 3 and again at 6 hr using a dissecting microscope.

Statistical Design and Analysis. Experiments were designed as randomized complete blocks (RCB) and analyzed using analysis of variance (ANOVA) (SAS, 1982). A split plot analysis, where sex was the main unit and treatment the subunit, was incorporated if "sex × treatment" interactions were significant ($P < 0.05$). Regardless of how the treatments were analyzed, treatment means, either combined or averaged over sex, were compared by the least significant difference (LSD). After statistical treatment, the data were normalized by conversion of punctures/disk to punctures, percent of the control. A test fraction or compound was termed a feeding repellent or deterrent if feeding was reduced relative to controls or a feeding stimulant if feeding was elevated relative to control at statistically significant levels ($P < 0.05$).

Procurement and Processing of ROS Buds. Fresh buds were gathered during the summers of 1984 and 1985 and stored at −18°C until the calyxes were quickly harvested by hand and lyophilized.

For test 1, fresh ROS buds were bioassayed using cotton buds with and without calyxes removed.

For test 2, lyophilized calyx tissue (1–10 g) was Soxhlet-extracted with either water, methanol (MeOH), chloroform ($CHCl_3$), or hexane. Extracts from the specified solvent were combined and concentrated so that 25 μl, when applied to the cotton bud surface in the cotton bud bioassay, contained 1.5 mg.

For test 3, lyophilized calyx tissue (50 g) was Soxhlet-extracted 3× with 500-ml quantities of MeOH (yield of extract 6.0 g). The extract was redissolved in MeOH–water (6:4) and applied to a Polyamide® (Brinkmann Instruments

Co., Westbury, New York) column (5.0 cm diam., 30.0 cm length), and eluted with the same solvent, then MeOH–water (7:3, 8:2), and 100% MeOH (500 ml each). Eluates were concentrated so that 1.5 mg was present on the surface of each cotton bud used in bioassay.

For test 4, the MeOH–water (6:4) fraction (test 3 above; 2.8 g of solids) was partially dissolved in ethyl acetate (EtOAc) and applied to a Biosil A column (2.0 cm diam., 17 cm length). Two yellow pigmented fractions (EtOAC-1 and EtOAC-2) were collected upon extended elution of the solids from the column with EtOAC. The remaining solids not soluble in ethyl acetate (1.5 g) were dissolved in MeOH and applied to the column. Subsequent application of MeOH to the column eluted these solids in a fraction designated MeOH-3. Eluates were concentrated so that 20 μl contained 200 μg of solids when applied to the filter paper disks in the plate bioassay. Assuming uniform distribution, the concentration on the disk was 1.12 $\mu g/mm^2$.

For test 5, a number of commercially procured fatty acids and their methyl esters were evaluated by the plate bioassay at 100 μg/disk.

For test 6, palmitic acid and methyl linoleate were evaluated by the plate bioassay at 0, 0.1, 1.0, 10, 100, and 1000 μg/disk.

Mass Spectrometry. Active fractions were analyzed by GC-EI-MS employing a Hewlett Packard 5985-B® quadrupole mass spectrometer interfaced with a DB-5 fused silica capillary column (15 m $\times$ 0.322 mm ID, film thickness 1 μm, J&W Scientific Co., Cordova, California). The GC was programmed from 70 to 250°C at 10°C/min (injector temperature = 200°C, detector temperature = 250°C, carrier gas = He at 40 cm/sec). Structural assignments were made by comparison with those of commercially procured samples (Sigma Chemical Co., St. Louis, Missouri), and by comparison with standard spectra (Stenhagen et al., 1974). Eleostearic acid [(*Z,E,E*)9,11,13-octadecatrienoic acid] and its methyl ester were gifts of Mr. Martin Jacobson, USDA-ARS, Beltsville, Maryland.

Tissue Fatty Acid Analyses. ROS calyxes (10 g dry wt) and buds less calyxes (20 g dry wt) were extracted 3$\times$ with $CHCl_3$–MeOH–water (2:1:1). After filtration, the resulting chloroform layer was extracted 3$\times$ with 5% $NaHCO_3$, and the combined extracts added to the water layer. The aqueous fraction was acidified and extracted with $CHCl_3$ to yield the free fatty acids. Aliquots of the free fatty acid fraction and the $CHCl_3$ layer less free fatty acids were esterified with boron trifluoride methanol to yield the fatty acid methyl esters which were then determined quantitatively in triplicate by GC-FID employing the conditions outlined by Thompson and Sikorowski (1979). The methyl esters were isolated by chromatography on a 2 $\times$ 16-cm Biosil A® column. The methyl esters were eluted with hexane–methylene chloride (1:1) and analyzed by GC-FID. No special attempt was made to determine the *cis* or *trans* geometry of the unsaturated fatty acids except to note that the GLC retention

volumes were coincident with those of the respective *cis* standards. All standards for bioassay were of the *cis* configuration.

RESULTS AND DISCUSSION

The boll weevil demonstrated a significant ($P < 0.05$) preference (Table 1, test 1) for cotton squares over ROS buds (18–39% of control), as previously reported by Parrott et al. (1966). Their earlier findings that the feeding deterrent was a calyx-localized (ROS buds less calyx = 56–86% of control; $P < 0.05$) and water-soluble fraction (aqueous ROS extract = 17–37% of control; Table 1, test 2, $P < 0.05$) were also confirmed. Removal of the calyx resulted in a level of acceptance of the ROS buds that fell between the cotton square and the ROS bud with calyx (56–86% of control; Table 1, test 1). Exhaustive hexane, $CHCl_3$, MeOH, or water Soxhlet extracts of lyophilized ROS calyx tissues coated onto cotton squares indicated that the MeOH fraction (6–50% of control) and water fractions (17–37% of control) were both inhibitory to control ($P <$ 0.05, Table 1, test 2) at 3 and 6 hr for both males and females. The MeOH

TABLE 1. FEEDING BY BOLL WEEVILS ON ROSE-OF-SHARON (ROS) BUDS AND ON COTTON BUDS COATED WITH SEVERAL ROS CALYX EXTRACTS[a]

	Punctures (% of control)			
	Males		Females	
	3 hr	6 hr	3 hr	6 hr
Test 1[b]				
Rose-of-Sharon buds	38-D	39-D	18-D	22-D
ROS buds less calyx	84	86	56-D	61-D
Test 2[c]				
Aqueous extract	23-D	17-D	17-D	37-D
Hexane extract	77	69-D	34-D	63-D
Chloroform extract	54	107	40-D	49-D
Methanol extract	15-D	21-D	6-D	50

[a] Data expressed as the percentage of feeding punctures on ROS buds or extract-treated cotton squares relative to fresh untreated cotton squares. Each test consisted of either 10 male or 10 female boll weevils plus bioassay material in a 10-cm Petri dish held in the dark at 28°C, 50 ± 5% relative humidity. Test designed as randomized complete blocks with analysis of variance (ANOVA). Numbers in each column followed by a "D" are feeding deterrents ($P < 0.05$); and "S" are feeding stimulants ($P < 0.05$); or no letter, no effect on feeding ($P < 0.05$).

[b] Three choice test: ROS bud, ROS bud with calyx removed, fresh debracted cotton square.

[c] Five-choice test: four extract-coated cotton buds (1.5 mg total solids/bud surface) and a fresh, debracted cotton bud. Extracts prepared by exhaustive Soxhlet extracts of 10 g lyophilized ROS calyx tissue.

TABLE 2. FEEDING BY BOLL WEEVILS ON POLYAMIDE AND BIOSIL A COLUMN CHROMATOGRAPHIC FRACTIONS OF ROSE-OF-SHARON METHANOL EXTRACT COATED ON COTTON BUDS.[a]

	Punctures (% of control)[b]			
	Males		Females	
	3 hr	6 hr	3 hr	6 hr
Test 3, polyamide fractions[c]				
MeOH–H_2O				
6:4	26-D	45-D	11-D	28-D
7:3	52-D	82	100	103
8:2	96	94	93	92
MeOH	96	68	109	67
Test 4, Biosil A fraction[d]				
EtOAC-1	2-D	2-D	0-D	0-D
EtOAC-2	4-D	6-D	3-D	3-D
MeOH-1	52-D	58-D	35-D	35-D

[a] Data presented as the percentage of feeding punctures found on extract treated feeding sites relative to untreated sites.
[b] Test designed as randomized complete blocks with analysis of variance (ANOVA). Numbers in each column followed by a "D" are feeding deterrents ($P < 0.05$); "S" are feeding stimulants ($P < 0.05$); or no letter, no effect on feeding ($P < 0.05$).
[c] Five-choice bioassay conducted with 1.5 mg solute per fresh cotton bud and one untreated bud. Ten males or females per dish. The methanol fraction (Table 1) was the starting material.
[d] Biosil A fractions tested on plate bioassay at 200 μg solute per feeding site. Ten males or 10 females per bioassay chamber. The starting material was the MeOH–H_2O 6:4 fraction from polyamide 6 C column (above).

fraction was selected for further chemical work. Most of the antifeedant activity eluted from the polyamide 6C column using 60% MeOH in water as eluant (11–45% of control; Table 2, test 3).

When this MeOH–water (6:4) fraction was redissolved in ethyl acetate, and applied to a Biosil A column, two light yellow bands were eluted with ethyl acetate. These bands, designated EtOAC-1 and EtOAC-2, were found to be highly deterrent to both sexes when evaluated by the plate bioassay (0–6% of control; Table 2, test 4). The MeOH eluate, MeOH-1, was less active (35–58% of control).

GC-MS of EtOAC-1 and EtOAC-2 (Table 3) resulted in the identification of three fatty acids (pelargonic, myristic, and palmitic) and the methyl esters of palmitic, linoleic, linolenic, and stearic acids. The chromatograms were very limited in other peaks, these listed compounds accounting for 74.1 and 93.0% of the chromatographic profile, respectively. Electron impact mass spectrome-

TABLE 3. COMPOUNDS IN BOLL WEEVIL FEEDING DETERRENT FRACTIONS EtOAC-1 AND -2 FROM ROSE-OF-SHARON CALYX (GC-MS ANALYSIS)

Peak No.	Total volatiles (%) 1	2	M^+	Identity
1	9.6	20.2	158	Pelargonic acid
2	—	6.5	228	Myristic acid
3	27.9	7.3	270	Methyl palmitate
4	—	11.7	256	Palmitic acid
5	9.7	2.9	266	
6	31.1	8.0	294	Methyl linoleate
7	8.6	10.2	292	Methyl linolenate
8	2.1	—	298	Methyl stearate
9	—	2.2	310	
10	4.0	5.1	294	
Recovery (%)	93.0	74.1		

try via solid probe suggested that some sterols and wax esters may be present in these two fractions, but the fragmentation patterns of the fatty acids and their methyl esters were prominent and accounted for most of the compounds in the fractions. IR spectra were compatible with the structural assignment of fatty acids and methyl esters, showing a strong carbonyl absorption at 1710–1712 cm^{-1}.

In order to determine the significance of these candidate compounds, a paired series of eight saturated and four unsaturated fatty acids (Table 4) and their respective methyl esters (Table 5) were evaluated by testing 100-μg quantities in the plate bioassay. Of the saturated acids, pelargonic, myristic, and palmitic acids were stimulatory (120–188% of control); capric and hendecanoic acids were deterrent (13–69% of control). The unsaturated acids (oleic, linoleic, and linolenic) were deterrent (17–35% of control) while eleostearic acid appeared to be slightly inhibitory.

With the saturated methyl esters, results were somewhat erratic. Although, methyl caprate and methyl hendecanoate were consistently deterrent (34–73% of control) and methyl laurate was highly deterrent (30–36% of control); methyl stearate and methyl arachidate were mostly stimulatory (90–237% of control). All of the unsaturated esters, methyl oleate, methyl linoleate, methyl linolenate, and methyl eleostearate, were deterrent (20–84% of control).

Finally, a representative stimulant, palmitic acid, and a representative deterrent, methyl linolenate, were tested by the plate bioassay at 0.1–1000 μg/disk. The results in Figure 1 show that the responses varied linearly with the log of the concentration, that there were no significant response differences be-

TABLE 4. EFFECTS OF FATTY ACIDS ON FEEDING BY COTTON BOLL WEEVIL IN PLATE BIOASSAY[a]

	Punctures (% of control)[b]			
	Males		Females	
Acids	3 hr	6 hr	3 hr	6 hr
Saturated				
Pelargonic	130	159-S	121	188-S
Capric	64-D	63-D	69-D	64-D
Undecanoic	13-D	20-D	16-D	22-D
Lauric	120	173-S	64	84
Myristic	120	141-S	145	125
Palmitic	127	131	174-S	160-S
Stearic	104	99	85	105
Arachidic	80-D	87-D	80	80-D
Unsaturated				
Oleic	36-D	32-D	17-D	20-D
Linoleic	25-D	35-D	21-D	34-D
Linolenic	33-D	34-D	23-D	21-D
Eleostearic	72	86	80	98

[a] Data expressed as the percent of feeding punctures observed on test sample feeding sites relative to control sites. Six-well plate bioassay disks were prepared with three randomly placed choices per dish: fatty acid, its methyl ester, and a control. Each feeding site was treated with 100 μg of the appropriate can didate or solvent, air-dried 30 min, and bioassayed with either 10 males or 10 females per dish. Puncture counts from like sites on each dish were pooled; 25 replicates were conducted for each sex.

[b] Test designed as randomized complete blocks with analysis of variance (ANOVA). Numbers in each column followed by a "D" are feeding deterrents ($P < 0.05$); "S" are feeding stimulants ($P < 0.05$); or no letter, no effect on feeding ($P < 0.05$).

tween males and females, and that the thresholds were similar at about 0.4–1.0 μg/disk in the plate bioassay.

Fatty acids and their methyl esters have previously been found to influence insect feeding. Palmitic acid was found to be stimulatory for the confused flour beetle *Tribolium confusum* (J. Du Vac) (Loschiavo, 1965). Vinson et al. (1967) reported that linoleic acid was a stimulant for the black imported fire ant *Solenopsis richteri* (Foral). Vinson et al. (1975) identified methyl myristate and methyl palmitate as repellent pheromones from the Dufour gland of the bee, *Xylocopa virginica texana*.

Considering that ROS calyx tissue was found to be deterrent while the remaining bud tissue was not, it was of interest to determine whether the fatty acid content was higher in calyxes than in buds. Accordingly, the total lipid fatty acids, the methyl ester fatty acids, and the free fatty acids were determined

TABLE 5. EFFECTS OF FATTY ACID METHYL ESTERS ON FEEDING OF BOLL WEEVILS[a]

	Punctures (% of control)[b]			
	Males		Females	
Methyl esters	3 hr	6 hr	3 hr	6 hr
Saturated				
Pelargonic	57	107	62-D	121
Capric	63-D	73	72	67-D
Hendecanoic	48-D	45-D	38-D	34-D
Lauric	30-D	36-D	36-D	36-D
Myristic	73	82	110	156-S
Palmitic	93	109	89	112
Stearic	152	237-S	90	155-S
Arachidic	196	168	149	151
Unsaturated				
Oleic	41-D	41-D	28-D	41-D
Linoleic	20-D	26-D	32-D	56-D
Linolenic	33-D	52-D	23-D	21-D
Eleostearic	56	84	26-D	39-D

[a] Six-well plate bioassay disks were prepared with three randomly placed choices per dish: fatty acid, its methyl ester, and a control. Each feeding site was treated with 100 μg of the appropriate candidate or solvent, air dried 30 min, and bioassayed with either 10 males or 10 females per dish. Puncture counts from like sites on each dish were pooled; 25 replicates were conducted for each sex.

[b] Test designed as randomized complete blocks with analysis of variance (ANOVA). Numbers in each column followed by a "D" are feeding deterrents ($P < 0.05$); "S" are feeding stimulants ($P < 0.05$); or no letter, no effect on feeding ($P < 0.05$).

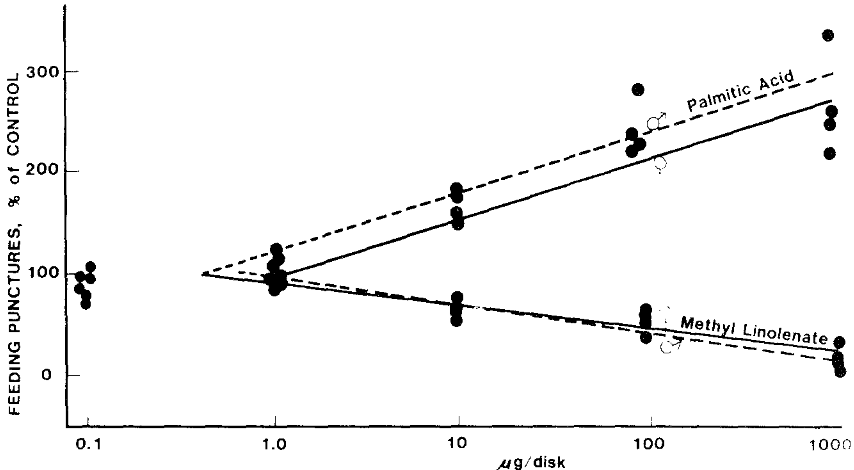

FIG. 1. Effect of concentration of palmitic acid and methyl linolenate of boll weevil feeding and estimation of thresholds, average at 3 and 6 hr.

TABLE 6. FREE FATTY ACIDS, FATTY ACID METHYL ESTERS, AND TOTAL LIPID FATTY ACIDS IN ROSE-OF-SHARON BUDS AND CALYXES

	Fatty acids (mg/g dry wt basis)				
Sample	Palmitic	Stearic	Oleic	Linoleic	Linolenic
Buds					
Total lipids	0.85	0.16	0.33	2.43	1.04
Methyl esters	0.08	0.01	0.03	0.21	0.18
Free fatty acids	0.02	0.01	0.01	0.05	0.02
Calyx					
Total lipids	0.57	0.05	0.14	1.31	0.67
Methyl esters	0.07	0.07	0.02	0.19	0.08
Free fatty acids	0.08	0.02	0.04	0.25	0.10

by GC-FID according to procedures described in the experimental section. The results, which are summarized in Table 6, show that while the percent total fatty acids was somewhat higher in buds than in calyxes, the free fatty acid content of calyxes was several-fold higher. The higher fatty acid content in calyxes supports the hypothesis developed through the isolational work and testing of standards that the unsaturated fatty acids and their methyl esters, present in relatively high concentrations in ROS calyx tissue, are responsible for feeding deterrency in the boll weevil.

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PHEROMONAL BASIS OF COURTSHIP BEHAVIOR IN TWO GYPSY MOTH PARASITOIDS: *Brachymeria intermedia* (Nees) and *Brachymeria lasus* (Walker) (Hymenoptera: Chalcididae)

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Abstract—The pheromonal basis of the courtship behavior of two gypsy moth parasitoids, *Brachymeria intermedia* and *Brachymeria lasus*, was traced to a single component in each case. This component was isolated by a combination of absorption and gas-liquid chromatography and shown to elicit some of the courtship behavior typical of these species. Bioassays of extracts from several independent techniques for sequestering pheromones, as well as interspecific assays, support this conclusion. Comparative analyses of both male and female extracts by capillary chromatography show the uniqueness of the pheromonal peak to the female volatile profile.

Key Words—*Brachymeria intermedia, Brachymeria lasus*, courtship behavior, chromatography, sex pheromone, Chalcididae, Hymenoptera.

INTRODUCTION

The mating behavior of many parasitic insects consists of a series of discrete, highly complex courtship responses (Matthews, 1975; Leonard and Ringo, 1978). Chemical, visual, auditory, and tactile cues have been implicated. However, with the exception of two species of ichneumonids (Robacher et al., 1976; Eller et al., 1984) little is known of the pheromonal basis of parasitoid courtship behavior. Considering the wealth of evidence existing for other groups of insects (Lepidoptera, for instance), and that parasitoids account for approximately 15% of Insecta (Askew, 1971), with possibly the greatest species diversity within a group of animals, it is of obvious import to any comprehensive knowledge of pheromones that this lacuna be explored. Biocontrol programs involving

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the release of exotic or native beneficials in new habitats require knowledge of their establishment and spread (Coppel and Mertins, 1977). This can be achieved with pheromones. Integrated pest-management programs can be better practiced by knowledge of the flight activity of parasitoids which may enable directed application of pesticides to avoid any detrimental impact on beneficials. Further, pheromone trapping of both host and its parasitoid may provide information on the degree of control exerted by the latter (Morse and Kulman, 1985).

Within this context, a study was initiated to define the pheromonal basis of the mating behavior of two species of gypsy moth endoparasitoids, *Brachymeria intermedia* (Nees) and *Brachymeria lasus* (Walker).

METHODS AND MATERIALS

Adult parasitoids were reared on the pupal stage of the factitious host, the greater wax moth, *Galleria mellonella* (L.). To this end, a small-scale mass-rearing technique was developed to provide a continuous and reliable source of the pupal stage (Mohamed and Coppel, 1983). Cocooned pupae reared singly, or in sheets of 50–100 of the wax moth were allowed to be parasitized by stock cultures of both species of parasitoids. The parasitized cocoons were placed singly in vials sealed with perforated plastic caps. They were incubated at 28.5°C, 16:8 light–dark, and 60 ± 5% relative humidity. Upon eclosion (males in 5–6 and females in 8–9 days), the adults were sexed and either treated to obtain crude pheromone or returned to stock cultures.

Several techniques were employed to collect crude pheromone samples. Some were based on literature reports on other species (Young and Silverstein, 1975) while others were designed to incorporate elements of the biology of these species. Within this framework, crude samples were collected from the parasitoids from the time of eclosion to death. Each technique was sufficiently distinct to allow several independent means of pheromone isolation and maximization of collection of crude samples. These techniques were as follows: (1) Emerging male or female parasitoids from cocoons in sheets were placed singly in 4-ml shell vials and sealed with a perforated cap; and, similarly, a male and female were placed in each vial. (2) Parasitoids emerging from singly parasitized cocoons previously held in vials were allowed to remain in these vials. The vials with parasitoids were incubated at 28.5°C and 16:8 light–dark for 24 hr. They were then removed and transferred to screened cages holding either males, females, or both sexes. The empty vials were stored until treatment to obtain crude pheromone. (3) Within these cages the parasitoids were allowed to aggregate daily on filter paper (Whatman No. 40) -lined vials (55 × 25 mm) during scotophase. The filter paper was removed and extracted monthly. (4) Dead parasitoids from all cages, of either sex, or a combination of the sexes were collected and refluxed in a solvent. (5) Virgin females from rearing cages

were placed in a glass cylinder (22.5 cm) with a tapering funnel-shaped entrance and an exit fitted with a stopcock. A heated air stream, filtered through calcium sulfate (Drierite) and charcoal was allowed to enter one end of the glass cylinder, and exit through a bed of Porapak Q at the other end, to adsorb volatiles. Approximately 5000 females and 1000 males of each species were processed.

Crude extracts from these sources were obtained in the following manner. To each vial 0.5 ml of a solvent mixture of hexane–ethyl acetate (75:25) was added and vortexed for 15 sec. Extracts from each source were then pooled separately (that is, male in vials, female in vials, male and female in vials). The filter papers from each source (technique 3) were placed separately in 250-ml Erlenmeyer flasks and agitated with the solvent mixture for 24 hr on a shaker. Volatiles from the Porapak Q were similarly desorbed. Dead bodies of each sex, or a combination thereof, were refluxed for 24 hr in the solvent mixture mentioned. The pooled extracts from each source were filtered separately over anhydrous sodium sulfate and concentrated with a Büchi evaporator. They were then transferred to 2-ml vials and stored at 0°C until further usage.

Crude extracted pheromone from each source was fractionated and/or analyzed by column, thin layer, and gas–liquid chromatography (GLC). Adsorption column chromatography was carried out on silica gel (1.1 g) packed in 40% ether–hexane in a 11.5 × 1 cm ID glass column. The elution series consisted of 4-ml aliquots of hexane; 0.1%, 1.0%, 10%, 20%, and 40% ether–hexane; and methanol. Initial separation was monitored by thin-layer chromatography using silica gel impregnated with 5% ammonium sulfate (Analtech Uniplates, self-charring) using 25% ethyl acetate in hexane as developing solvent. Further fractionation was achieved by GLC using sequentially, 1.8-m glass columns (2 mm ID), (1) 10% OV-1, and then (2) 10% OV-101, on 80/100 Gas Chrom Q and a volume flow rate (He) of 30 ml/min. The temperature program used was as follows: for column (1): 75°C (3 min), 15°C/min to 130°C (5 min); and for column (2): 40°C (10 min), 3°C/min to 65°C (10 min) with the injection port temperature at 40°C with a flash injector assembly.

Analyses of both crude and fractionated samples were facilitated with a 15-m DB-5 capillary column, using helium at a flow rate of 4 ml/min, and a temperature program of 40°C (5 min), 15°C/min to 240°C (5 min); 15°C/min to 285°C (35 min) with an injection port temperature of 195°C.

Gas–liquid chromatography was done on a Spectra Physics 7100 equipped with flame ionization detectors. The gas chromatograph was fitted with a splitter modified from Brownlee and Silverstein (1968). Components from the packed columns were shunted via the splitter to a glass capillary tubing. The tubing was encased in finely crushed Dry Ice held in place by a glass cylinder tapered at one end. The capillary tubing and tapered glass cylinder were held in place with Teflon tape. Condensed effluents within the tubing were desorbed with several washes of ether into a 4-ml vial using a 10-ml Cornwall glass syringe.

Samples were concentrated under argon and then bioassayed or further analyzed.

Bioassays were conducted on crude, column, and GLC fractions. Each sample, in concentrations from 0.5 to 2.0 female (or male) equivalents per 10 μl hexane, was applied to one spot on the perimeter of a 1.27-cm filter paper disk (Schleicher and Schuell Inc.). The disk was placed in the middle of a glass Petri dish (9 cm diam.) on a white cardboard background and the solvent allowed to evaporate for 5 min. Five male parasitoids (3–7 days posteclosion) were transferred to the Petri dish. Their behavior was observed for 15 min under a light that generated a temperature of $29 \pm 1.0°C$ at the base of the Petri dish.

The criteria for a sample's activity were based on qualitative observations on the courtship behavior of these parasitoids, and those previously described (Simser and Coppel, 1980a, b; Leonard and Ringo, 1978). The following discrete behaviors were observed and their numbers tallied on several hand counters: wing flexing (WF), wing vibrations (WV), sway (S, movement of the body from side to side, as the male moves, at a few degrees inclination from the vertical), sway in contact with another male (SCM), disk location (DL) followed by a brief antennation of the disk lasting for less than 5 sec and disk location with antennal probing of the spot where the sample was located for greater than 15 sec. Each assay, for each species, was conducted between 1 and 4 hr after the onset of photophase which was either at 0800–1100 or 1500–1900 hr depending on the incubators' light regime. Behaviors not readily quantifiable were recorded descriptively. Bioassays were conducted with samples drawn at random, or with observers who had no prior knowledge of the sample source or content.

Field bioassays were attempted with *B. intermedia*, which is established against the gypsy moth. Samples were pipetted onto 4-cm-long dental cotton wicks, air dried for 30 min, and suspended in white Pherocon II traps (Zoecon Corp., Palo Alto, California). The prepared traps were suspended at breast height on scrub oak trees (located near Otis Air Force Base, Massachusetts). This site consisted of pitch pine and a moderate understory which previously had been defoliated 50–60% by the gypsy moth. The traps were randomized and monitored daily for three weeks from mid-July to early August.

The techniques and procedure used are summarized in Figure 1. All data were analyzed for normal distribution, whereupon parametric tests were employed to assess significant differences of means. These tests include analyses of variance (ANOVA) and least significant difference (LSD).

RESULTS

The activity of crude extracts over several replicates from a given source, or between different sources, did not elicit a concerted series of discrete courtship responses in either species. However, occasional observations of male par-

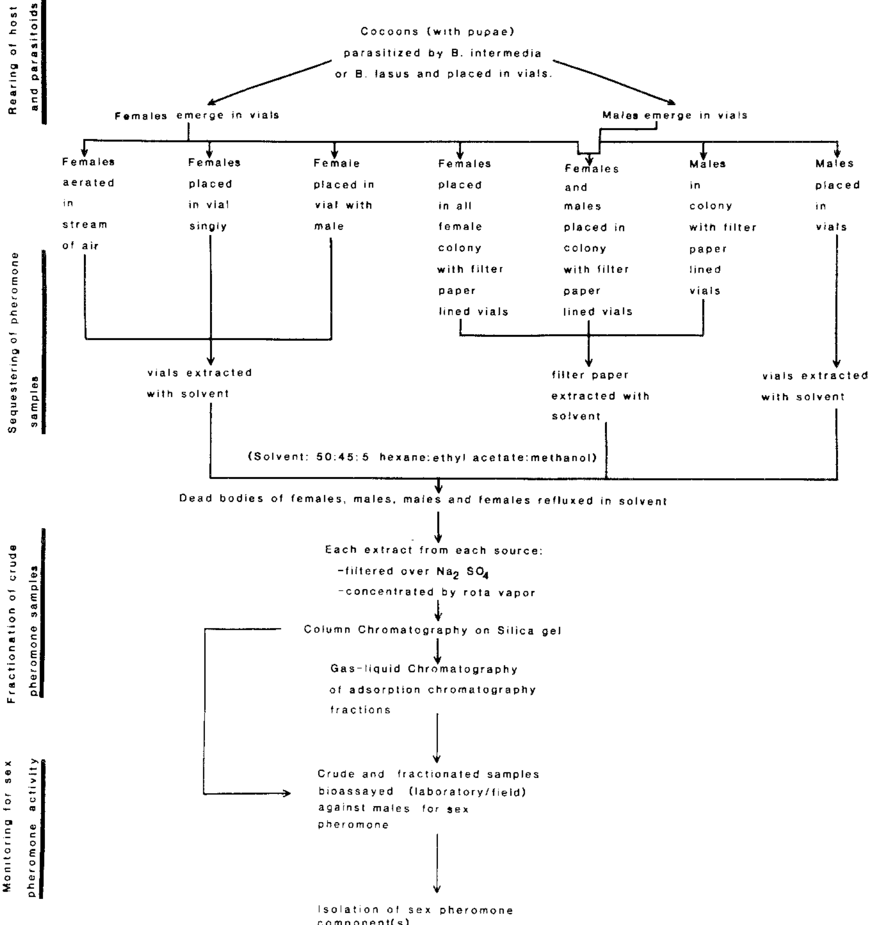

FIG. 1. Flowchart summarizing the techniques and sequence of steps used in sequestering and analyzing the sex pheromones of the parasitoids, *Brachymeria intermedia* and *Brachymeria lasus*.

asitoids locating the disk and wing pulsation on exposure to the crude fractions did allow us to infer the existence of a pheromone, with its effect possibly being inhibited by other components. This proved to be the case as adsorbtion chromatography with silica gel of the crude sample led to the isolation of a 0.1% ether–hexane fraction which was active. Responses elicited were typical of mate location such as disk location, antennation of the disk, and courtship behaviors such as wing flexing, wing vibration, and swaying of the parasitoids (Tables 1 and 2). Bioassays of this fraction in combination with other adsorption column chromatography fractions did not elicit any increased activity. Thin-layer chromatography of these active fractions, for both species, gave bands at $R_f = 0.9$.

TABLE 1. COURTSHIP RESPONSES OF *B. intermedia* MALES TO COLUMN AND GAS–LIQUID CHROMATOGRAPHIC FRACTIONS OF CRUDE SAMPLES COLLECTED FROM CONSPECIFIC VIRGIN FEMALES, MALES OR COMBINATION OF THE TWO SEXES, USING VARIOUS TECHNIQUES[a]

	$\bar{X} \pm$ (SE) of courtship responses of 5 male parasitoids[b]					
Source of extract	WR	WV	S	SCM	AD[a]/DL	AD[b]/DL
Intraspecific tests						
Vials with virgin females and coccons*	21 (4)[a]	2 (0.4)[b]	2 (1)[c]	4 (1)[d]	23 (5)[e]	24 (7)[f]
Vials with virgin females	14 (4)	3 (1.3)	1 (0.4)	4 (2)	15 (3)	21 (3)
Vials with males and females	16 (2)	0 (0)	1 (0.4)	0	13 (3)	22 (3)
Aeration of virgin females	16 (3)	0 (0)	1 (1)	1 (1)	18 (2)	22 (6)
Filter paper from virgin female colonies	7 (1)	1 (0.4)	2 (0.4)	1 (0.4)	10 (2)	22 (6)
Refluxed bodies of virgin females	21 (1)	0 (0)	2 (0.4)	2 (0.4)	14 (3)	22 (7)
GLC fraction[c]	24 (4)[a]	7 (2)[c]	6 (1)[c]	10 (2)[d]	19 (2)[c]	8 (1)[g]

Controls						
Vials with males and cocoons	0.3 (0.2)	0	0	0	0	0
Filter paper from male colonies	0.8 (0.5)	0	0	0	0	0
Refluxed bodies of males	1 (1)	0	0	0	0	0
Solvent	3 (1)	0	0	0	0	0
Interspecific tests[c]						
B. lasus female sources	2 (1)	0.2 (0.1)	0	0	1.3 (0.3)	0

[a] A 0.1% ether–hexane fraction collected from adsorbtion chromatography was used, and for the GLC fraction a peak at 13.4 min from an OV-101 column.

[b] Each source of extract was bioassayed $N = 6$ replicates. The extracts elicited behaviors clearly different from controls and interspecific sources. Symbols used are abbreviations for discrete behaviors of males: WR, wing raising; WV, wing vibration; AD[a]/DL, disk location followed by antennation of disk for less than 5 sec; AD[b]/DL, disk location followed by antennation of disk for greater than 10 sec; S, sway; SCM, sway on contact with a male.

[c] The responses elicited within the interspecific tests and GLC fractions are an average of $N = 12$ replicates based on two replicates per technique. Statistical similarity between means (in a column) are indicated by similar letters and vice versa. The means were compared with that of our most active source (asterisked). Statistical tests were conducted with the unequal sample size t test at $P \leq 0.01$.

TABLE 2. COURTSHIP RESPONSES OF *B. lasus* MALES TO VARIOUS COLUMN AND GAS–LIQUID CHROMATOGRAPHIC FRACTIONS OF CRUDE SAMPLES COLLECTED FROM CONSPECIFIC VIRGIN FEMALES, MALES, OR COMBINATION OF THE TWO SEXES, USING VARIOUS TECHNIQUES[a]

	$\bar{X} \pm$ (SE) of courtship responses of 5 male parasitoids[b]					
Source of extract	WR	WV	S	SCM	AD[a]/DL	AD[b]/DL
Intraspecific tests						
Vials with virgin females and cocoons*	22 (5)[a]	10 (2)[b]	7 (2)[c]	7 (1)	17 (4)[d]	46 (5)[e]
Vials with virgin females	29 (5)	3 (1)	4 (1)	5 (1)	18 (3)	29 (4)
Vials with males and females	42 (11)	14 (3)	4 (1)	4 (2)	17 (3)	46 (8)
Aeration of virgin females	38 (4)	4 (1)	2 (1)	3 (1)	21 (4)	36 (6)
Filter paper from virgin female colonies	17 (4)	6 (2)	4 (3)	4 (3)	17 (4)	17 (6)
Refluxed bodies of virgin females	25 (4)	0	3 (1)	4(1)	12 (2)	23 (4)
GLC fraction [c]	20 (3)[a]	6 (1)[b]	6 (1)[c]	5 (1)	18 (3)[d]	18 (4)[f]

Controls						
Vials with males and cocoons	2 (0.2)	0	0	0	2 (1)	0
Filter paper from male colonies	2 (1)	0	0	0	3 (1)	0
Refluxed bodies of males	4 (1)	0	0	0	3 (1)	0
Solvent	1 (0.3)	0	0	0	0.3 (0.2)	0
Interspecific tests[c]						
B. intermedia female sources	5 (1)	1 (0.5)	0.2 (0.2)	0.3 (0.3)	6 (2)	1 (0.4)

[a] A 0.1% ether–hexane fraction collected from adsorption chromatography was used; and for the GLC fraction a peak at 14.6 min from an OV-101 column.
[b] Each source of extract was bioassayed $N = 6$ replicates. The extracts elicited behaviors clearly different from controls and interspecific sources. Symbols used here are abbreviations for discrete behaviors of males: WR, wing raising; WV, wing vibration; AD[a]/DL, disk location followed by antennation of disk for less than 5 sec); AD[b]/DL, disk location followed by antennation of disk for greater than 10 sec); S, sway; SCM, sway on contact with a male.
[c] The responses elicited within the interspecific tests and GLC fractions are an average of $N = 12$ replicates based on two replicates per technique. The means were compared with that of our most active source (asterisked). Statistical tests were conducted with the unequal sample size t test at $P \leq 0.05$. Statistical similarity between means (in a column) are indicated by similar letters and vice versa.

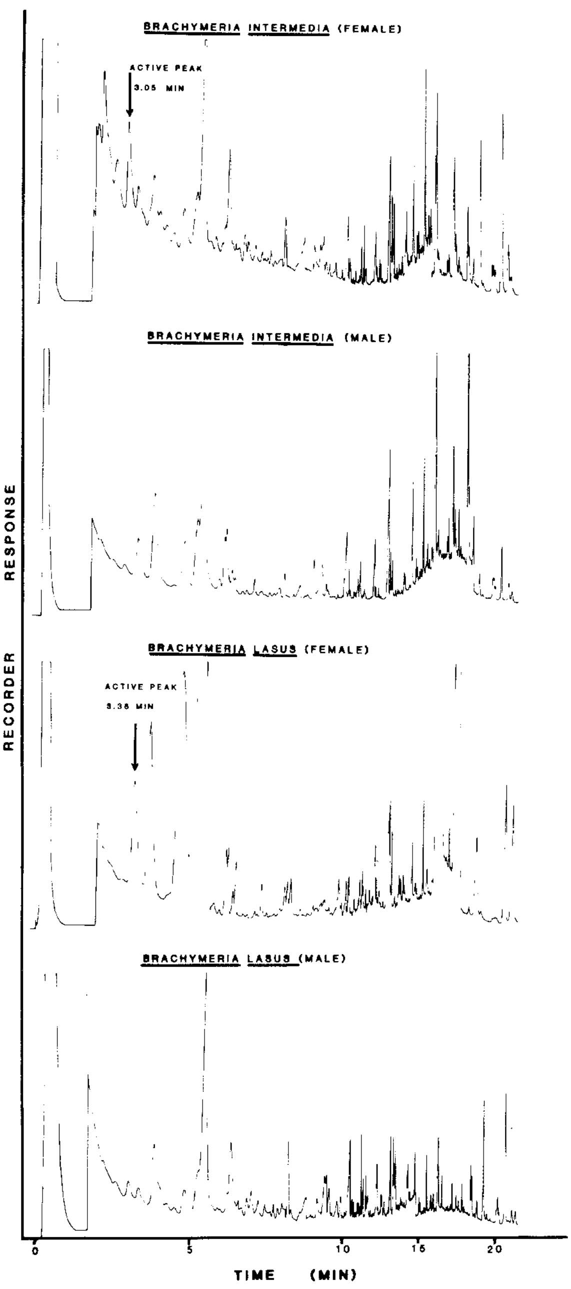

FIG. 2. Comparative analyses, by capillary chromatography of crude extracts, from both sexes of *B. intermedia* and *B. lasus*. The source was from vials in which they had enclosed from parasitized cocoons. A 15-m DB-5 capillary column was used with detector at 64 × 10^{-10} attenuation and range (1.5 min) 2 × 10^{-11} attenuation and range to the end of run.

No courtship responses could be elicited from control experiments in either species. That is, solvents, samples derived from male sources in a manner identical to that of females, and interspecific tests of males of one species against female sources of the other species yielded no responses typical of a pheromone (Tables 1 and 2). The lack of response in the latter two instances is consistent with the uniqueness of some components of the GLC profile of the female when compared to the male and that of the female of each species (Figure 2). This is further borne out by GLC analyses which revealed that the active peak was only present in the 0.1% either–hexane fraction.

Further bioassays of adsorbtion chromatography samples were conducted against *B. intermedia* in the field. Since the occurrence of the sex pheromone of this species was extremely low (ng/female), a limited number of traps was deployed. Although female-derived sources of extract did attract low numbers of male parasitoids (Table 3), further assays were not attempted so as to conserve our material for spectrometric analysis.

Preparative GLC on an OV-1 column of the active adsorbtion column chromatographic fractions yielded an active area for each species between retention times of 2.5 and 6.5 min. Separation of this region on a more polar column (OV-101) led to the isolation of an active peak for each species (Figure 3). The peaks were defined on the DB-5 capillary column (Figure 2) at 3.05 min for *B. intermedia* and 3.38 min for *B. lasus*. Courtship behavior was similar to that observed from the adsorbtion column chromatographic fraction (Table 1 and 2, GLC fraction). This component occurred in all sources of extracts, although in varying concentrations. The most productive source, extracts from vials which had newly eclosed females only, showed the component in concentrations for either *B. intermedia* or *B. lasus* at 1–3 ng/female, respectively. Other sources had concentrations ranging from 0.1 to 0.5 ng/female.

TABLE 3. FIELD RESPONSE OF *Brachymeria intermedia* TO ADSORPTION COLUMN CHROMATOGRAPHY FRACTIONS FROM SEVERAL DIFFERENT SOURCES[a]

Source of extract	Total number of males trapped[b]
Control (solvent)	0
Vials with virgin females and cocoons	4
Filter paper from virgin female colonies	3
Refluxed bodies of virgin females	2
Refluxed bodies of male and females	1

[a]Bioassays were conducted with 0.1% ether–hexane fraction obtained from adsorption column chromatography. Other fractions were also bioassayed but were inactive. Each trap was baited with 10 female equivalents.

[b]Numbers of males caught are from pooled results of three traps per source of extract.

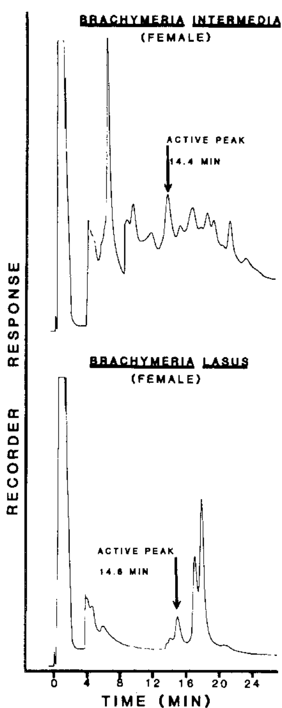

FIG. 3. Packed column chromatograms of active adsorption chromatography fraction. Analysis (and fractionation) was done with a 1.8-m, 2-mm-ID glass column with 10% OV-101. Detector was at 32×10^{-10} (3 min), and 4×10^{-11} to end of run.

DISCUSSION

It is evident that the courtship behavior of these parasitoids has a pheromonal basis, this being established in several ways. First, several independent methods of pheromone collection were used, all of which proved to be active, although to varying extents. GLC analyses and preparations of samples from the various techniques resulted in demonstrating pheromone-mediated behaviors for both species. In contrast, bioassays of other fractions obtained by these preparative methods, solvents used in various phases of sample collection, interspecific bioassays (*B. lasus* males vs. *B. intermedia* female sources, and vice versa), and male extracts (collected under similar conditions for females) all proved inactive.

Additionally, the nature of the behavioral responses was consistent with what was known of the courtship responses of males to the females of these species. This was evident in the occurrence and frequency of discrete behaviors

to the pheromone source, such as wing flexing (FW), wing pulsation (WP), disk location (DL), etc., which were clearly different from various controls. More importantly, for establishing a pheromonal basis of these behaviors, the discrete values were observed within a context of a series of mutually reinforcing responses. That is, the parasitoid, on entry to the Petri dish would first examine the dish's surface with the antennae. In the presence of an active sample, such examinations usually led to disk location, whereupon the male would antennate exactly the spot where our pheromone sample was placed (usually the diameter of 10-μl pipet). As this occurred, the body arched, and rapid wing vibration followed which usually lasted for 15–60 sec. An alternative pathway of behavior by the male on the disk was by turning 360° several times. Either behavior usually results in swaying of the body in a lateral plane as the male moves away. This sequence was repeated several times during the observation period. The sway component of the courtship behavior, in the presence of an active sample, also occurred when a male either approached the disk or as a male approached another male (SCM). Although the recorded values are apparently discrete, they are a reflection of an underlying pattern of a concerted series of behaviors which constitute the courtship response.

Several additional types of male behaviors of either species were observed but occurred infrequently. These included: kicking other males with the forelegs in what seemed an attempt to monopolize the pheromone area, swaying at the disk, attempting to antennate disk from the top of the Petri dish, and aggregation of males above the disk during and after the end of our observation period. Although infrequent, most of these behaviors have been observed in colonies containing both sexes. Given the rarity of their occurrence, no attempt was made at quantification.

The courtship responses to adsorption column chromatography or GLC fractions do have their bases in the volatile chemistry of these species. Comparison of the capillary chromatograms between sexes and species indicated that the most volatile region (first 10 min) had the most variability in both the number of components as well as the occurrence and nonoccurrence of certain peaks. This observation acquired significance as the active peaks for *B. intermedia* and *B. lasus* were found at 3.05 and 3.38 min, respectively. If a generalization is permitted from these observations, it is possible that, given active extracts, regions of variability found in comparing GLC profiles between sexes of a species could provide a clue as to where to initiate a search for a pheromone.

The field bioassays for *B. intermedia* trapped very few parasitoids but were significant against the controls and consistent with the activity to the 0.1% ether–hexane fraction observed in the laboratory. Since the pheromone components occurred in very low concentrations, and were extremely volatile, more field bioassays have been deferred until synthetic material is available.

Although our research showed clearly a single-component pheromone elic-

iting all major courtship responses for these species, we do not rule out the possibility of other minor component(s). The frequency of behaviors were designed to allow the assessment of a second component(s). However, the high variability obtained when combining fractions did not allow a definitive assessment of this possibility.

Given the size of the male parasitoids (*B. intermedia*, 4.5 ± 0.4 mg and 4.5 ± 0.1 mm; *B. lasus*, 4.2 ± 0.4 mg and 4.8 ± 0.2 mm; $N = 12$, in weight and length, respectively), the discrete nature of each behavior in the courtship sequence, the simplicity of the Petri dish for observation and manipulation, and the availability of a synthetic pheromone, it can be readily appreciated that such a combination could lead to a system that allows for developing a model of how parasitoids use chemical information against or with a background of auditory, tactile, or visual cues. The availability of pheromones for these species should allow for the rapid acquisition of information on establishment, dispersal, distribution, and abundance, which in turn can provide a more reliable picture of their impact on the gypsy moth, one of their hosts.

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TEMPORAL PATTERNS OF SEX PHEROMONE TITERS AND RELEASE RATES IN *Holomelina lamae* (LEPIDOPTERA: ARCTIIDAE)

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Abstract—Six hydrocarbon components (2-methylhexadecane, *n*-heptadecane, 2-methylheptadecane, 2-methyloctadecane, *n*-nonadecane, and 2-methylnonadecane) were identified in sex-pheromone gland extracts and in airborne collections of *Holomelina lamae*. Low variability in the ratio of components among individuals indicates tight regulation of blend composition. Minor changes were evident in the quantity and ratio of the blend as a function of time of day. Based on gland extracts, the total quantity of the six components increased from day 1 ($\bar{X}$ = 6299 ng) to day 4 ($\bar{X}$ = 7498 ng) and subsequently decreased. No significant correlations were found between total gland contents and wet or dry weights of moths. Emission rates of *H. lamae* females were determined from pheromone adsorbed onto Porapak Q. Quantities released peaked shortly after the onset of calling and decreased rapidly as calling continued. Peak release rates ranged from 13 to 350 ng/10 min, and from 37 to 835 ng/60 min. Noncalling females did not emit detectable quantities of pheromone. Based on release rates and the rate of pulsation of the abdominal tip, the average amount released per pulse is not constant. The mean ratio of components released (0.78:7.45:84.80:2.84:2.59:1.53) was not very different from the ratio of components in gland extracts of 2-day-old females (0.70:4.19:90.12:1.65:1.91:1.42). We propose that the blend is atomized rather than volatilized from the gland, thus retaining nearly the same ratios in the female's effluvium as in her gland.

Key Words—Sex pheromone, pheromone release rates, pheromone titer, airborne pheromone collection, *Homomelius lamae*, Lepidoptera, Arctiidae, 2-methylheptadecane.

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INTRODUCTION

Most moths that employ pheromones for sexual recruitment employ blends of several pheromone components. A persistent problem in research on pheromones has been the correlation of surface washes or gland extracts with volatile components. Olsson et al. (1983) summarized the problem as follows: "Discussion of the relative amounts of compounds in a pheromone blend in terms of major and minor components are generally based on the relative amounts found in extracts of female glands. When the vapor pressures of the pheromone components are significantly different, the relative amounts in the vapor phase differ from those in the liquid phase." They showed that the pheromone components of the turnip moth, *Agrotis segetum*, decyl, (Z)-5-decenyl, (Z)-7-dodecenyl, and (Z)-9-tetradecenyl acetate, which were found in a ratio of 5.7:4.3:62.7:27.3 in abdominal tip extracts, were represented as 28.6:25.5:43.2:2.7, respectively, in the vapor phase. Few studies have combined airborne collections of pheromone components with gland extracts, and few behavioral evaluations of multicomponent pheromone blends have been based on the blend actually emitted by the female. Further, little work has been directed at determining how the ratios are modulated by factors such as age and time of day.

Roelofs and Cardé (1971) determined that 2-methylheptadecane was a major component of the sex pheromone of several sibling species in the *Holomelina aurantiaca* complex. Pulsing of the abdominal tip in this group yields a higher pheromone release rate than that obtained from artificially extruded glands (Schal and Cardé, 1985), and peak pheromone release occurs soon after the onset of calling even though calling in *H. lamae* persists for several hours (Schal and Cardé, 1986). Here we report on the composition of a multicomponent blend in *H. lamae* pheromone glands and airborne collections and constituent quantities and ratios as a function of time of day and age.

METHODS AND MATERIALS

Insects. The main *Holomelina lamae* colony (MI) originated from insects collected in Manistique, Michigan (R16W, T42N, sec 14) on August 10, 1978, and was maintained in the lab for ca. 60 generations. A second colony (ME) originated from 12 females collected at Seawall Bog, Acadia National Park, Maine, on August 12, 1984; second generation females were used. Larvae were reared individually on pinto-bean diet in 30-ml clear plastic cups at 27°C and 15:9 light–dark regime until the last instar, whereupon they were transferred to 24°C and 16:8 light–dark regime. Light intensity during photophase was 1400 lux. Insects were checked daily for pupation, and female pupae and adults were kept in clear plastic cups. For airborne volatile collections, females following ecdysis were kept in screen cages in constant 40 cm/sec wind.

Pheromone Extraction. Abdominal tips of females were excised with forceps and immersed for 1 hr in 150 μl of a 0.5 ng/μl solution of 2-methylpentadecane (2me-15c, as internal standard) in redistilled *n*-hexane. A subsequent extraction of the same gland for 24 hr liberated an additional 4.7% of 2-methylheptadecane (2me-17c), the major pheromone component, but also greatly increased the amount of debris which interfered with GLC analysis. In both extracts, all six components were liberated in similar proportions. Smaller volumes of solvent increased losses associated with removal of the extracted glands from the solvent; although quantitative analysis was unaffected because an internal standard was used, we attempted to minimize these losses in order to permit quantification of minor extractable components. Samples were stored in conical glass tubes made by drawing Pasteur pipets over a flame. They were placed in hexane-containing glass vials with Teflon-lined caps and stored at −20°C.

Airborne Collections. Two types of collection devices were used. For 10-min aeration of the abdominal tip, collection tubes were modified Pasteur pipets with one flared and one constricted end; they were silanized and filled with 200 mg of preconditioned Porapak Q between glass wool plugs (Schal and Cardé, 1985). Glass beads, glass wool, and charcoal adsorbants were unsatisfactory because the short-chain hydrocarbons were not retained as well as the long-chain compounds. Conditioning of Porapak Q was modified from Byrne et al. (1975). The adsorbant was washed with redistilled *n*-hexane, N_2 dried, heated at 180°C in a N_2 stream for 24 hr, packed into tubes, and washed again with hexane. Tubes thus treated were reused after extraction without further conditioning.

Females were placed on horizontal wooden dowels (0.5 cm diameter) where they assumed vertical calling positions with the abdomen hanging below the dowel. The collection tube was positioned such that the abdominal tip was ca. 3–5 mm below the outer rim of the flared end of the tube. Thus, tubes could be exchanged readily with no disturbance to the moth. Readsorption onto the female's body was minimized by turbulence-free unidirectional flow as determined with titanium tetrachloride "smoke."

For 60-min collections, the apparatus described by Schal and Cardé (1985) was employed. Individual females with clipped wings were placed on a screen perch within the upper portion of a chamber composed of two modified ground-glass joints (14/20) whose lower portion was packed with 150 mg of preconditioned Porapak Q. The inlet was packed with glass wool (prewashed with hexane and heated at 350°C) as an air filter.

For both 10- and 60-min collections, 210 ± 10 ml/min of room air was pulled through the collection tube with a vacuum pump. Room temperature was 23 ± 2°C. Pheromone was eluted with 800 μl of *n*-hexane in 100-μl aliquots, and 100 ng 2me-15c was immediately added as internal standard.

To determine the collection efficiencies for both devices, 5 μl of a hexane

solution, consisting of 1 μg of each of the six components [2-methylhexadecane (2me-16c), *n*-heptadecane (*n*-17c), 2-methylheptadecane (2me-17c), 2-methyloctadecane (2me-18c), *n*-nonadecane (*n*-19c), 2-methylnonadecane (2me-19c); all from K & K Laboratories] was applied to the glass-wool plug above the Porapak and aerated for 10 or 60 min. No breakthrough occurred in either set-up when two collection tubes were arrayed in series for 3 hr.

Quantitative Analysis. Extracts in hexane were reduced to 2 μl with a stream of prefiltered N_2. Absolute losses of the six components ranged from 9 to 30%, but their ratio relative to the internal standard remained the same. Gas-liquid chromatographic (FID) analysis was conducted on 2-m × 2-mm-ID and 1-m × 2-mm-ID glass columns packed with 3% SP-2100 (methyl silicone) on 100–120 mesh Supelcoport. Oven temperature was 155°C for 5 min and programmed to 210°C at 3°/min. To examine for possible trace amounts of other compounds, some runs were made on a polar 2-m × 2-mm-ID Silar 10CP column (10% Silar 10C on acid-washed 100–120 mesh Chromasorb W).

RESULTS

Gland Titers. In addition to 2me-17c, the major component reported in *Holomelina* (Roelofs and Cardé, 1971), five other hydrocarbons were extracted. Abdominal tips of newly ecdysed MI females ($N = 9$) averaged a total of 6299 ng of all six components combined (Figure 1, Table 1). Quantities and ratios of components extracted from pharate adults ($N = 5$) 24 hr before eclosion were not significantly different from freshly eclosed females. Two-day-old ME females (one generation in lab) had similar ratios of the six components (Figure 1, Table 1), but the titers were significantly lower than those of 2-day-old MI females (Table 1).

Relatively small diel changes in pheromone gland titers were evident over 13 days (Figure 1). The levels of all components decreased around 10 hr after lights on, coinciding with onset of calling (Schal and Cardé, 1986), but with the exception of 3-day-old females, these changes were not statistically significant ($P > 0.05$, SNK test). In 3-day-old females, the titer of all components rose to its highest level by 14 hr after lights on (Figure 1), although calling continued until lights off (Schal and Cardé, 1986).

The ratio of the six components in the gland did not vary on a diel basis ($P > 0.05$, SNK test; Figure 1). Small standard errors indicate tight regulation of the composition of glands in different females as do significant ($P < 0.01$) correlation coefficients (Pearson) among quantities of all six components.

Quantities of 2me-16c and *n*-17c increased with age up to 13 days, based on a linear model ($P < 0.01$) (Figure 1); 2me-19c decreased with age ($Y = -0.14X + 87.0$, $P = 0.03$), while the change in other components did not differ significantly from zero. For the first four days of calling, the titer of each

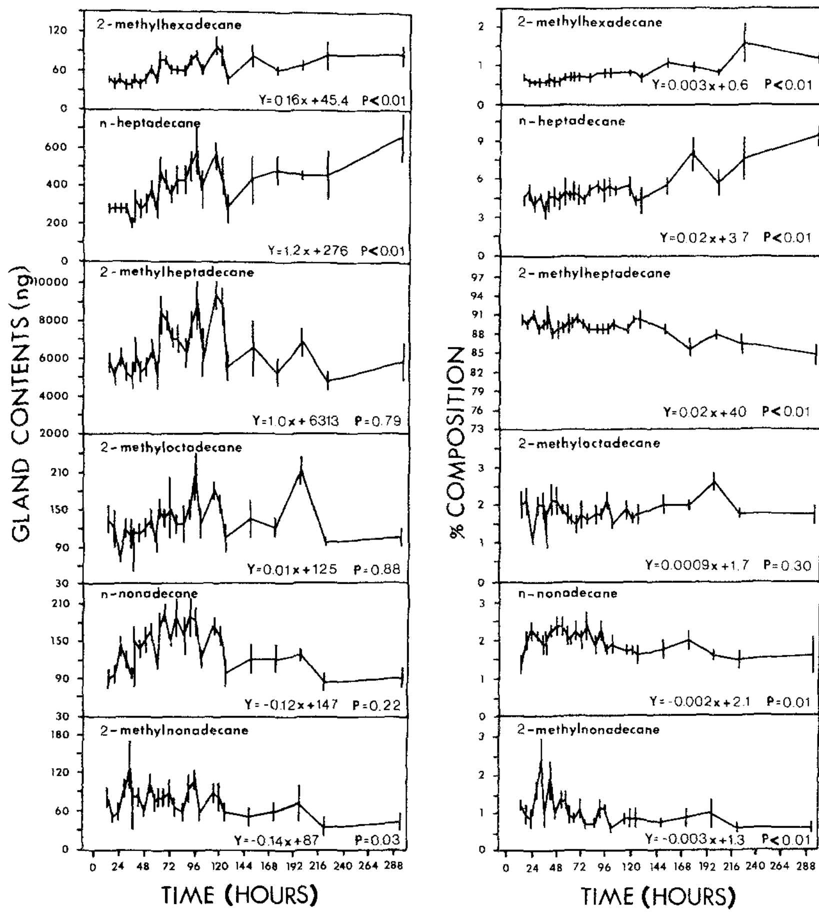

FIG. 1. Changes in quantities (left) and proportions (right) of the six components (±SEM) extracted from pheromone glands of 0- to 14-day old *H. lamae* females. An 8-hr scotophase occurred between 1600 and 2400 hrs daily. Samples sizes range from 8 to 14 for each time.

component except 2me-19c, and the titer of all components combined increased with age ($P < 0.05$) (Figure 1). On subsequent days, the quantities of all components did not change significantly until day 13.

Although only small changes were seen in the ratios of components as females aged (Figure 1), significant ($P < 0.01$) increases with age occurred in 2me-16c and *n*-17c, and significant decreases ($P < 0.01$) occurred in the ratios of 2me-17c, *n*-19c, and 2me-19c; the proportion of 2me-18c did not change with time ($P = 0.30$). In the first four days, the ratios of only 2me-16c and *n*-17c increased significantly; the proportions of the other components remained unchanged (Figure 1). On subsequent days (5–13), the proportion of *n*-

TABLE 1. QUANTITIES AND PROPORTIONS (±SD) OF SIX COMPONENTS IN PHEROMONE GLANDS OF MI AND ME *H. lamae* AT VARIOUS TIMES AND RELEASED BY 2-DAY-OLD MI FEMALES

	At emergence ($N = 9$)		Mean first 4 days ($N = 164$)	
Component	ng	%	ng	%
2-Methylhexadecane	45 ± 10	0.72 ± 0.05	55 ± 25	0.75 ± 0.11
n-Heptadecane	281 ± 103	4.36 ± 1.01	355 ± 186	4.90 ± 1.43
2-Methylheptadecane	5668 ± 1267	90.24 ± 2.33	6418 ± 2331	89.31 ± 2.19
2-Methyloctadecane	135 ± 77	2.06 ± 0.88	127 ± 76	1.79 ± 0.82
n-Nonadecane	90 ± 44	1.40 ± 0.54	147 ± 67	2.06 ± 0.67
2-Methylnonadecane	80 ± 40	1.22 ± 0.44	82 ± 50	1.18 ± 0.75

TABLE 2. PEARSON CORRELATION ANALYSIS OF CHANGE IN PERCENT COMPOSITION OF EACH EXTRACTED COMPOUND WITH SUM OF SIX COMPOUNDS[a]

	Day 2 ($n = 19$)		13 Days ($N = 234$)		Released ($N = 39$)	
	r_p	P	r_p	P	r_p	P
2-Methylhexadecane	0.31	0.19	−0.04	0.58	−0.17	0.31
n-Heptadecane	0.07	0.76	−0.01	0.99	−0.36	0.02
2-Methylheptadecane	0.11	0.64	0.10	0.13	0.08	0.65
2-Methyloctadecane	−0.24	0.33	−0.09	0.15	0.19	0.26
n-Nonadecane	0.03	0.92	−0.17	0.01	−0.01	0.99
2-Methylnonadecane	−0.18	0.47	−0.10	0.14	0.28	0.09

[a] r_p is the Pearson correlation coefficient, P is probability level.

17c increased ($P < 0.01$), that of 2me-17c decreased significantly ($P < 0.01$), and the others remained unchanged.

To determine whether gland composition changed with the total amount of extractable material, the proportion of each component was plotted against the total amount extracted for 2-day-old females and for all females over 13 days. With the exception of *n*-19c, which decreased in representation as the total blend increased, all correlations (Pearson) were insignificant (Table 2), indicating that the composition of the pheromone blend is independent of the total titer.

Correlation with Body Weight. Pupae and newly eclosed females were

Mean first 13 days (N = 234)		Day 2 (32–39 hr, N = 19)		ME females (day 2, N = 5)		Release ratio (N = 39)
ng	%	ng	%	ng	%	%
61 ± 31	0.84 ± 0.34	45 ± 16	0.70 ± 0.08	16 ± 5	0.73 ± 0.08	0.78 ±, 0.22
391 ± 231	5.45 ± 2.24	262 ± 112	4.19 ± 1.04	125 ± 46	5.82 ± 1.85	7.45 ± 1.93
6461 ± 2644	88.87 ± 2.77	5726 ± 1751	90.12 ± 1.90	1861 ± 569	83.20 ± 3.52	84.80 ± 3.19
129 ± 72	1.80 ± 0.76	101 ± 39	1.65 ± 0.62	77 ± 40	3.91 ± 2.42	2.84 ± 1.40
140 ± 66	1.97 ± 0.70	123 ± 62	1.91 ± 0.65	123 ± 46	5.44 ± 0.67	2.59 ± 0.89
76 ± 49	1.06 ± 0.69	90 ± 65	1.42 ± 1.07	21 ± 10	0.90 ± 0.37	1.53 ± 0.85

weighed, their glands extracted, and the dry weights obtained after 24 hr in a drying oven (70°C). No significant relations were evident between total gland titer and either wet weight (r = 0.16, P = 0.50, N = 19) or dry weight (r = 0.61, P = 0.11, N = 8) for 2-day-old females or for 0- to 4-day-old females (r = 0.12, P = 0.15, N = 147; r = 0.11, P = 0.22, N = 136, respectively) (Figure 2A, B). Also, the quantities and proportions of all individual components did not vary significantly with body weight.

Release Rates. All components isolated from gland extracts were also collected from freely calling 2-day-old females (Table 1). Three collection tubes were changed successively every 10 min in the first 35 min of calling; subse-

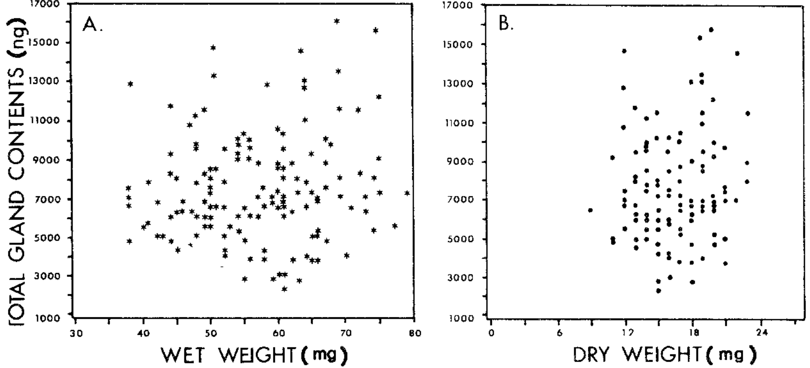

FIG. 2. Relation of total gland content of *H. lamae* with wet and dry weights.

quently, 10-min collections were made once every hour. An initial increase in the main component, 2me-17c, after the first 10 min of calling was followed by a rapid decrease, indicating that most of the material was released early in the calling period (Figure 3). This was borne out in successive 60-min collections from freely calling females where a rapid decline in the amount of pheromone released occurred after the first hour of calling (Figure 4).

The six components were represented in similar proportions in both air-

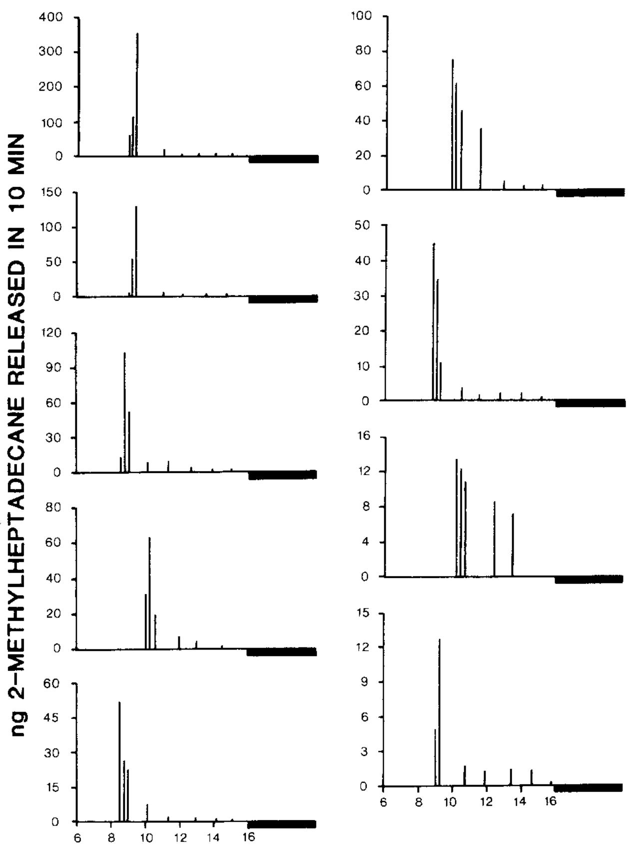

FIG. 3. Release rates per 10 min of nine *H. lamae* females throughout the scotophase (dark bars). Only 2-methylheptadecane is shown. Three collections of 10 min each were conducted in the first 35 min of calling; subsequently, 10-min collections were conducted once every hr.

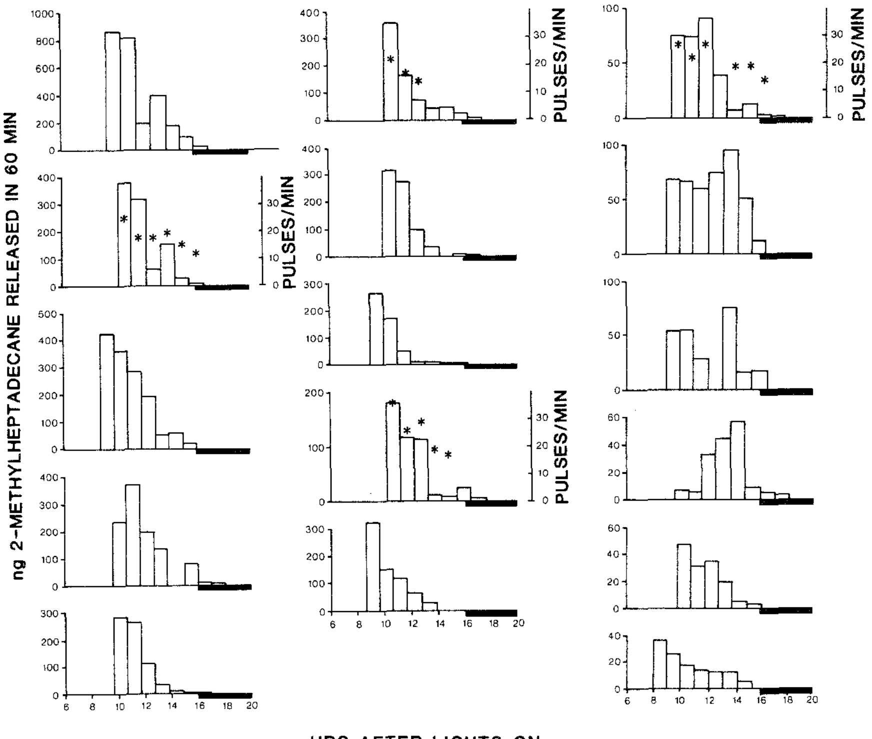

FIG. 4. Release rates per 60 min of 16 *H. lamae* females throughout the calling period. Only 2-methylheptadecane is shown. Successive 60-min collections were conducted from the onset of calling to its termination. For four females, the rates of extrusion of the terminal abdominal segments are also noted (asterisks).

borne collections and gland extracts (Table 1). Only the proportion of n-17c varied inversely with the total blend ($r = 0.36$, $P = 0.02$, $N = 39$; Table 2). The proportions of other components did not change as the total blend increased.

Pulsing Rate. H. lamae, like other arctiid moths, rhythmically extends and retracts the last two abdominal segments during calling. We determined the rate of pulsation of the abdominal tip for freely calling females in still air by counting extrusions per 1 min at 1-hr intervals throughout the calling period. At the onset of calling, pulsation frequency averaged 26/min (Figure 5) and duration of exposure of the terminal segments was long. The pulse rate rapidly peaked at 40/min where it remained for several hours. Toward the termination of calling, the pulse rate decreased gradually to a mean of 13/min (Figure 5).

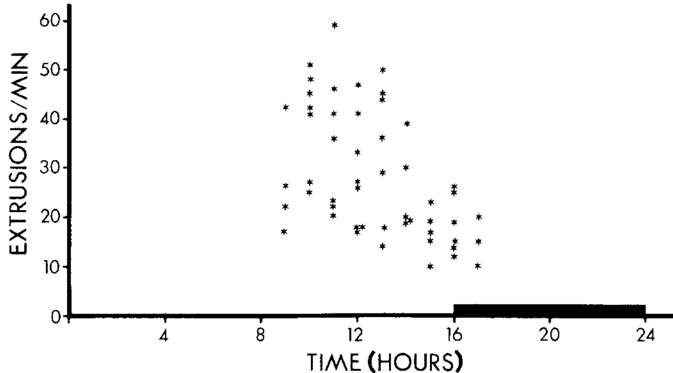

FIG. 5. Rate of extrusion and retraction of the terminal abdominal segments of *H. lamae* as a function of time of day.

DISCUSSION

Pheromone Profile. Roelofs and Cardé (1971) identified 2-methylheptadecane as the major component of the sex pheromone of a group of *Holomelina* sibling species. Males of several species were attracted to and trapped with baits of 2me-17c, but few *H. lamae* males were trapped, even though males oriented to within short distances of the traps. They suggested that missing components, greater release rates of the primary attractant, or both could be important in effecting close-range orientation (Roelofs and Cardé, 1971). We found five related components (2me-16c, *n*-17c, 2me-18c, *n*-19c, 2me-19c)[2] in gland extracts of females reared in the laboratory for ca. 60 generations and in first-generation offspring of feral females; the six components were also collected in air passing over calling females (Figure 1, Table 1). It is possible that other components are also volatilized, but at proportions below 1% of the total blend.

Among the hydrocarbon analogs of 2me-17c identified in *H. lamae* (Cardé et al., 1987), *n*-17c increases male trap catch two to threefold over 2me-17c alone, when released from separate rubber septa at charges of 1000 and 10 or 1000 and 1 μg, respectively. At charges of 100 μg with 1000 μg 2me-17c, *n*-17c diminishes trap catch twofold (Cardé et al., 1987). Thus, relative emission of *n*-17c within a range relative to 2me-17c is important to male attraction in *H. lamae*.

ME females had similar ratios of the six components in gland extracts (Table 1), but the quantity of pheromone extracted was significantly lower in ME compared to MI females ($P < 0.01$, t test). Minks (1971) reported a de-

[2] Prof. W. Roelofs and R. Cardé originally characterized these components by GLC and MS in 1970 at the New York Agricultural Experiment Station in Geneva.

crease in sex pheromone production in inbred strains of the summerfruit tortrix moth, *Adoxophyes orana*, and Miller and Roelofs (1980) showed that a lab colony of *Argyrotaenia velutinana* had significantly lower pheromone titers than field-collected moths. The differences observed in the two strains of *H. lamae* could be attributed to inherent differences in the two source populations or to inbreeding in the laboratory of the Michigan strain.

Diel and Age Effects on Gland Content. In the first four days, total gland content in *H. lamae* exhibited a diel periodicity only in 3-day-old females, decreasing abruptly at the onset of calling, but returning to its noncalling level within 2–4 hr while females still called (Figure 1). Means at different times in the diel were not significantly different from each other for 1-, 2-, and 4-day-old females. Thus, high emission rates at the onset of calling (Figures 3 and 4; Schal and Cardé, 1985) and lack of significant diel fluctuations in pheromone titer suggest rapid biosynthesis of pheromone during calling.

Similar results were found with *Trichoplusia ni* which does not have a daily rhythm in pheromone titer (Shorey and Gaston, 1965; Sower et al., 1972). However, Webster and Cardé (1982) related an abrupt drop in pheromone titer to the onset of calling in *Platynota stultana*; gland titer increased throughout the day and peaked at the initiation of calling. Similar relationships were obtained for surface washes of pheromone glands of *Plodia interpunctella* and *Ephestia cautella* (Coffelt et al., 1978), and *Heliothis zea* (Raina et al., 1986) in which peak calling coincided with maximal pheromone titer in the middle of the scotophase.

The pheromone content of virgin females changed little with age (Figure 1). However, the quantity of all components increased over the first four days, and subsequently decreased or remained unchanged. A similar pattern was observed by Lawrence and Bartell (1972) in *Epiphyas postvittana* based on male response to female extract. Miller and Roelofs (1977), Webster and Cardé (1982), and Raina et al. (1986) showed that pheromone titers in *A. velutinana*, *P. stultana*, and *H. zea* increased for the first two, four, and three days respectively, but decreased over the next few days.

The narrow coefficients of variation in the ratio of the pheromone components (Figure 1, Table 1) were independent of age (as in Miller and Roelofs, 1977) and indicate tight regulation of the blend. In *H. lamae*, although relative amounts of four of the six components changed with time, these changes are probably insignificant to behavioral response. For example, the change in 2me-16C was from ca. 0.7% on day 1 to 1.3% on day 13. Although statistically significant, it is doubtful that males detect such slight changes.

Correlations between total gland titers and proportion of individual components resulted in insignificant r values, indicating that blend composition is independent of blend quantity (Table 2).

Gland Content vs. Body Weight. Pheromone gland titer is unrelated to the wet or dry weights of pupae or adult females (Figure 2). Similar results were

obtained by Miller and Roelofs (1980) and by Charlton and Cardé (1982) for *A. velutinana.* and *Lymantria dispar*, respectively. Fitzpatrick et al. (1985) found that pupal weight did not correlate to male hairpencil contents (benzaldehyde) in *Pseudaletia unipuncta.* However, Shorey and Gaston (1965) reported that pheromone content in 4-day-old *T. ni* was correlated with the weight of females at emergence.

Release Rates and Ratios. Freely calling *H. lamae* females release all six components of the pheromone blend only during the calling period (Figure 1, Table 1); no detectable pheromone was collected from noncalling females either before or after calling. Although calling persisted for several hours in *H. lamae* (Schal and Cardé, 1986), the majority of the daily pheromone release occurred early in the calling period. A similar pattern is suggested in *Utetheisa*, in which females called for up to 200 min (Figure 6 in Conner et al., 1980), but males were attracted to calling females for an average of only 29 min, depending on the season. *T. ni* releases pheromone in calling bouts of ca. 20 min. Pheromone is released at a high rate early, but decreases asymptotically over the course of a calling bout (Bjostad et al., 1980). The strategy implicated is one of maximization of early pheromone release in some noctuids and arctiids, with concomitant rapid rates of pheromone biosynthesis as evidenced by the minor changes in gland content. Presumably, females tend to be located and mated early in the calling period or bout.

Although release rates later in the calling period may have little significance in the field because females may be mated early, they do have important implications for studies of pheromone release. Many such studies make the tacit assumption that release rates are constant throughout the calling interval, and therefore various periods of aeration of the gland can be corrected to yield a per minute or per hour release rate. The patterns exhibited by *H. lamae*, *H. aurantiaca*, and *T. ni* clearly question such extrapolations. Similarly, other studies have documented large variability in the amount and temporal pattern of pheromone emission from individual females (e.g., Morse et al., 1982).

In situations where glands are forcibly extruded (chemically or mechanically) for 10 min, and it is known that a subsequent 30 min of aeration yields little additional material (e.g., Pope et al., 1984), volatilization from the gland surface declines exponentially. Release rates (ng/min) thus obtained will differ when averaged for all 10 min or for any interval shorter or longer than 10 min. It is not known whether actual release rates approximate the maximum rate obtained from forcibly extruded glands (probably the first minute), or an average over a certain interval. For instance, the single highest 10 min rate of release for *H. lamae* was 353 ng, which would extrapolate to 2118 ng/hr or double the single highest release rate obtained from 60-min collections.

H. lamae exhibits a relatively high rate of pheromone emission (Figures 3 and 4; Schal and Cardé, 1985). Why such high release rates? An obvious explanation is to increase the distance of communication. Furthermore, high re-

lease rates may function in reproductive isolation, but ratios of components, diel activity patterns, and geographic and microhabitat distributions (Roelofs and Cardé, 1971) are probably more important partitioning factors.

The rates of emission of a single pheromone component depend upon its vapor pressure, environmental factors (e.g., temperature, wind), and the size and form of the emitting surface. Steck et al. (1984) showed that 3% of Z9-14:Ac and 97% of Z11-16:Ac provided for sustained trapping of the noctuid *Mamestra configurata* over several weeks. But, because the Z9-14:Ac was released and thus depleted more rapidly than Z11-16:Ac, its proportion in the trap declined so that the lure then attracted *Enargia infumata*. Similar effects on ratios were obtained by Olsson et al. (1983) in a study of vapor pressures of sex pheromones. The differential release of various components from artificial sources and interactions among loaded compounds suggest some important cautionary notes in formulating pheromones. First, since different substrates release materials differentially, release rates and ratios must be determined for each substrate before field tests are conducted. Second, as compounds are added or omitted from rubber septa, as in studies of the roles of each component, release rates and ratios must be determined for each new combination of components to account for interaction among components. In fact, in a comparison of three substrates, Linn et al. (1984) found that Z7-14:Ac and Z9-14:Ac were released at a much lower rate relative to the loading when compared to the 12-carbon compounds, resulting in an inappropriate ratio and poor response by *T. ni*.

A central concern is how the blend of different components is regulated. Both emitted and extracted blends of *H. lamae* contained hydrocarbons ranging from 16 to 19 carbons in length which differ in vapor pressure. Yet, the ratio of volatilized and extracted components was nearly the same, and for both, blend quality did not change with blend quantity. The remarkable similarity between the profiles of the six-component blend in the female's gland and her effluvium indicates that evaporation of the blend from the gland surface is not responsible for the ratio of components emitted. Species in the *Holomelina aurantiaca* complex examined to date possess paired tubular pheromone glands (unpublished). We propose that upon each pulse of the abdominal tip, the blend is atomized in a fine mist, thus retaining the same ratio of components as found in the gland (Cardé et al., 1987). This mechanism could also account for the conspicuously high rate of emission. This novel interpretation of blend regulation allows the ratio of components biosynthesized and present in the gland to reflect the ratio released.

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ALLOMONAL FUNCTIONS OF STEROID HORMONE, ANTHERIDIOL, IN WATER MOLD *Achlya*[1]

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Abstract—The steroid hormone antheridiol has previously been shown to play a number of key roles in regulating development of the male sex organs, antheridia, of the water mold *Achlya ambisexualis*. We now demonstrate that synthetic antheridiol can inhibit both sexual (determined by oogonial counts) and asexual reproduction (determined by counts of asexual spores) in an isolate of the homothallic species *A. heterosexualis*. The implications of this dual role of antheridiol as hormone and allomone are discussed.

Key Words—*Achlya ambisexualis*, *A. bisexualis*, *A. heterosexualis*, Oomycetes, Saprolegniales, allomone, antheridiol, asexual sporulation, hormone, oogoniogenesis, pheromone, steroid, water mold.

INTRODUCTION

Sexual organogenesis in the water mold *Achlya* (Oomycetes: Saprolegniales) is initiated and coordinated by the steroid horomones antheridiol and oogoniol (Raper, 1951; Barksdale, 1969; Arsenault et al., 1968; Preus and McMorris. 1979). Most studies of this system have focused on heterothallic *Achlya ambisexualis* Raper isolates such as 734 (female) and E87 (male). Briefly, the female produces antheridiol (Figure 1) continuously under appropriate nutritional conditions. Males respond to antheridiol by producing antheridial branches and synthesizing oogoniol. This steriod in turn induces females to produce sex organs, oogonia, which attract the antheridial branches in a chem-

[1] Oomycetes: Saprolegniales.

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FIG. 1. Structure of antheridiol.

otropic response probably involving antheridiol (Barksdale, 1967). A further stage of male sexual organogenesis, the production of the antheridium which is delimited from the remainder of the antheridial hypha by a septum, is also regulated by antheridiol (Barksdale, 1967). In this classical example of chemical communication between sexually compatible mating types, the hormonal exchange is via the intervening aqueous matrix, so that antheridiol and oogoniol have been termed pheromones.

Homothallic strains of *Achlya* can, by definition, produce both antheridia and oogonia. Conjugation tests between heterothallic and homothallic isolates have shown that these organisms use mutually similar or identical hormonal mechanisms (Raper, 1950; Barksdale, 1965).

Asexual reproduction in *Achlya* induced by nutritional stress, involves the development from vegetative hyphae of sporangia within which asexual spores are formed.

Because *Achlya* is a coenocytic (aseptate) organism capable of translocating cytoplasm to reproductive structures such as asexual sporangia (Dick, 1973) or oogonia (Hintz and Horgen, 1983), it would seem reasonable to hypothesize that the reproductive modes are mutually interrelated. In other words, conditions favoring one pathway of reproductive development might be expected to diminish the resources available for an alternative pathway. However, no such interactions between reproductive modes have been documented. Riehl and Toft (1985) commented that the asexual mode is considered to be independent of the steroid hormones.

In the course of characterizing the conjugation potential of several newly collected homothallic isolates (Thomas et al., 1984), we confirmed earlier observations (Raper, 1950) that strong females, when juxtaposed with homothallics, can suppress the production of oogonia in the latter. Barksdale (1967) reported inhibition of oogonia in homothallic *Achlya* strains by antheridiol isolated from culture filtrates of *A. bisexualis*. We now report that synthetic an-

theridiol inhibits both oogoniogenesis and asexual sporangiogenesis in a homothallic *A. heterosexualis* isolate.

METHODS AND MATERIALS

Fungus Material. Achlya ambisexualis Raper strains E87 (male) and 734 (female) were gifts of J.T. Mullins. The homothallic *Achlya* strain used (American Type Culture Collection number 52875) was isolated as strain B4 by Thomas et al. (1984) and should not be confused with the B4 isolate of Barksdale (1965). We identified the isolate as *A. heterosexualis* Whiffen-Barksdale, primarily because of the monoclinous origin of antheridial hyphae, which develop from points very near the oogonium, usually on the same hypha as the oogonium (Barksdale, 1965). The isolate produced antheridial hyphae when juxtaposed with female tester strains representing *A. ambisexualis*, *A. bisexualis*, and *A. heterosexualis*, but failed to demonstrate sexual compatibility with any of the tester females (Thomas et al., 1984). Spores were prepared by transferring agar plugs from Petri plate cultures to 100 ml peptone–yeast extract–glucose liquid medium (PYG) (Cantino and Lovett, 1960). After 24 hr incubation on a reciprocating shaker, the medium was decanted and replaced with two successive changes of 5×10^{-4} M $CaCl_2$ (Griffin, 1966). After a further 12 hr incubation on a shaker, the resulting spore suspension was decanted to a sterile receiver flask. Spore suspensions were stored at 4°C and used within three weeks.

Asexual Sporulation. Spores were inoculated to PYG and grown on a reciprocating shaker at room temperature for 24 hr. Colonies were collected by filtration, washed, and transferred to 2 ml 5×10^{-4} M $CaCl_2$ in each of 25×5-mm Petri plates with varying concentrations of antheridiol. After 24 hr, spore production was determined by hemacytometer counts of two aliquots from each dish. Treatments were in duplicate, and experiments were repeated at least once.

Sexual Reproduction. Cultures of *A. heterosexualis* 52875 were prepared and treated as described above, except that liquid M medium (Mullins and Barksdale, 1965) replaced the $CaCl_2$. After two days oogonia were counted in six colonies from each plate.

Chemicals. Antheridiol, synthesized by the protocol of McMorris et al. (1974), was applied in ethanolic solutions. All treatments including the controls, contained ethanol at a concentration not exceeding 1% (v/v) of the treatment medium.

RESULTS

In all three *Achlya* isolates, asexual spore release occurred within 4–6 hr after transfer to initiating medium. The effects of antheridiol on asexual repro-

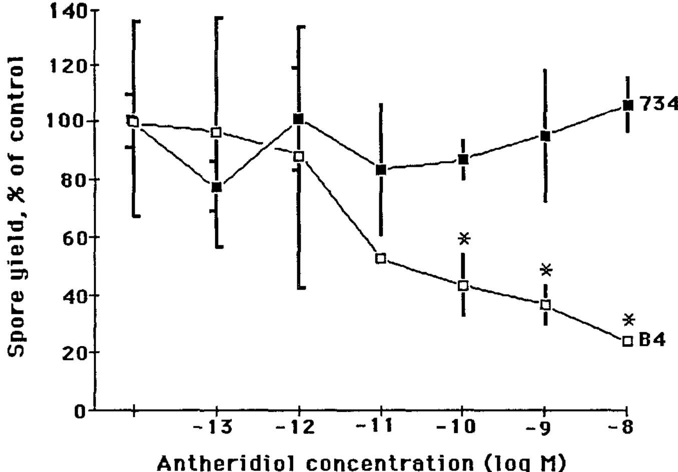

FIG. 2. The effect of antheridiol on asexual sporulation in the homothallic *A. heterosexualis* 52875 and in the female *A. ambisexualis* strain 734, which does not produce antheridial branches in response to the hormone. The vertical bars represent ± standard deviation of each mean; the asterisks denote means significantly different from the controls (two-tailed t-test, $P < 0.001$).

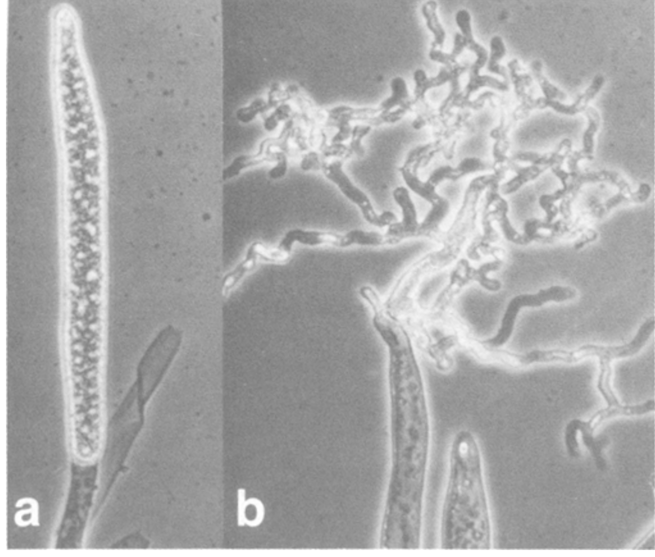

FIG. 3. Hyphal tips of *A. heterosexualis* 52875, modified to produce a terminal sporangium (a), or induced by 10^{-8} M antheridiol (b). In the presence of hormone, a hyphal tip which might otherwise have differentiated to produce a sporangium has produced numerous antheridial branches. Magnification, 300X.

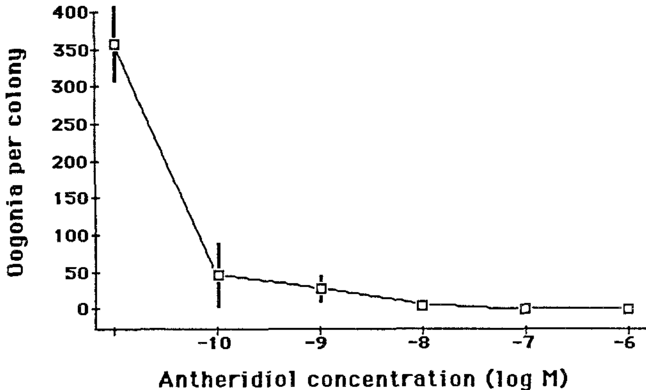

FIG. 4. The effect of antheridiol on oogonium production in *A. heterosexualis* 52875. The vertical bars representing ± standard deviation of each mean are omitted when they fall within the marker squares; treatment means are significantly different from that of the control (two-tailed t test, $P < 0.001$).

duction varied with the isolate. The homothallic isolate *A. heterosexualis* 52875, which responded to antheridiol by producing an abundance of antheridial hyphae starting 1.5 hr after treatment, showed a marked reduction of asexual spore production (Figure 2). In contrast, asexual sporulation in the *A. ambisexualis* female strain 734, which produces no antheridiol receptor (Riehl and Toft, 1985) and which shows no morphological response to the hormone, was unaffected by antheridiol (Figure 2). In the *A. ambisexualis* male strain E87, antheridial hyphal primordia became visible 3 hr after treatment and the antheridial hyphae were fewer than in *A. heterosexualis*. The effect of antheridiol on sporulation in E87 (not shown) was variable, ranging from moderate to nonsignificant inhibition. The morphological effects of antheridiol on *A. heterosexualis* 52875 (Figure 3) include massive induction of antheridial branches on hyphae which might otherwise have differentiated to produce sporangia.

In *A. heterosexualis* 52875 oogonia are produced 12–24 hr after transfer from PYG to M medium. The inhibition of oogonium production in the presence of antheridiol is illustrated in Figure 4.

DISCUSSION

Inhibition of asexual reproduction in the various strains by antheridiol appears to be directly proportional to the abundance of antheridiol receptor. The receptor is undetectable in *A. ambisexualis* 734 (Riehl and Toft, 1985), and the receptor level in *A. ambisexualis* E87 is approximately 10–20% of that present

in *A. heterosexualis* 52875 (D. des S. Thomas and T.C. McMorris, unpublished).

The vigorous production of antheridial hyphae by *A. heterosexualis* with consequent reduction of resources available for asexual and sexual spore formation may help to account for the inhibitory effects of antheridiol on asexual sporulation and on oogonium formation. These results suggest the possibility that under natural conditions females of *A. ambisexualis* or *A. bisexualis* (Coker and A. Couch), both of which secrete antheridiol, could suppress the reproduction of neighboring *A. heterosexualis* colonies. Barksdale (1965) has pointed out that these three cross-conjugating species of *Achlya* can be interpolated on a gradient ranging from low hormone productivity and high sensitivity at one end (*A. heterosexualis*) to high productivity and low sensitivity at the other end (*A. bisexualis*). Antheridiol concentrations in a culture of the *A. bisexualis* female strain T5 can exceed 5×10^{-8} M (Barksdale, 1969) and should be even higher than this near the source mycelium in an unstirred culture. The lower hormone levels shown in the present work to inhibit reproduction in *A. heterosexualis* can therefore be considered to be physiological concentrations.

Allelochemicals are chemical signals between a sender organism and a receiver belonging to a different species (Whittaker and Feeny, 1971), and include allomones, which give adaptive advantage to the sender. Among the subcategories of allomone are inductants, which modify the growth of the receiver. Where a chemical signal acts, as does antheridiol, to decrease the reproductive fitness of a member of another species, it can be classified as an allomone. The literal meaning of this term, derived from the Greek *allos* (other) and *hormon* (to excite), is appropriate in the case of antheridiol when it elicits a futile sexual response in a target organism such as *A. heterosexualis*.

The hormone/allomone duality in antheridiol function is likely to constitute an important constraint on natural selection within the genus *Achlya* and may help to elucidate observations such as the high female-to-male ratios reported in natural populations (Raper, 1950; Barksdale, 1960; Thomas et al., 1984), and the aforementioned inverse relationship between hormone sensitivity and productivity in a *A. heterosexualis*. As far as we are aware, antheridiol is unique among steroids in its potential for the dual functions of hormone and allomone within a single genus.

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MISLEADING THE COLORADO POTATO BEETLE WITH AN ODOR BLEND[1]

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Abstract—Walking tracks of Colorado potato beetles, *Leptinotarsa decemlineata* Say, were recorded on a locomotion-compensator in response to wind, odors of host plants *Solanum tuberosum* L. and nonhost plants *Lycopersicon hirsutum* f. *glabratum* C.H. Mull, and to mixtures of these plant species. Host-plant odor induced positive anemotactic responses in starved females, whereas odor of the nonhost *L. hirsutum* was neither repellent nor attractive. The attractiveness of host-plant odor, however, was neutralized in the odor blend of plant species. Masking the attractive host-plant odor will hinder the beetle's searching for host-plant patches, and this principle may be exploited in pest control by mixed cropping.

Key Words—Colorado potato beetle, *Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae, *Solanum tuberosum*, *Lycopersicon hirsutum* f. *glabratum*, chemoattraction, masking, mixed cropping, olfactory orientation, searching behavior.

INTRODUCTION

Insect pest levels are often lower in mixed cropping systems than in monocultures, and it has been suggested that the searching behavior of phytophagous insects is disrupted by the confusion of signals from intercropped plants (Cromartie, 1981; Kareiva, 1983; Stanton, 1983). There is, however, no direct evi-

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dence that when the odor of a nonhost plant is added to a plume of host-plant odor this will disrupt the insect's olfactory orientation. The purpose of the present study was to see whether odor blending affects the olfactory orientation of the Colorado potato beetle, *Leptinotarsa decemlineata* Say, towards its host-plant potato *Solanum tuberosum* L.

The walking Colorado potato beetle responds to potato-plant odor by an odor-conditioned positive anemotaxis (Visser, 1976; Visser and Thiery, 1985). The composition of the potato leaf odor was previously studied (Visser et al., 1979; Visser, 1983), and electrophysiological experiments revealed the characteristics of the antennal olfactory receptors (Visser, 1979, 1983).

Although in laboratory studies experimental conditions are precisely controlled, which makes a comparison with field conditions difficult, this approach is essential to study the behavioral patterns that underlie the insect's colonization of host-plant patches in the field. This paper presents data on the odor-conditioned anemotaxes of Colorado potato beetles as released by host-plant odor, and the effects of nonhost-plant odor on these responses. We used as a source of nonhost-plant odor the strong-smelling wild tomato *Lycopersicon hirsutum* f. *glabratum* C.H. Mull.

METHODS AND MATERIALS

Recordings of Behavior. Experiments were previously described on the orientation of Colorado potato beetles towards a source of windborne potato-plant odor in a low-speed wind tunnel (Visser, 1976). In the present study, behavioral responses of beetles were recorded using a locomotion-compensator in front of this wind tunnel. The insect test section of the original tunnel was removed, and the flow leaving the contraction passed over the beetle walking on the locomotion-compensator. This instrument, which was designed by E. Kramer and P. Heinecke (Max-Planck-Institut fuer Verhaltensphysiologie, Seewiesen, FRG), has been used in a number of studies on insect orientation (Kramer, 1976; Weber et al., 1981).

In brief, the locomotion-compensator operates as follows: an insect walks freely on top of a large sphere while its positional change is monitored by means of a beam of light reflected by a small mirror which is glued to its elytra. Walking activity is continuously compensated by two motors rolling the sphere in the opposite direction. The rotations of the sphere are detected by two pulse generators in contact with the sphere, and pulses are recorded and analyzed by a computer. In this way, the beetle's position was recorded every second, and walking speed and direction were calculated. Full technical details of this equipment will be presented elsewhere (Visser and Thiery, in preparation).

Insects and Plants. Beetles were obtained from laboratory stock culture.

Newly emerged females were fed for 2 hr on potato foliage and then starved for at least 12 hr prior to the experiments. At the time of the experiments, the females were about one day old. Plants were reared in greenhouses: potatoes *Solanum tuberosum* cultivar Eigenheimer, and wild tomatoes *Lycopersicon hirsutum* f. *glabratum*.

Experiments. Individual females were exposed for 10 min to each of four subsequent stimuli: (1) a clean air flow of 80 cm/sec as control, and the same air flow carrying (2) the odor of wild tomato *L. hirsutum* f. *glabratum*, (3) the odor of potato *S. tuberosum*, and (4) the odor from a mixture of the two plant species. At least 1 hr elapsed between successive exposures of individual females. For each plant species, three pots of fully grown plants were placed in the dark upwind section of the wind tunnel, and contained approximately an equal volume of foliage. The experiments were conducted at 24°C. Light intensity on top of the sphere was set at 1750 lux by means of two high-frequency illumination units (2500 Hz). The air speed was measured at the outlet of the wind tunnel. Other conditions were previously described (Thiery and Visser, 1986).

Analyses of Responses. Five variables are used to describe the beetle's responses: (1) walking speed as the mean of 599 instantaneous speeds per individual, (2) vector length as the resultant displacement from the origin after 600 sec, (3) straightness of the track as the quotient of the vector length and the total length of the walking track, (4) time spent walking upwind corresponds to the proportion of angle observations with deviations of less than 60° from the wind direction, and (5) upwind length as the upwind displacement after 600 sec. Variables were compared between treatments using nonparametric statistics (Siegel, 1956): sign test (variable 3) and Wilcoxon's test (variable 1, 2, 4, and 5). In this way the variables from the treatments were tested as related samples which compensates for the large variations in walking speeds between individual beetles.

RESULTS AND DISCUSSION

Orientation Responses. Representative walking tracks of an individual female in response to wind, nonhost-plant odor, host-plant odor, and odor of the mixture of host and nonhost plants are shown in Figure 1(a–d). Circular distributions of the beetles in the four treatments are depicted in Figure 2, and Table 1 shows the corresponding values of the variables that quantify orientation.

In a clean air flow, Colorado potato beetles walked with a weak preference for upwind directions which is manifested by their net upwind displacement (Table 1). In this stimulus condition the beetles did not walk very straight (straightness is 0.38). Odor of the wild tomato did not elicit an increase in

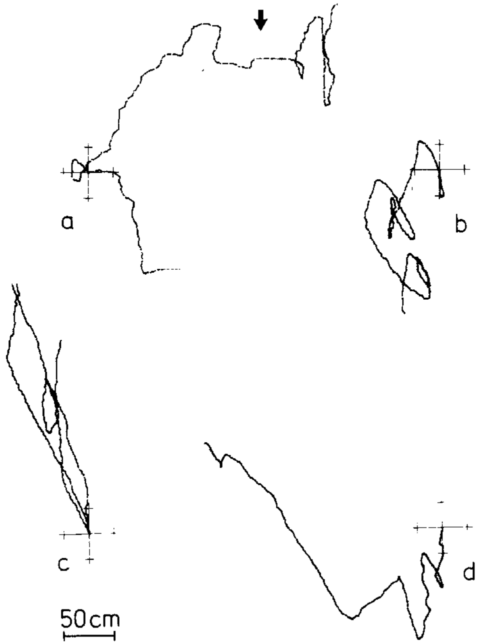

FIG. 1. Tracks of one female Colorado potato beetle in four successive treatments of 10 min each: (a) wind control, (b) *Lycopersicon hirsutum* odor, (c) *Solanum tuberosum* odor, and (d) odor blend of *L. hirsutum* and *S. tuberosum*. Arrow indicates wind direction. The plotter was reset to the origin automatically at reaching the edge of the paper.

upwind responses of the beetles. Vector length, straightness, upwind time, and upwind length were not changed as compared with clean air. However, walking speed decreased significantly relative to that in clean air.

When stimulated by potato-plant odor, all beetles orientated completely upwind (Figure 2, Table 1). They spent most of their time walking straight upwind towards the odor source, as can be seen in an increased straightness and upwind time. The resultant vector measured about twice the vector length from the previous experiments. The upwind length equaled approximately the vector length since the latter was pointed strictly upwind.

The odor blend consisting of *L. hirsutum* and *S. tuberosum* volatiles, however, caused a decrease of all variables. Vector length, straightness, upwind time, and upwind length of the track were comparable with those in the clean air treatment. The significant decrease of walking speed did not cause a further reduction in vector length or upwind length (Table 1). The presence of nonhost-plant odor in the air flow apparently inhibits the positive anemotactic responses which are observed with potato plant odor; the beetles do not walk upwind.

Masking by Nonhost-Plant Odor. When compared with the wind stimulus

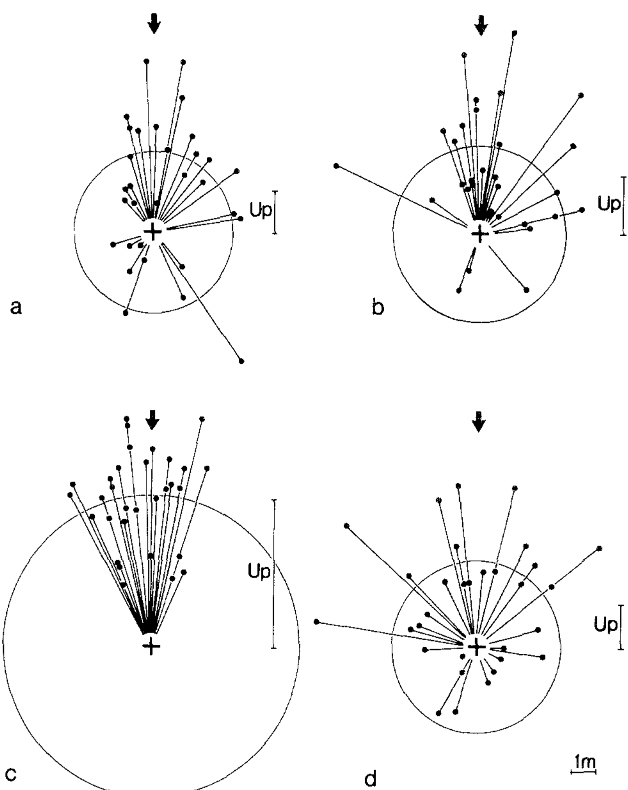

FIG. 2. Circular distribution of 31 female Colorado potato beetles in four successive treatments: (a) wind control, (b) *Lycopersicon hirsutum* odor, (c) *Solanum tuberosum* odor, and (d) odor blend of *L. hirsutum* and *S. tuberosum*. Each line represents the vector of displaçement of one beetle after 10 min of walking. The mean vector length in each treatment is shown as the radius of the circle. Up: upwind component of the mean vector. Arrows indicate wind direction.

alone, wild tomato is neither repellent nor attractive. The beetle's walking speed is reduced on stimulation by both the odor of wild tomatoes, and the blend of odors. Nevertheless, in these conditions vector length and upwind length are comparable with those measured in pure wind. This implies that repellency is not involved since this term is defined as a movement being directed away from the odor source (Dethier et al., 1960), and would be directed downwind. The neutralization of orientation responses in the Colorado potato beetle is interpreted as the result of olfactory camouflage of the host plant by combining it with a different odor. An essential part of the attractive potato leaf odor consists of a complex of C_6 alcohols, aldehydes, and corresponding derivatives generally distributed in green leaves (Visser et al., 1979). The specificity of this so-

TABLE 1. RESPONSES OF FEMALE COLORADO POTATO BEETLES TO ODORS OF *Lycopersicon hirsutum*, *Solanum tuberosum*, AND MIXTURES OF PLANT SPECIES[a]

Stimulus	Walking speed (mm/sec)	Vector length (mm)	Straightness	Time walking upwind (%)	Upwind length (mm)
Wind	14.2 ± 0.8a[b]	3289 ± 626a	0.38 ± 0.06a	57 ± 7a	1675 ± 1054a
L. hirsutum	11.8 ± 0.7b	3559 ± 733a	0.49 ± 0.09a	65 ± 7a	2442 ± 924a
S. tuberosum	13.8 ± 0.7a	6210 ± 682b	0.75 ± 0.06b	86 ± 4b	6014 ± 692b
Odor blend	12.3 ± 0.6b	3420 ± 702a	0.46 ± 0.09a	57 ± 7a	1823 ± 918a

[a]Data represent means ±95% confidence intervals (two-tailed). Number of beetles is 31.
[b]Different letters in a column indicate statistical differences between treatments at $P < 0.001$ (two-tailed).

called green odor is set by the ratios of its individual components, since changing their ratios, by addition of pure volatile chemicals, interrupts the upwind movements of the beetles (Visser and Avé, 1978). In the present study it is shown that addition of the odor of wild tomato plants to the potato-leaf odor, likewise blocks the release of positive anemotactic responses in this beetle.

It is noteworthy that an accession of *L. hirsutum* f. *glabratum* that contains high levels of 2-tridecanone, is resistant to the Colorado potato beetle (Kennedy and Sorenson, 1985). In other experiments, the camouflage of potato plants was also obtained by adding the odor of cabbage *Brassica oleracea* L. var. *gemmifera* DC. (Thiery and Visser, 1986). For that reason, masking of host-plant odor is thought to be caused by the change in green odor composition, although further experiments are needed in order to exclude 2-tridecanone as a masking component.

Potato plant odor releases odor-conditioned positive anemotactic responses in females as well as in males and postdiapause beetles (Visser, 1976; Visser and Nielsen, 1977). The disruption of olfactory orientation by the mixing of odors occurs independently of the beetle's feeding experience since its emergence; the same effects were found when "naive" females were used (Thiery and Visser, 1986).

Mixed Cropping. The translation of the present findings on odor blending to field conditions requires consideration of the relevance of the beetle's walking behavior to field conditions and the extent of odor blending in the atmosphere. In Western Europe host-plant finding by the Colorado potato beetle happens mainly while the beetle is walking (the late J. de Wilde, unpublished field observations 1979, 1981). Newly emerged and postdiapause adults are unable to fly until 8–10 days of age when flight muscles are fully developed (De Kort, 1969). In the field, wind turbulence will blend volatiles from a mixed stand of plants (Stanton, 1983; Visser, 1986). It is expected, therefore, that the

beetle's range of attraction to host-plant odor is reduced in mixed cropping systems. Masking the attractive host-plant odor will then hinder the beetle's searching for host plants.

Bach (1982) reported recaptures of Colorado potato beetles which were released between small field plots containing different arrangements of host and nonhost plants. In contrast with the conclusions of the present laboratory study, she emphasized that the colonization of these beetles did not appear to be negatively affected by the presence of nonhost plants, like melon. Apart from not mentioning sex, age, and prior starvation period of the beetles, information is lacking on relevant abiotic factors such as temperatures and the prevailing wind direction. Since Bach noted that statistics were not justified with the small sample size used, this study is insufficient to conclude that plant diversity does not hinder the colonization of host plants by Colorado potato beetles.

Panasiuk (1984) and Schearer (1984) reported volatiles of tansy *Tanacetum vulgare*, that repelled Colorado potato beetles. Their studies were undertaken in order to isolate components which cause the observed reductions of beetle populations when potatoes were interplanted with tansy. These two investigations applied nearly the same methodology; however, their results differed strikingly. For example, Panasiuk reported that α-pinene attracted beetles, whereas Schearer's data suggested repellency of this compound. These observations contradict EAG data showing that the antennal receptors of the Colorado potato beetle are insensitive to this compound; small EAG responses were solely obtained to high doses that are not present in the headspace of plants (Visser, 1979). Moreover, the reports of Panasiuk and Schearer do not contain substantial data on beetle responses, and the applied doses exceeded many times their concentrations in natural plant-odor blends. The suggestion that the compounds which they identified as repellents are responsible for the reductions of beetle populations in interplantings is premature since an extreme dose of any volatile compound will be repellent (Visser, 1986).

Conclusions. Plant odors are composed of general as well as specific components (Visser, 1986). The present study shows evidence that the attractive host-plant odor is masked on blending with nonhost-plant odor. In heterogeneous ecosystems and in mixed croppings, it is expected, therefore, that host-plant odors are frequently camouflaged. This principle needs further experimentation to develop intelligent methods of intercropping for insect pest control.

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AIRBORNE DIFFERENCES IN ODORS EMITTED BY *Rattus norvegicus* IN RESPONSE TO REWARD AND NONREWARD

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Abstract—To test the hypothesis that rats (*Rattus norvegicus*) emit airborne, differential odors in response to reward and nonreward, donor rats received random sequences of rewarded and nonrewarded placements in small compartments and an airstream transported odors from these compartments to test rats in a separate chamber. When donors remained in the compartments during, or were removed just prior to, air transport, test rats utilized transported odors as discriminative cues signaling their own reward and nonreward for a lever-press response. When the airstream was passed through a clean compartment containing paper flooring extracted from donor compartments, test rats were not able to discriminate. Test trials to assess for control by food odors suggest that donor-produced odors, rather than food odors per se, provided the discriminative signals for test rats. Results confirm the existence of somewhat volatile, although apparently stable, odors emitted in response to reward and nonreward, and implicate a differential in amount and/or type of odor produced by donors to these two events as the source of discriminative control.

Key Words—Reward odor, nonreward odor, olfactometer, *Rattus norvegicus*.

INTRODUCTION

In 1966, independent reports from two laboratories suggested that rats (*Rattus norvegicus*), experiencing intermittent food reward, might emit quantitatively

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and/or qualitatively different odors in response to reward and nonreward (McHose and Ludvigson, 1966; Spear and Spitzner, 1966). In the ensuing years, numerous studies have produced evidence for an odor differential, generated under these circumstances, as a controlling source of differences in both unlearned and learned responses of conspecifics (e.g., Bloom and Phillips, 1973; Collerain, 1978; Ludvigson et al., 1979; Ludvigson and Sytsma, 1967; Mellgren et al., 1973; Morrison and Ludvigson, 1970). Furthermore, such odor differences have been shown to control differential responding in conspecifics when: (1) the rewarding event consists of water (e.g., Davis et al., 1982); (2) food is always present, but access is sometimes prevented (e.g., Voorhees and Remley, 1981); and, (3) food is always provided, but in differing quantities (e.g., Ludvigson et al., 1985), or at differing delays (e.g., McHose and Ludvigson, 1966). Thus, the phenomenon clearly seems attributable to odors contributed by the rats experiencing intermittent differences in reward rather than the odor of the reward itself. Recently, the generality of the phenomenon was extended in a demonstration that wild wood rats (*Neotoma floridana osagensis*) both emit and respond to such odors (Davis et al., 1985). Although the ecological significance of these odors is presently unclear (cf. Davis et al., 1985; Ludvigson et al., 1985; Voorhees and Remley, 1981), an understanding of their adaptive functions would be aided by analyses of the odorants themselves as well as the reactions of conspecifics, including the basis upon which conspecifics can discriminate between the odors.

All of the aforementioned studies were conducted using alleyways, T mazes, or similar types of apparatus, and all except Voorhees and Remley (1981) brought the test rats to the odor source. Generally speaking, the most common procedure involves administering a rewarded or nonrewarded treatment to one or more food-deprived rats (donors) in the apparatus, followed by the introduction of test rats (one at a time) whose behavior is assessed for relevant response differences to the prevailing odors. Although this approach has been useful for the study of conspecific reactions to the odors, inferences about the nature of the odors and source of their discriminable differences have been less readily achieved. Relatively little is known about the olfactory stimuli themselves, aside from the fact that they accumulate as successive donors are administered treatments (Prytula et al., 1981; Taylor and Ludvigson, 1983). Since the test rats are permitted access to the location in which donors received a treatment, they could be responding to high-, low-, and/or nonvolatile components available on the surfaces or in the air of the apparatus; discriminable differences in one or more of these components could be based on qualitative, quantitative, and/or distributional differences in the components.

The present study addresses the nature of discriminable differences in the odors which rats produce to reward and nonreward. Test rats received reward and nonreward for a lever-press response contingent upon rewarded and nonre-

warded treatments given to donors in small compartments. An airstream, passed through these compartments, delivered available odors to serve as discriminative cues signaling reward and nonreward for the test rats. The acquisition of differential rates of lever-pressing to the odors, and subsequent changes in these rates as manipulations were made to alter the odors, provided the basis for inferences about the nature of and control by transported odors.

METHODS AND MATERIALS

Experiment 1

Voorhees and Remley (1981) demonstrated that an airstream passed over paper flooring extracted from an alleyway in which donors had received reward or nonreward elicits differential responses in mitral cells of test rats' olfactory bulbs as a function of the treatment received by the donors. However, in a study of Taylor and Ludvigson (1986), rats provided with an airstream passed over clean paper flooring or paper flooring from an alleyway in which donors had either received reward or nonreward were able to discriminate between the clean papers and donor-exposed papers, but gave no behavioral evidence of differentiating between papers from the reward, as opposed to nonreward, of donors. Perhaps only small quantities of odorants can be transported from the paper, and these quantities are insufficient to support a behavioral discrimination although capable of producing differences in neurological responses. If, however, test rates were allowed to sample these same odorants through direct contact with the paper, as is possible in the alleyway situation, then subtle differences might control behavioral responding in that situation. The present experiment tested the hypothesis that rewarded and nonrewarded rats provide airborne, discriminatively different odors in response to these two events.

Subjects. Eight male rats served as subjects. Upon arrival from the supplier, the rats were individually housed in wire-mesh cages and provided free access to food and water until 132 days of age. At this time they were deprived of food and gradually reduced in body weight until each rate stabilized to between 80 and 85% of its free-feeding weight. Two of the eight rats were randomly selected to serve as test rats, and the remaining six were randomly divided into two donor squads of three rats each. The test rats were randomly designated as test rat 1 and 2, and the donor squads as squads 1 and 2.

Apparatus. A small-animal olfactometer consisting of an air pump and purification system, a donor-treatment component, and a test chamber, all connected by an airstream, served as the experimental apparatus. The olfactometer is schematically depicted in Figure 1. Room air was pumped (DeVilbiss Suction Pump No. 701), via a polyethylene hose, through a series of three glass columns containing, in order, anhydrous calcium sulfate, charcoal granules, and distilled

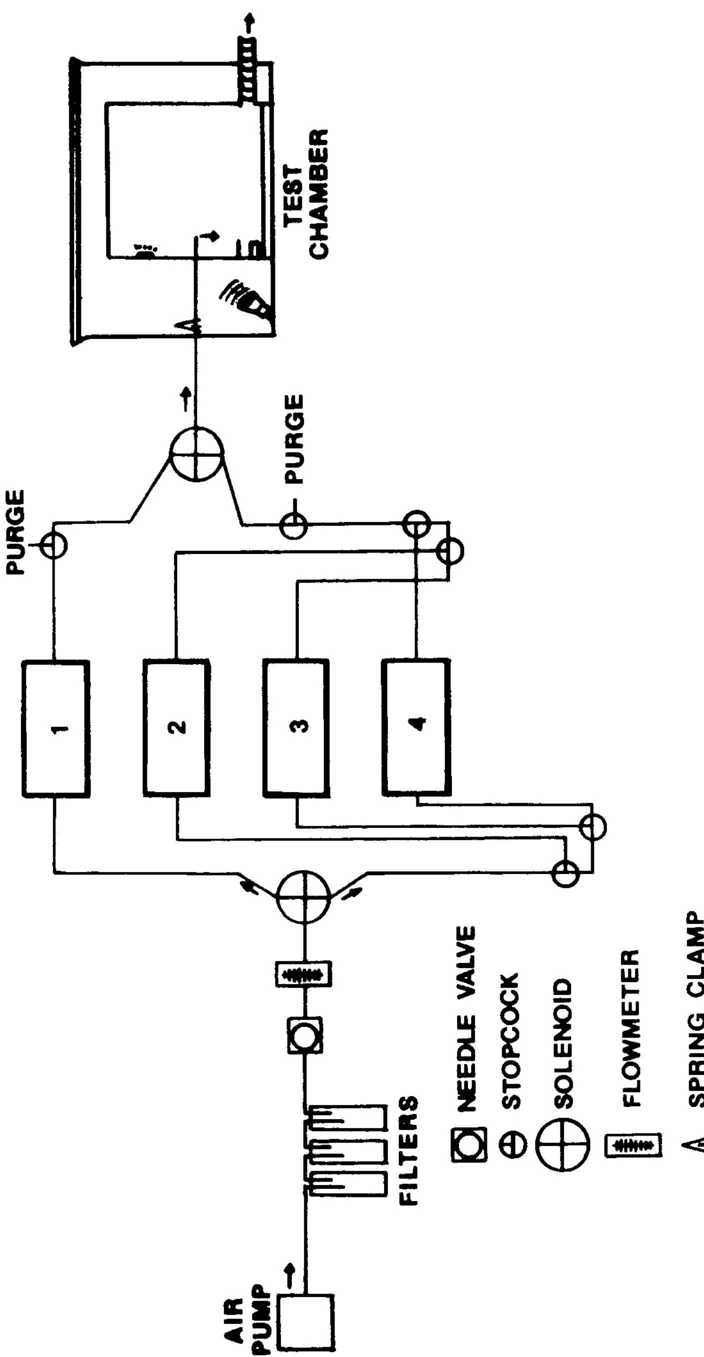

FIG. 1. Schematic diagram of the olfactometer. Room air was pumped through the filter system and the flow rate regulated before passing through one or more placement boxes (2–4) containing the odorant for delivery into the test chamber.

water. Thus, room air was dehydrated in the first column, deodorized in the second, and partially rehydrated in the third. The filtered air was carried through glass tubing to a standard needle valve and Dwyer flowmeter (RMA 16), and the flow rate regulated to 5 liters/min.

The air then passed into the donor-treatment component composed of glass tubing, Teflon couplings, glass and Teflon three-way stopcock valves, two three-way solenoid valves (ASCO No. 8320A85), and four placement boxes (box 1, 2, 3, and 4). The air flow could be directed through box 1, or through boxes 2, 3, and 4 separately or in any combination. Each placement box was made of stainless steel and measured 26.5 cm long, 8.5 cm wide, and 10.3 cm high. The top of each box was covered with a 0.64-cm-thick glass sheet extending the length and width of the box. Boxes 2, 3, and 4 were used for the placement and treatment of donors, while box 1 provided a source of clean air. Each of boxes 2, 3, and 4 was equipped with a removable, stainless-steel food cup designed to minimize the spillage of food crumbs onto the floor of the box. Each cup measured 34 mm, inside diameter, and was 29 mm deep. The base consisted of a flat trough, 10 cm × 7 cm, with edges folded up to form a 7-mm-high lip. With this design, rats would eat from the food cup and crumbs fell into the trough rather than onto the floor.

The air emerging from the donor-treatment component followed a final common path of glass tubing for delivery into the test chamber. The odor-delivery tube protruded 1.27 cm into the chamber and terminated in a 3-mm-wide opening in the bottom of the tube. The test chamber consisted of a modified Lehigh Valley Electronics operant conditioning chamber situated inside a sound-attenuating enclosure. A response lever, centered 3.81 cm below the odor-delivery tube, a food tray, and a houselight were mounted on the front wall. The length of the chamber was 22.86 cm, and the width was restricted to 10.8 cm by exchangeable glass plates which extended the length and height of the chamber. On the back wall, a round portal, 3.81 cm in diameter, allowed the chamber air to be constantly evacuated with the aid of an attached hose and exhaust fan. The sound-attenuating enclosure contained a speaker through which white noise was emitted. Together, the speaker and the exhaust fan provided a constant masking sound inside the chamber.

Preliminary Training. The donor rats were given three days of habituation to the placement boxes, consisting of four 1-min placements each day. Beginning on the fourth day, and continuing for nine days, the donors were trained on an intermittent schedule of reward and nonreward consisting of four rewarded and four nonrewarded placements each day. The order of these placements was randomly determined each day with the restriction that not more than three like events occur in succession. To administer a placement, each donor was inserted into a separate placement box at roughly the same time. Each box contained a strip of clean paper flooring and a food cup. The glass tops were

quickly placed over the boxes and weights were set on top to prevent the rats from dislodging the tops. On rewarded placements, the food cups contained fifteen 45-mg Noyes pellets which the rats were allowed 45 sec to consume; on nonrewarded placements the cups were empty and the rats were confined for 45 sec. After the 45-sec period, the tops were briefly removed to extract the food cups with needle-nose pliers and were then replaced. With the donors in the boxes, filtered air was simultaneously circulated through all three boxes for 1 min. Then donors were removed from the boxes and transported from the experimental room while preparations were made for the next trial.

During a day, each donor received all placements in the same box; however, across days, donors were systematically rotated among the boxes. Between placements, any fecal matter or traces of moisture were removed, clean flooring installed, and clean air circulated through all three boxes for 3 min. Squad 1 received all eight placements before any were administered to squad 2. The placement boxes were rinsed in hot water and dried between squads.

The two test rats were adapted to the test chamber, given pellet habituation, and trained to perform the lever-press response. Subsequently, they were trained to utilize houselight-onset as a signal for rewarded lever-pressing and houselight-offset as a signal for nonrewarded lever-pressing. Once both test rats were consistently responding during light-on periods, and showing little, or no, responding during light-off periods, the olfactory discrimination procedure was initiated.

Olfactory Discrimination Procedure. The olfactory discrimination procedure required each test rat to differentiate between two odorants, one arising from the reward of donors and one from the nonreward of donors. On any given trial, one of the two odorants was delivered by airstream during the light-on period of the trial. Across trials, for each test rat, lever-pressing was consistently rewarded in the presence of one of these odorants, but not rewarded in the presence of the other odorant. To administer a trial, the donor squad was given a rewarded or nonrewarded placement, and then the test rat was carried into the experimental room and placed in the test chamber.

Following a 5-sec delay, the houselight came on and air flowed through box 1 and into the chamber for 3 sec. The solenoid valves then switched and directed air through boxes 2, 3, and 4, and into the test chamber. Following a 2-sec delay, to ensure adequate odor transport, the differential reward period began. On a rewarded trial for a test rat, fifteen 45-mg Noyes pellets were dispensed automatically for the first lever-press response following the lapse of a variable interval which ranged from 3.75 to 36.25 sec. As the pellets were dispensed, the houselight went off and the airstream delivered into the chamber was passed through box 1. The rat was allowed 45 sec to consume the pellets before removal from the chamber. On a nonrewarded trial for a test rat, no pellets were dispensed and the rat was confined to the chamber for 45 sec.

Each test rat received four rewarded and four nonrewarded trials per day, with the order dictated by the random sequence of rewarded and nonrewarded placements assigned to the respective donor squad. Test rat 1 received a rewarded trial when its donors received a rewarded placement, and a nonrewarded trial when its donors received nonreward. For test rat 2, reward and nonreward occurred when its donors received nonreward and reward, respectively. In this fashion, odors transported from rewarded donors could serve as a signal of impending reward for test rat 1 and nonreward for test rat 2, while odors transported from nonrewarded donors could signal impending nonreward for test rat 1 and reward for test rat 2.

Between trials, a test rat was isolated in an adjacent waiting room. A white noise source in the waiting room provided a masking sound for intertrial activities going on in the experimental room. During the intertrial interval of approximately 15 min, the test chamber walls and grid floor were swabbed with clean water and dried, the two glass plates in the chamber were replaced with clean ones, and clean paper was inserted beneath the grid floor. These measures were taken to minimize the presence of residual odors from the preceding trial. Meanwhile, the donor boxes were flushed with clean air, and another placement was administered to the donors. Test rat 1 received all trials of a day before test rat 2 received any.

Experimental Manipulations. The olfactory discrimination task described above remained fixed throughout the experiment, while manipulations were made to alter the available odors delivered to the test rats in various phases as described below. In all phases, except phase B, squads 1 and 2 served as donors for test rats 1 and 2, respectively. Test rats were progressed through four distinguishable phases in the sequence A B A C D C.

During phase A, the donors remained in their boxes on each trial as filtered air was passed through all three boxes. This procedure minimized disturbance of the air in the boxes and, presumably, maximized the opportunity for transporting available donor-produced odors to the test rats. In phase B, squad 1 served as donors for test rat 2, and squad 2 served as donors for test rat 1, while all other aspects remained unchanged from phase A. This phase was introduced in an attempt to conceptually replicate observations from alleyway studies (e.g., Eslinger and Ludvigson, 1980) wherein interchanging donors appear to produce little, if any, disruption in test rats' responses to reward and nonreward odors. By inference, the controlling components of such odors would appear similar across rats rather than unique to individual rats.

In phase C, donors were removed from the boxes and the experimental room when the food cups were extracted. This phase assessed whether controlling cues remain despite removal of the donors, and thus provided for inferences about the stability of the odors and potential extraneous control by nonolfactory cues. Nonolfactory cues might include differential movements and/

or noise by the donors conducted through the glass tubing to the test rats. Continued differential responding by test rats would argue against such extraneous control and suggest a degree of stability in the olfactory cues, defined by their ability to withstand disruption during donor removal.

In phase D, manipulations were made to assess whether controlling odors are preserved on the paper flooring extracted from the boxes. On each trial of this phase, one of the three donors in a squad was given a placement in box 2 and then removed. The strip of paper flooring was transferred, with a pair of forceps, to box 4 and the box covered. After placing clean paper in boxes 2 and 3, the remaining two donors of the squad were concurrently given placements in these two boxes. The paper flooring from these boxes was subsequently added to the one previously transferred to box 4. The airstream delivered to the test rat was circulated through box 4 containing the three strips of paper flooring.

Experiment 2

This experiment was designed to further examine and clarify the ability of transferred papers to provide a functional source of discriminative cues for test rats. In order to avoid the introduction of major procedural changes when testing transferred papers, a new donor procedure was implemented for this experiment. In addition, this experiment provided a more straightforward test of potential discriminative control by food odors.

Subjects. The donor and test rats, as well as all aspects of their care and maintenance, remained unchanged from experiment 1.

Apparatus. The olfactometer remained unchanged; however, box 3 was not used at all in this experiment. The flowmeter was adjusted to yield a flow rate of 6.5 liters/min.

Olfactory Discrimination Procedure. The discrimination procedure for test rats remained unchanged from experiment 1, although each test rat only received three rewarded and three nonrewarded trials each day.

A new procedure was instituted for the treatment of donors. To administer a trial to a donor squad, the donors were successively given 45-sec placements in a single box without changing the paper flooring or cleaning the box between donors. Following the removal of the final donor, the test rat was presented with air circulated through this single box. In all phases, squads 1 and 2 served as donors for test rats 1 and 2, respectively.

Experimental Manipulations. The test rats were progressed through three distinguishable phases in the sequence A B A C. During phase A donors were given rewarded and nonrewarded placements in box 2. The performance of test rats in this phase was used as a baseline for evaluating the effects of manipulations in phase B. In phase B, donors were administered placements in box 4 in the same manner in which placements were given in box 2 during phase A.

On each trial, following the removal of the final donor in the squad, the single strip of paper flooring was quickly transferred, with a pair of forceps, to box 2. Air was circulated through this box and delivered to the test rat. This phase served as a further attempt to assess the efficacy of paper flooring as a source of controlling odors. On day 10, test trials were conducted to assess responding to air samples passed over clean paper flooring in a clean placement box. Accordingly, donors were given placements in box 4, as on the other days of phase B, but instead of transferring the paper flooring to box 2, a clean strip of paper was inserted into box 2 and the air circulated over this clean paper. Test rats were rewarded on half of these trials, with rewarded trials randomly determined.

Following a return to phase-A conditions, phase C was instituted. In phase C, donors were given placements in box 2; however, following the removal of the last donor on nonrewarded placements, two 45-mg Noyes pellets were inserted into the middle of the box onto the paper flooring. Pellets were not inserted in box 2 following rewarded placements. The two-pellet quantity was selected because inspection of the placement boxes throughout all phases of both experiments indicated that observable quantities of food crumbs were not present on the floor. Hence, two intact food pellets were judged likely to provide an odor of food in the nonreward odor sample that was at least as intense as any food odor provided by potential residual crumbs in the reward odor sample. If a food-odor differential were responsible for the test rats' discriminations, then this manipulation would be expected to eliminate, if not reverse, this differential, and hence, test rats' characteristic rates of responding to nonreward and reward odors should be reversed or, at least, differences in rate eliminated. In contrast, if a donor-produced odor differential were responsible for discrimination, then considerable differences in rate of responding to reward and nonreward odors should be maintained in this phase.

RESULTS AND DISCUSSION

Experiment 1

Total responses and total time in the presence of odors from rewarded and from nonrewarded donor treatments were computed each day for each test rat, and these values were used to calculate daily rates of responding (Figure 2). Although the two test rats displayed rather different acquisition curves, both achieved substantial levels of differential responding over the initial 16 days of phase A. Thus, test rats clearly learned to utilize differential cues as signals for their own reward and nonreward. Interchanging donor squads in phase B did not appreciably alter the degree of differential responding by either test rat, indicating that the cues utilized by test rats were rather similar across the two

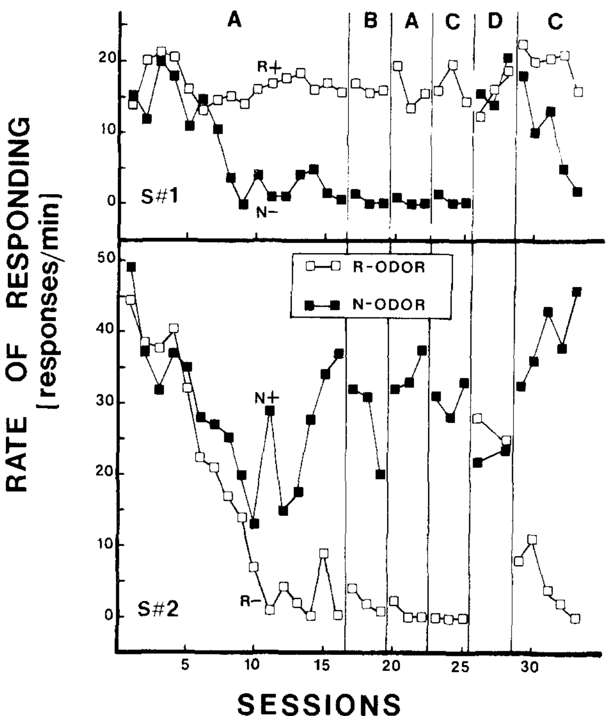

FIG. 2. Lever-presses/minute during experiment 1 by test rat 1 (top) when rewarded in presence of reward odors (R+), but nonrewarded in presence of nonreward odors (N−); and by test rat 2 (bottom) when rewarded in the presence of nonreward odors (N+), but nonrewarded in the presence of reward odors (R−).

donor squads. This serves to conceptually replicate the observations of Eslinger and Ludvigson (1980) made in the alleyway situation and to confirm their conclusion that the relevant odorants possess commonality across rats.

In phase C, the removal of donors before passing air through the boxes had little influence on the magnitude of differential responding exhibited by either test rat. Since donors were not in the boxes at the time the test rat was in the chamber, differential odors transported from the placement boxes, rather than extraneous, nonolfactory cues, must have controlled test rats' behavior. Further, although the odors are sufficiently volatile to be transported by air, they appear not to be so volatile or unstable as to be lost or altered in the process of removing donors from the boxes. Were the latter to be the case, then appreciable diminution in differential responding should have occurred.

In phase D, air passed over transferred paper flooring did not maintain differential responding. Upon returning to phase-C conditions, the test rats recovered their discriminations, although some degree of reacquisition was re-

quired. This suggests that the controlling odors are not transferable on paper, or at least not in sufficient quantity to maintain the test rats' discriminations. However, it might be argued that procedural changes introduced in phase D disrupted odor production by donors or altered the odors produced. While this seems unlikely, it cannot be ruled out. One might also argue that a food-odor differential, arising from food having been in the boxes on rewarded, but not on nonrewarded, trials, could have mediated discrimination by test rats through all previous phases, while in phase D, food odors failed to be transferred on paper from rewarded placements, resulting in the loss of the controlling cues. However, if this were true, test rat 1 should have responsed at a low, nondifferential rate to all papers presented in phase D, since according to this food-odor hypothesis, the absence of a food odor had been consistently nonrewarded during previous phases. Clearly, test rat 1 did not respond in this fashion, but rather responded at a high, nondifferential rate, suggesting control by odorants other than food.

Experiment 2

Daily rates of responding to odors from rewarded and nonrewarded placements were computed and are displayed in Figure 3. Both test rats discriminated quite well on all days of phase A. Thus, the change in the donor procedure from experiment 1 to experiment 2 did not alter the odor cues enough to interfere with discrimination. In phase B, transferred papers again failed to maintain the discriminations of test rats, but the discriminations were recovered upon a return to phase-A conditions. This observation confirms the results of phase D in experiment 1. Further, rates of responding to clean paper flooring on day 10 were similar to rates displayed to transferred papers on the other days of phase B. This supports the conclusion that no behaviorally controlling differential in reward and nonreward odors was preserved on the paper flooring upon which donors received treatments, and by inference, argues that discriminable differences are based on airborne and somewhat volatile olfactory components.

In phase C, test rats maintained substantial levels of differential responding, reflecting considerable ability to discriminate between odors from rewarded and nonrewarded donors, despite the addition of a rather strong food odor to the nonreward samples. Rates of responding to nonreward odor plus food odor were, however, elevated for test rat 1 and depressed for test rat 2, suggesting that the food odor either partially masked the typical odor from nonreward of donors or increased its similarity to the odor from reward of donors. However, assuming the odor of two intact food pellets was sufficient to cancel, or reverse, any prior and consistent food odor differential favoring reward odor, these data indicate that the airborne, controlling differences between the odor of rewarded and the odor of nonrewarded donors derive from donor-produced, treatment-engendered olfactory components.

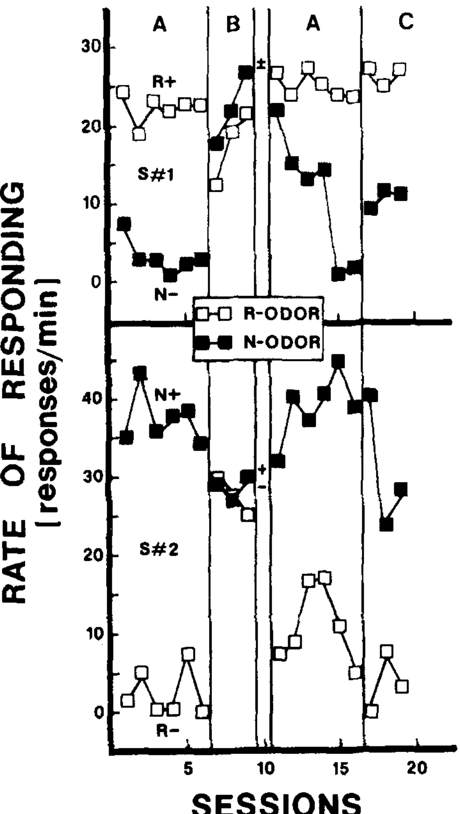

FIG. 3. Lever-presses/minute during experiment 2 by test rat 1 (top) when rewarded in presence of reward odors (R+), but nonrewarded in presence of nonreward odors (N−); and by test rat 2 (bottom) when rewarded in the presence of nonreward odors (N+), but nonrewarded in the presence of reward odors (R−). Day 10 = odor of clean paper presented and test rats 1 and 2 rewarded (+) and nonrewarded (−) on 50% of the trials.

CONCLUSIONS

Although this study leaves open the question of individual differences in, and the generality of, both odor production and odor discrimination in rats, the results do indicate that at least some rats can discriminate between the odors remaining in a location where conspecifics have found a food reward and the odors remaining when conspecifics have failed to find a food reward. The results of this study are consistent with the results of others, obtained with quite different methodologies, in showing that the relevant odorants are emitted by

the conspecifics in response to their reward and nonreward experiences. Very little has been known about the nature of these odors or the dimensions along which they are differentiated by the rat. Results of experiments reported here suggest some conclusions relevant to this void.

First, although the odors might provide various discriminable differences, depending on physical features of the location and spatial proximity of the discriminating rat, readily discriminable differences can be transported by an airstream. Thus, both reward and nonreward odors must contain volatile olfactory components which differ from one another. These components are the most likely source of olfactory cues observed to mediate discriminations in numerous alleyway studies. Other differences in the odors might arise in the alleyway, such as a differential in spatial distribution, and could be used by rats, but such differences are apparently not necessary for a discrimination to occur.

Second, the data suggest that the volatile components emitted by donor rats in response to reward and nonreward differ, at least in part, qualitatively, although quantitative differences could also exist. The differential, however, cannot simply be due to a difference in amount, or concentration, of otherwise qualitatively equal odorants. If, for example, the odorants produced in response to nonreward were simply more concentrated than those produced in response to reward, then test rat 1, for whom nonreward odor was a signal for nonrewarded lever-pressing, would have learned to inhibit responding to "high" concentrations of the odorant, while test rat 2, for whom nonreward odor was a signal for rewarded lever-pressing, would have learned to inhibit responding to "low" concentrations. This is, however, not what was observed when test rats were presented with transferred paper flooring in phase D of experiment 1 and phase B of experiment 2.

Transferred papers retain very little, if any, of the respective odorants, as demonstrated by the inability of such papers to maintain the test rats' discriminations. When presented with air passed over transferred papers, both test rats responded at a high rate similar to that typically shown to the cue signaling their reward. Since reward odor and nonreward odor served as signals for rewarded lever-pressing for test rats 1 and 2, respectively, the notion of "quantitative differences alone" would require the further, unlikely, assumption that reward odor was the less concentrated of the two when squad 1 received treatments, but nonreward odor was the less concentrated when squad 2 received treatments. In fact, phase B of experiment 1 suggests that the odorants were much the same across these two donor squads. Hence, it is more likely that a difference in type of odorant served to mediate test rats' discriminations. The fact that both test rats responded to all transferred papers, and even clean papers (day 10 of experiment 2), at a rate similar to that typically shown to the respective cue signaling reward for lever-pressing, may simply indicate relatively greater discriminative control by the cue signaling nonreward to the respective

test rat (i.e., nonreward odor and reward odor for test rats 1 and 2, respectively).

Beyond implicating the existence of some qualitative differences in the odorants, the present data do not address the nature of this qualitative difference. Odors produced in response to nonreward might contain many, or all, of the components present in reward odor, plus additional, possibly treatment-unique, components. On the other hand, reward odor, or both reward and nonreward odors, might contain treatment-unique volatile components.

Third, it is worth noting that these volatile components, whether arising from reward, nonreward, or both, must not be too highly volatile or unstable. Some degree of air disturbance was inevitable in removing donors from the boxes (phase C of experiment 1 and phases A and C of experiment 2), but this removal had little effect on the magnitude of test rats' discriminations established without removing donors (phases A and B of experiment 1).

As to the ecological significance of the odorants, several possibilities exist. Rats might emit nonreward odorants and/or reward odorants as signals for guiding the foraging activities of conspecifics, with the former odors signaling the absence of a previously available food source and the latter odors signaling the presence of a food source. In contrast, or in addition, one or both odorants might signal the emotional state of the emitting rat (e.g., "frustration" in the case of nonreward odor), thereby influencing such behaviors as social aggregation. Future work directed at further clarifying the nature of qualitative differences between reward and nonreward odors should help to identify and separate particular ecological functions of the respective odors.

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SEX PHEROMONE OF BLACKHEADED FIREWORM, *Rhopobota naevana* (LEPIDOPTERA: TORTRICIDAE), A PEST OF CRANBERRY

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Abstract—Splitless capillary gas chromatography indicated the presence of (Z)- and (*E*)-11-tetradecenyl acetate and (Z)-11-tetradecenyl alcohol in the washes of female abdominal tips of the blackheaded fireworm, *Rhopobota naevana* (Hubner). Gas chromatography combined with mass spectroscopy confirmed the presence of tetradecenyl acetate in extracts of female tips. The low levels observed in these extracts (<1 ng/female equivalent), prevented further chemical and spectroscopic identification. These materials were found to be stimulatory at low levels in electroantennogram studies. A combination of 9 μg of (Z)-11-tetradecenyl acetate and 3 μg of (Z)-11-tetradecenyl alcohol on rubber septa in wing traps provided an effective attractant. (Z)-9-Dodecenyl acetate, a previously reported attractant, did not significantly increase field trapping catches when added to the binary mixture, but was found to enhance trap catches when added to each of the primary components.

Key Words—Blackheaded fireworm, *Rhopobota naevana* (Hubner), *Rhopobota unipunctana* (Haw.), Lepidoptera, Tortricidae, Olethreutinae, sex pheromone, (Z)-11-tetradecenyl acetate, (Z)-11-tetradecenyl alcohol, (Z)-9-dodecenyl acetate.

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INTRODUCTION

The blackheaded fireworm, *Rhopobota naevana* (Hubner) (=*R. unipunctana*), is a serious pest of cranberry, *Vaccinium macrocarpon* (Ait.) in the United States and Canada. Damage is caused by the larvae which web together and destroy the fruiting uprights causing cranberry bogs to have a scorched appearance. The European species, *Rhopobota* (*Acrolita*) *naevana* has been reported from Sweden to Europe feeding on holly and a variety of fruit trees, especially apple. In North America, however, *R. naevana* appears to be specific to cranberry except for one other record from evergreen huckleberry, *Vaccinium ovatum* (Pursh.) (Breakey, 1960).

The life history of *R. naevana* in British Columbia is similar to that in Massachusetts (Franklin, 1948) and Washington (Plank, 1922). It overwinters as flat, yellowish eggs on the undersides of the cranberry leaves. The eggs hatch in mid-May, and the larvae, when fully grown, pupate in the debris beneath the vines. Small silvery-grey moths emerge in late June and persist until early August. The diurnal moths make short jerky flights above the vines and lay eggs on the undersides of the cranberry leaves. A few eggs overwinter, but most give rise to a second generation of larvae in early July. Second generation moths are present from early August to late September. They also lay eggs, most of which overwinter, although some give rise to a partial third generation of larvae, which perish during the winter.

The development of an effective, pheromone-based monitoring system could considerably reduce the present high dependence on pesticide spraying to control the damage caused by this insect. Survey traps containing (*Z*)-9-dodecenyl acetate (*Z*9–12 : Ac) were reported to attract male *R. naevana* (Ando et al., 1977). Reports of damage to local cranberry farms, and the possibility of developing an effective monitoring system led us to begin investigations into the sex pheromone of *R. naevana.* This paper reports the isolation, partial identification, and field bioassay of pheromone components from female *R. naevana.*

METHODS AND MATERIALS

Pheromone Collection. Larvae of *R. naevana* were collected from local cranberry bogs, reared to pupae, and sexed. The male pupae have eight abdominal segments, whereas the females have only seven segments (Forbes, 1923). Upon emergence, adults were kept at ambient temperature and humidity under a 12 hr light–12 hr dark photoregime. Females 2–3 days old were placed in dim light to simulate dusk conditions after being in bright light for 10 hr. The abdominal tips were extruded and excised into a few microliters of heptane which had been purified by distillation followed by filtration through alumina. The tip

wash of each individual was analyzed immediately. Larger samples were obtained by combining the excised tips of 50 insects. The pooled wash was carefully concentrated by gentle warming in a narrow neck ampoule prior to analysis. An extract was also prepared by leaving 10 excised tips in solvent for three days. The extract was then washed three times with an equal volume of 5% sodium bicarbonate to remove free fatty acids, once with an equal volume of water and concentrated for analysis.

Analyses. Splitless capillary gas chromatography was performed on a Hewlett-Packard 5890 using the following fused silica capillary columns: (1) a 25-m × 0.2-mm-ID Carbowax 20 M and (2) a 25-m × 0.2-mm-ID methyl silicone, both from Hewlett-Packard, Avondale, Pennsylvania; (3) a 30-m × 0.25-mm-ID DB-1 cross-linked polydimethyl siloxane from J & W Scientific, Rancho Cordova, California; and (4) a 30-m × 0.25-mm-ID SPB-5 cross-linked 5% vinyl, 5% phenyl, polymethyl siloxane from Supelco, Bellefonte, Pennsylvania. Column 1 was programmed at 100°C for 2 min, 30°/min to 140°C, then 1.5°/min to 170°C; columns 2, 3, and 4 were programmed at 80°C for 2 min, 10°/min to 180°C, then 2°/min to 240°C. The carrier gas was helium at 1 ml/min utilizing an injector temperature of 200°C and a flame ionization detector maintained at 250°C. Selected ion mass spectra were obtained on a HP5985 GC-MS fitted with a 30-m DB-1 column using electron impact ionization.

Chemicals. All materials used were prepared by standard routes and purified as their acetates by column chromatography on silica gel impregnated with silver ion. The materials were analyzed on appropriate columns and shown to contain less than 1% of any geometric or positional isomer.

Electroantennograms. Antennal responses of male insects were measured using an electroantennographic detector similar to that described by Arn et al. (1975). The test substance, 10 ng, was applied to a small piece of filter paper as a solution in 1 μl of hexane. On evaporation of the solvent, the paper was transferred to a 10-ml disposable syringe and a 3-ml pulse was injected manually into a stream of humidified air which passed continuously over the antennal preparation at 300 ml/min. Trimmed antennae were suspended across chloridized silver electrodes, with electrical contact established with a drop of 0.8% aqueous NaCl. Fourteen separate observations were recorded for each compound with a low solvent response subtracted for each observation. The mean response and standard error was then calculated for each compound.

Field Tests. Chemicals were applied in hexane to the inside of rubber septa (Arthur H. Thomas No. 8753-D22). The septa were pinned to the upper inside of wing traps similar in design to the Pherocon 1CP trap (Zoecon Corp., Palo Alto, California), the bottoms of which were coated on the inside with Stikem Special (Seabright Enterprises Ltd., Emeryville, California).

Sites were selected which had been sprayed to provide low infestations of fireworm and thus enhance discrimination between competitive lures. Earlier

field trials in sites of high fireworm population density (Raine and Clements, unpublished) had shown very high trap catches, with little or no discrimination between lures.

The traps were suspended on stakes or on irrigation sprinkler risers about 0.3–0.6 m above the cranberry vines. The distance between traps varied from about 30 to 90 m in 1984 and from 30 to 35 m in 1985. The traps were arranged in randomized complete blocks at each of two sites in both 1984 and 1985. Eight replicates of each lure combination were tested for attractiveness to male moths at two sites within a 16-hectare commercial cranberry bog in 1984. Four replicates of each lure combination were tested at two separate bogs of 4 and 8 hectares about 2 km apart in 1985. Counts were made weekly in 1984 and twice weekly in 1985. Captured moths were scraped from the trap bottoms at each count and the bottoms were replaced when the sticky surface became dirty. Examination of the genitalia of 250 moths removed from selected traps showed that only male moths were trapped. Specimens collected were identified as *R. naevana* (=*R. unipunctana*) by P.T. Dang, Biosystematics Research Institute, Ottawa, Ontario, Canada.

Data Analysis. Variances in all experiments were significantly heterogenic as determined by the Bartlett and Hartley test ($\alpha = 0.05$) and the data were in the form of counts with some zeros. Therefore, the data were transformed into $\sqrt{x + 0.5}$ and subjected to ANOVA (Sokal and Rohlf, 1969). The analysis indicated that there was no significant difference between counts at each site in each of 1984 and 1985. Therefore, counts at both sites for each year were pooled, and the data presented as the mean number of male moths caught per trap during each trapping period. Multiple comparisons among treatment means were made using Duncan's multiple-range test ($\alpha = 0.05$).

RESULTS

Analyses. Only five of 100 individual females analyzed by splitless gas chromatography from 1981 to 1983 yielded sufficient material to be detected (>0.1 ng). The five positive traces revealed peaks corresponding to tetradecenyl acetates and alcohols. The two positive washes run on column 1 showed correspondence with *E*11–14:Ac (9.39 min, only one trace), *Z*11–14:Ac (9.55 min), and *Z*11–14:OH (10.95 min). The three traces recorded on column 2 revealed correspondence with *E*11–14:Ac (12.28 min, one trace), *Z*11–14:Ac (12.37 min), and *Z*11–14:OH (10.57 min, one trace). There were no detector responses at retention times corresponding to 12-carbon acetates and alcohols.

Subsequent investigations on a further 85 female tips, involving 25 different combined tip washes revealed the consistent presence of *Z*11–14:Ac in amounts varying from less than 0.1 ng up to almost 1 ng/female. The retention

times for Z11–14:Ac were 15.19 min on column 3, and 17.47 min on column 4. In 15 of the 25 combined tip wash traces, responses were recorded at these times. Seven traces indicated the corresponding peak for Z11–14:OH (column 4, 14.87 min), in varying amounts, averaging 30% of the quantity of Z11–14:Ac. The *E*11–14:Ac isomer (column 3, 15.06 min; column 4, 17.37 min) was represented in just three traces, averaging 10% of the peak area relative to Z11–14:Ac. A barely detectable peak corresponding to the retention time of Z9–12:Ac (column 4, 13.76 min) was observed in two traces in which the other components were clearly present. These observations, in addition to the report of Ando et al. (1977) that Z9–12:Ac was an attractant, prompted our inclusion of the 9–12:Acs in field testing.

A portion of soaked tip extract containing 10–20 female equivalents contained no detectable peaks at the retention times of the previously observed compounds when analyzed by capillary gas chromatography. No enhancement of a possible aldehyde component (Z11–14:Ald) was observed, as has been detected in other insects (Struble and Richards, 1983; Gillespie et al., 1984). A mass spectrometric analysis of 50 female equivalents of this extract, monitoring the ions of *m/e* 61, 194, and the total ion current, indicated the presence of a tetradecenyl acetate, most likely Z11–14:Ac, as judged from retention time correspondence. There was a small peak in the *m/e* 194 ion trace coincident with a very small inflection of the total ion trace at the retention time of Z11–14:OH. Ions of *m/e* 61, 166, and total ion current were monitored to examine the possible presence of Z9–12:Ac. Only the *m/e* 61 trace showed a detectable peak at the retention time of dodecenyl acetates, hardly confirming their presence in this extract.

Electroantennograms. The male antenna responded very strongly to Z11–14:Ac (0.98 ± 0.10 mV), with Z11–14:OH producing the next greatest response (0.25 ± 0.07 mV). A weak but positive response (0.15 ± 0.03 mV) was obtained with Z9–12:Ac. A moderate response (0.24 ± 0.07 mV) was obtained with *E*11–14:Ac, but all other compounds tested (*E*9–12:Ac, 0.08 ± 0.03 mV; Z11–14:Ald, 0.08 ± 0.03 mV; *E*11–14:Ald, 0.12 ± 0.02 mV; *E*11–14:OH, 0.09 ± 0.03 mV) were at best only slightly active.

Field Trapping. Trapping results in 1984 (Table 1) clearly indicated that Z9–12:Ac was not significantly more attractive to male fireworm moths than the control. The addition of Z11–14:Ac resulted in a significantly increased attraction. Addition of the corresponding *E* isomers indicated by chemical analysis to be present in the female produced no enhancement of attraction when added to the binary mixture.

Trapping in 1985 included the alcohol, Z11–14:OH, which appeared to be present in female extracts and gave a good electroantennogram response. This alcohol was as effective an attractant alone as Z11–14:Ac and strongly increased the attractancy of Z11–14:Ac. The addition of Z9–12:Ac gave sig-

TABLE 1. CAPTURES OF MALE *Rhopobota naevana* IN RESPONSE TO VARIOUS LURES AT RICHMOND, B.C.

Dates	Lures (μg)	Mean No. Moths/Trap[a]	Standard Error
1984	Z9-12:Ac(50) + Z11-14:Ac(100)	6.00	±0.46a
Aug 15 to Sept. 7	Z9-12:Ac(50) + Z11-14:Ac(100) + *E*9-12:Ac(10) + *E*11-14:Ac(20)	3.87	±0.79a
	Z9-12:Ac(50)	0.67	±0.20b
	Hexane control	0.42	±0.17b
1985	Z9-12:Ac(1) + Z11-14:Ac(9) + Z11-14:OH(3)	533.57	±31.89a
June 7 to July 15	Z9-12:Ac(1) + Z11-14:Ac(9)	118.75	±15.07b
	Z11-14:OH(3)	25.41	±7.02c
	Z11-14:Ac(9)	25.10	±6.98c
	Hexane control	0.27	±1.21c
1985	Z9-12:Ac(1) + Z11-14:Ac(9) + Z11-14:OH(3)	115.28	±5.92a
Aug. 1 to Sept. 25	Z11-14:Ac(9) + Z11-14:OH(3)	98.30	±5.47a
	Z9-12:Ac(1) + Z11-14:OH(3)	8.68	±1.67b
	Female(s)[b]	0.94	±0.66c
	Hexane control	0.64	±0.63c

[a] Means followed by the same letter are not significantly different at the 5% level (Duncan's multiple-range test).
[b] One to three virgin females in small cages pinned inside traps.

nificantly improved trap catches when added to either the acetate or the alcohol. Although the total number of males caught increased slightly during the last trapping period when the Z9-12:Ac was added to the highly attractive binary mixture of Z11-14:Ac and Z11-14:OH, this increase was not statistically significant.

There was no significant difference between counts in traps baited with one to three females and the controls. The concentration of pheromones as determined in female gland washes was routinely subnanogram and may well be too low to compete effectively with synthetic lures containing a few micrograms of attractant. The relative consistency of the catch in control lures compared to the substantial increase in traps containing lighter loadings suggests that low microgram loadings may be more effective. This would be consistent with the low levels of pheromones observed in the female glands.

DISCUSSION

The retention time correspondence on all capillary columns of volatiles obtained from female glands, combined with discriminating field trapping results, strongly indicates that Z11-14:Ac and Z11-14:OH are the primary pheromone components of *R. naevana.* A 3:1 mixture of these materials at low microgram levels constitutes an effective lure for the trapping of male blackheaded fireworm moths. Traps containing Z9-12:Ac in addition to the binary mixture of primary components were competitive, if not superior, to the binary mixture. Our inability to fully characterize Z9-12:Ac, if present, was due to its very low concentration in female washes or extracts. Comparison of synthetic lures with live females was hampered by the apparent short lifetime of the restrained females.

During the summer of 1985 we became aware of a very similar study (H.G. Davis and L.M. McDonough[5]) and their results are presented in the accompanying paper.

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EVIDENCE FOR CHEMICAL DEFENSE IN TROPICAL GREEN ALGA *Caulerpa ashmeadii* (CAULERPACEAE: CHLOROPHYTA):

Isolation of New Bioactive Sesquiterpenoids

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Abstract—Results of field feeding preference studies with 12 species of tropical green algae of the genus *Caulerpa* showed that *C. ashmeadii* was preferred least by herbivorous fishes. Chemical investigations of *C. ashmeadii* demonstrated the presence of high concentrations of sesquiterpenoid metabolites. The chemical isolation and structural elucidation of five major *C. ashmeadii* metabolites, as well as the results of field feeding preference, antimicrobial, and ichthyotoxicity assays demonstrating the biological activities of these metabolites are reported here.

Key Words—Herbivory, chemical defense, marine algae, allelochemicals, *Caulerpa*, sesquiterpenoids.

INTRODUCTION

Some secondary metabolites from tropical algae have been implicated in chemical defense against grazing fishes and invertebrates in herbivore-rich tropical waters (Sun and Fenical, 1979; McConnell et al., 1982; Paul and Fenical, 1982, 1983). Other investigators have shown distinct feeding preferences in laboratory

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and field feeding assays (Ogden, 1976; Hay, 1981, 1984; Littler et al., 1983; Lewis, 1985). However, few studies have tried to relate these known feeding preferences with the presence of chemical deterrents in algae (Norris and Fenical, 1982; Hay, 1984). In this study, *Caulerpa ashmeadii* was the most resistant alga to herbivorous fishes of 12 *Caulerpa* species and, in some cases, was almost completely avoided in feeding preference assays. Chemical investigation of this same alga led to the isolation of five new sesquiterpenoid metabolites which showed a high degree of biological activity in antimicrobial and ichthyotoxicity assays. The presence of these biologically active terpenoids, which have not been found in any other species of *Caulerpa*, appears to explain the resistance of *C. ashmeadii* to grazing fishes.

Other species of *Caulerpa* have been studied and found to contain terpenoid metabolites, many of which possess the novel bisenol acetate functionality unique to green algae of the related families Caulerpaceae and Udoteaceae (Blackman and Wells, 1978; Amico et al., 1978; Capon et al., 1981, 1983; Paul and Fenical, 1982, 1985). A major sesquiterpenoid metabolite, caulerpenyne (**6**) has been isolated from 10 different species of *Caulerpa* in varying concentrations (Amico et al., 1978; Paul, 1985). Other nonterpenoid nitrogenous compounds such as caulerpin (**7**) and caulerpicin have also been isolated from several different species of *Caulerpa* and have been proposed to be responsible for the biological activity of these algae (Maiti et al., 1978; Mahendran et al., 1979: Doty and Aguilar-Santos, 1966, 1970; Lobel and Ogden, 1981; Vest et al., 1983).

In this study, we posed the following questions: (1) Can the low susceptibility of *Caulerpa ashmeadii* to grazers be related to the presence of bioactive secondary metabolites? (2) How do the biological activities of caulerpenyne and caulerpin compare with the activities of the *C. ashmeadii* metabolites? (3) What are the molecular structures of the *C. ashmeadii* metabolites and can the structural features of these molecules explain the observed biological activities?

METHODS AND MATERIALS

Study Area. Looe Key (24°37′18″N, 81°24′24″W) is located 12.9 km southwest of Big Pine Key, Monroe County, Florida, and was established as a National Marine Sanctuary in 1981. Within the 18.2-km^2 sanctuary lies an inner "core" area of about 1.7 km^2 that includes rich seagrass, coral, and macroalgal assemblages. There are several distinctive habitats: (1) the seaward-most intermediate fore-reef at about 15 m depth; (2) the fore-reef including the spur and groove system (10 m deep to intertidal); (3) the reef flat (1–2 m deep); and (4) the extensive back-reef system (1–10 m deep). Spearfishing is not permitted at Looe Key and, consequently, herbivorous fish populations (especially the parrotfishes and surgeonfishes) are both exceptionally large and diverse (Bohnsack

et al., NOAA draft report). Littler, Littler, and LaPointe (NOAA draft report) were of the opinion that herbivory by reef fishes is the dominant direct controller of algal standing stocks throughout the reef flat and fore-reef slopes.

Herbivory Studies. The suspended-line bioassay method of Littler et al. (1983) was used in the grazing experiments. Clumps of 12 *Caulerpa* species (~ 10 cm^2 in area), representative of the spectrum of available forms, were placed between twists of a three-stranded, 2-mm-thick, white nylon line at 0.5-m intervals in a randomized pattern. The lines were placed in three of the major reef habitat types (intermediate fore-reef, fore-reef, and reef-flat) which have been shown to have large herbivorous fish populations (Bohnsack et al., NOAA draft report), on June 19 and 21, 1984.

The technique yields insights into the differential resistances of the various species and forms of *Caulerpa* to herbivory. For all habitats on both days, three separate lines were used, each containing three clumps per species. A fourth control line was placed in the nearby back reef, where herbivores were few, as a control for losses other than by grazing. The lines were photographed, then suspended ca. 0.3 m above the bottom with each end tied to a coral head for a 3-hr daylight period. Surgeonfishes and parrotfishes were not wary of the lines and began feeding as soon as the divers moved away. Fish typically moved from clump to clump taking small bites, becoming more persistent as they located a particularly palatable clump. After 3 hr, the lines and algae were returned to the boat, rephotographed, and subsequently quantified in the laboratory by the point intercept method (Littler et al., 1983). Losses of all control algae were negligible over the identical 3-hr period. In this technique, the percent thallus area (two-dimensional) lost to grazing for each specimen was calculated from the color slides (Kodachrome 64) by projecting the transparencies onto a grid of dots (at stratified randomized intervals) that were directly related to surface area. Results were compared using Duncan's multiple-range test (Steel and Torrie, 1960).

Chemical Analyses. Samples of *Caulerpa ashmeadii* were collected near Big Pine Key, Florida, in November 1984. The algae were placed directly into ethanol and subsequently extracted with dichloromethane. Metabolites were purified by open-column Florisil chromatography and by silica gel high-performance liquid chromatography (HPLC) with ethyl acetate–isooctane mixtures.

IR spectra were recorded with a Perkin-Elmer model 137 spectrophotometer and UV spectra were recorded in methanol with a Beckman Mk IV instrument. Proton nuclear magnetic resonance (NMR) spectra were recorded with a 360-MHz Nicolet-Oxford Magnetics FT spectrometer and [^{13}C]NMR spectra were recorded at 50 MHz with a Nicolet-200 instrument. High-resolution mass spectra were obtained through the Mass Spectrometry Service Laboratory at the University of Minnesota.

Laboratory Assays. Each metabolite was examined for antimicrobial activity against three marine bacteria and one marine fungus by standard agar plate-

assay disk methods. Zones of inhibition (clear zones) were measured for each filter disk in triplicate against each microorganism tested.

Tropical damselfishes, *Pomacentrus coeruleus* and *Dascyllus aruanus* (ca. 10–15 g average weight), were used to assay for toxicity of the algal metabolites toward marine fishes (Paul et al., 1980). For each assay, compounds were dissolved in a small amount of ethanol and stirred into seawater ($\sim 25°C$) at known concentrations (100 μl EtOH in 200 ml seawater). A damselfish was placed into the seawater and observed for 1.5 hr. A solvent control was always run simultaneously. Toxicity was measured as the death of the fish within this time. Each compound was tested against at least three fish at the minimum effective concentrations.

RESULTS

Herbivory Studies. All loss of algal material from the lines resulted from grazing by herbivorous fishes. This was confirmed by the characteristic grazing scars and by extensive observations of the suspended thalli on the lines by divers. This method measured the relative vulnerability of each species to grazing by natural populations of herbivorous fishes. We could not identify the fish species responsible for grazing nor could we determine the preference of any one fish species.

The most palatable species (Table 1, Figure 1) were *Caulerpa prolifera* v. *obovata* and *C. lanuginosa*, forms that occur cryptically among beds of the seagrass *Thalassia testidinum* which grow on sand-covered rock. The most consistently herbivore-resistant alga across all habitats was *C. ashmeadii*, with less than half of its area being grazed by fishes (Figure 1). This contrasts with a mean 89.5% loss shown by the other 11 species. *Caulerpa ashmeadii* was significantly more resistant to fish herbivory ($P < 0.05$, Duncan's multiple-range test, Steel and Torrie, 1960) than all other species (Figure 1) with the exception of *C. sertulariodes* which lost 71.3% of its thallus area during the experiments.

Chemical Analyses. The major *Caulerpa ashmeadii* metabolite, **1** (Figure 2), was isolated as 20% of the organic extract after purification by silica gel HPLC (15% EtOAc–isooctane) ($[\alpha]_D^{25} = -48°$, $c = 1.4$, $CHCl_3$). This metabolite was analyzed for $C_{21}H_{30}O_6$ by high-resolution mass spectrometry (318.1846 for M^+-HOAc) in combination with consideration of its [^{13}C]NMR spectral features. This molecular formula required seven degrees of unsaturation. The presence of the *E,E*-1,4-diacetoxybutadiene functional group was evident based upon UV absorption at λ_{max} 248 nm ($\epsilon = 17{,}000$); IR absorptions at 2940, 1720, 1440, and 1210 cm^{-1}; and characteristic [^{1}H]- and [^{13}C]NMR spectral features (see Tables 2 and 3) (Blackman and Wells, 1978; Sun and Fenical, 1979). In addition to the bisenol acetate functionality, compound **1** was recognized to possess an additional secondary acetate group and a methyl-

TABLE 1. COMPARISON OF 12 TAXA OF *Caulerpa* TO GRAZER SUSCEPTIBILITY (MEANS ARE GIVEN IN PERCENT CONSUMED)[a]

Species	Mean	SD	CI	SE	*N*
Caulerpa prolifera (Forssk.) Lamour. v. *obovata* J. Ag.	97.4	5.6	3.2	1.5	14
Caulerpa lanuginosa J. Ag.	95.8	15.6	8.1	3.7	15
Caulerpa cupressoides (West) J. Ag. v. *cupressiodes*	95.1	7.3	4.1	1.9	15
Caulerpa racemosa (Forssk.) J. Ag.	93.7	5.9	3.8	1.7	12
Caulerpa verticillata J. Ag.	92.0	14.5	8.0	3.7	15
Caulerpa cupressoides (West) C. Ag. v. *mamillosa* (Montagne) Wever-van Bosse	90.4	13.8	7.6	3.6	15
Caulerpa mexicana (Sonder) J. Ag.	87.5	12.6	7.0	3.3	15
Caulerpa paspaloides (Bory) Greville v. *wurdemanni* Wevver-van Bosse	87.2	14.9	11.4	5.0	9
Caulerpa paspaloides (Bory) Greville v. *compressa* Weber-van-Bosse	87.0	14.2	7.8	3.6	15
Caulerpa mexicana (Sonder) J. Ag. F. *pectinata* (Kützing) Taylor	86.9	12.1	7.0	3.2	15
Caulerpa sertularioides (Gmel.) Howe	71.3	37.5	21.7	10.0	14
Caulerpa ashmeadii Harv.	46.9	37.6	20.8	9.7	15

[a] SD = standard deviation; CI = 95% confidence interval; SE = standard error; *N* = number of lines.

trisubstituted olefin as deduced by further [^{1}H]- and [^{13}C]NMR resonances (Tables 2 and 3). These functionalities accounted for six of the seven degrees of unsaturation, thus indicating one carbocyclic ring to be present in the structure. Comparisons of **1** with the *C. flexilis* var. *muelleri* metabolite **8** (Tables 2 and 3) showed both to possess similar spectral features and carbon skeletons. Proton NMR decoupling studies allowed the complete assignment of **1**, including a secure placement of the secondary acetate at C-4.

Compound **2** was isolated as 10% of the organic extract after final purification by silica gel HPLC (15% EtOAc–isooctane) ($[\alpha]_D^{25} = -98°$, $c = 1.4$, $CHCl_3$). The molecular formula of the metabolite was indicated as $C_{17}H_{24}O_3$ by high-resolution mass spectrometry ($M^+ = 276.1740$), a formula which requires six degrees of unsaturation. IR absorptions at 2940, 1760, 1680, 1650, 1440, 1210, 1110 cm^{-1} and UV absorption at λ_{max} 240, $\epsilon = 22{,}000$, when considered together with ^{1}H and ^{13}C spectral features, indicated the presence of an enol acetate, an unsaturated carbonyl, and a methyl-trisubstituted olefin.

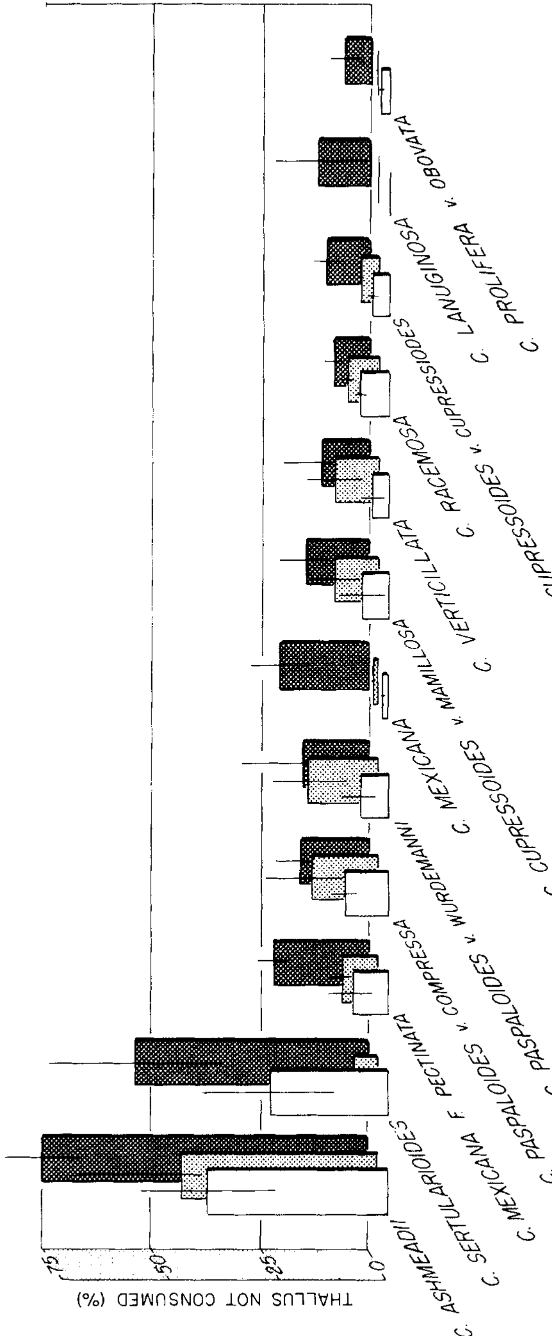

FIG. 1. Comparison of the three major habitats from deep to shallow (front, light = intermediate fore-reef; middle, gray = fore-reef; back, dark = reef flat) in terms of overall herbivory, and grazer resistance of 12 *Caulerpa* forms, in order from highest to lowest means for all habitats. Standard error is given by vertical lines.

FIG. 2. Metabolites from *Caulerpa ashmeadii* and related species of *Caulerpa*.

Spectral comparisons could be readily made with the related metabolites **1** and **8** and the known metabolite onchidal (**9**) (Ireland and Faulkner, 1978). Thus, compound **2** was assigned as a double-bond isomer of onchidal.

Sesquiterpenoid **3** was isolated as 5% of the organic extract after silica gel HPLC (15% EtOAc–isooctane) ($[\alpha]_D^{25} = -104°$, $c = 1.0$, $CHCl_3$). Compound **3** was determined to be $C_{15}H_{22}O_2$ by high-resolution mass spectrometry (234.1622 for M^+). The presence of two aldehydes (one α, β unsaturated) was readily apparent from IR absorptions at 2960, 1720, 1730, 1580, 1450, 1220 cm^{-1}; from UV absorption at λ_{max} 232, ϵ = 16,500; and by [^{1}H]- and [^{13}C]NMR spectral features (Tables 2 and 3). Full proton NMR decoupling and

TABLE 2. [^{1}H]NMR DATA FOR *Caulerpa ashmeadii* AND RELATED METABOLITES[a]

Carbon	Metabolite						
	1	2	3	4	5a	8[b]	9
1	7.60, d, J = 12.5 Hz	8.19, d, J = 12.5	9.53, bs	9.50, t, J = 2 Hz	7.34, d, J = 12.6 Hz	7.37, d, J = 13 Hz	8.26, d, J = 14 Hz
2	5.73, d, J = 12.5 Hz	5.97, d, d, J = 12.5, 1.7 Hz	3.40, 3.30, J_{AB} = 14.5 Hz	3.11, t, J = 2 Hz	6.13, d, J = 12.6 Hz	5.92, d, J = 13 Hz	6.10, dd, J = 14, 1 Hz
3							
4	5.90, t, J = 7 Hz	6.42, t, J = 7 Hz	6.78, t, J = 7 Hz	5.81, t, J = 7 Hz	5.58, t, J = 7 Hz	n.r.[c]	6.41, t, J = 7 Hz
5	1.90, mult	2.51, t, J = 7 Hz	2.43, mult	2.18, mult	2.20, mult	n.r.	2.05 m
6	1.90, mult	2.00, mult	1.71, mult	1.68, mult	2.18, mult	n.r.	2.57 m
7							
8	5.18, mult	5.41, mult	5.43, mult	5.33, mult	5.28, mult	5.33, mult	
9	1.50, mult	1.38, mult	1.40, mult	1.48, mult	1.99, mult	n.r.	1.3–2.05 m
10	1.29, mult	1.23, mult	1.21, mult	1.13, mult	1.42, 1.15, mult	n.r.	
11							
12	7.20, s	9.37, d, J = 1.7 Hz	9.39, s	4.45, s	4.54, mult	7.17, s	9.40, d, J = 1
13	1.61, s	1.68, s	1.69, s	1.65, s	1.61, s	1.71, s	4.81, s, 4.50, s
14	0.88, s	0.88, s	0.90, s	0.88, s	0.83, s	0.89, s	0.89, s
15	1.08, s	0.91, s	0.93, s	0.92, s	0.83, s	1.00, s	0.99, s
OAc	2.17, s	2.13, s		1.98, s	2.07, s	2.16, s	2.16, s
	2.03, s					2.14, s	
	2.01, s						
Ester					1.28, bs (28 H)		
					0.82, s (3 H)		

[a] ^{1}H spectra recorded at 360 MHz in CCl_4 solution.
[b] Compound 8 recorded at 90 MHz in $CDCl_3$.
[c] n.r., Values not reported in literature.

TABLE 3. [^{13}C]NMR DATA FOR *Caulerpa ashmeadii* AND RELATED METABOLITES[a]

	Metabolite					
Carbon	1	2	3	4	5a	8[b]
1	134.8	142.0	195.9	196.9	137.7	134.2
2	109.6	105.1	39.2	44.2	110.3	113.3
3	119.7	134.6	134.7	125.3	130.2	122.4
4	70.2	155.8	157.6	135.8	135.9	29.4
5	34.7	30.3	30.1	29.2	30.1	26.1
6	45.3	49.4	49.2	49.4	49.6	49.3
7	136.5	135.0	135.1	135.0	135.4	136.3
8	120.6	122.2	122.3	121.6	121.6	120.4
9	31.6	31.8	31.7	31.6	31.8	31.8
10	23.4	23.2	23.2	23.3	23.3	23.1
11	32.6	32.6	32.6	32.5	32.3	32.6
12	137.6	192.6	192.6	69.1	66.5	135.9
13	27.2	27.5	27.4	27.6	27.7	27.3
14	23.4	23.4	23.4	23.7	23.7	23.2
15	27.4	27.5	27.4	27.7	27.7	27.6
OAc	169.2	167.0		165.5	168.5	167.9
	167.2	19.9		20.3	19.9	167.5
	166.3					20.6 (2C)
	20.5					
	19.9 (2C)					
Ester					172.2	
					30.1 (11C)	
					34.4	
					29.0	
					25.4	
					14.3	

[a]Spectra recorded at 50 MHz in benzene-d6 solution. Multiplicities determined by SFORD and GASPE experiments.
[c]Compound 8 recorded at 20 MHz in $CDCl_3$.

spectral comparisons with the known compound rhipocephanal (Sun and Fenical, 1979) led to the final structure assignment of **3.** The geometry of the C-3–C-4 olefin was assigned as *E* based on the characteristic shielding of the C-12 aldehyde in the [^{1}H]NMR (Table 2) and on comparison with model compounds (Faulkner, 1971; Wehrli and Nishida, 1979). The saturated aldehyde showed coupling to an adjacent methylene group. The compound is a double-bond isomer of the African termite metabolite ancistrodial (Baker et al., 1978) which acts in chemical defense against ants.

Another sesquiterpenoid produced by *Caulerpa ashmeadii* was compound **4,** which was isolated as 8% of the organic extract after silica gel HPLC (10% EtOAc–isooctane) ($[\alpha]_D^{25}$ = $-111°$, c = 0.8, $CHCl_3$). Again, spectral com-

parisons with other related metabolites facilitated the structural elucidation of this compound (Tables 2 and 3). Five degrees of unsaturation were inherent in the molecular formula ($C_{17}H_{26}O_3$; 218.1653 for M^+-HOAc). These unsaturations could be accounted for by one aldehyde group, a primary acetate, two olefins, and a carbocyclic ring. These latter functionalities were identified by [^{1}H]- and [^{13}C]NMR spectral features as well as by IR absorptions at 2960, 1745, 1730, 1580, 1450, 1220 cm^{-1} and UV absorption at λ_{max} 233, $\epsilon = 2100$. Proton NMR decoupling studies showed that the aldehyde on C-1 was coupled only to a methylene at δ3.11. The methylene protons adjacent to the acetate group showed no coupling; therefore the olefin was placed at C-3–C-4. Protons on carbons C-4–C-6 were also interrelated through decoupling studies to further establish the structure of **4.**

Compounds **5a** and **5b** were isolated as a mixture as 5% of the organic extract by final silica gel HPLC (5% EtOAc–isooctane) ($[\alpha]_D^{25} = -62°$, c = 1.6, $CHCl_3$). The spectral features of these metabolites showed the presence of the familiar enol acetate functionality at C-1–C-2 and the same sesquiterpenoid monocyclic ring system as in compounds **1–4** (Tables 2 and 3). The presence of fatty acid ester residues positioned at C-12 were indicated by [^{1}H] and [^{13}C]NMR spectral features (Tables 2 and 3); IR absorptions at 2960, 1760, 1730, 1715, 1370, 1215, 1110 cm^{-1}; and UV absorption at λ_{max} 246, ϵ = 8200. High-resolution mass measurements of the acylonium ions (RCO^+) confirmed the presence of two fatty acid residues: 239.2392 for $C_{16}H_{31}O$ (17% intensity) and 237.2264 for $C_{16}H_{29}O$ (3.7% intensity). Compounds **5a** and **5b** were therefore estimated to compose 82% and 18% of the mixture, respectively. Other sesquiterpenoid esters possessing linear carbon skeletons have been reported from *C. prolifera* (De Napoli et al., 1983).

Laboratory Assays. All metabolites, except compounds **5a** and **5b,** showed antimicrobial activity toward at least one marine bacterium (Table 4). Only sesquiterpenoid **2** was active toward the marine fungus and compound **2** was the most active overall. Compounds **3** and **4** also showed activity toward all three bacteria.

All metabolites, except the fatty esters **5a** and **5b** and caulerpin **7,** were toxic to damselfish (Table 5) within 1.5 hr. The aldehydes **2, 3,** and **4** again showed the highest degree of biological activity in this assay.

DISCUSSION

This study demonstrates the strong avoidance of *Caulerpa ashmeadii* by reef herbivores and the presence of four biologically active compounds in the alga which are specific to this species of *Caulerpa* (Paul, 1985; Paul and Fenical, 1987). The unpalatability of *C. ashmeadii* relative to other *Caulerpa* species correlates with the presence of highly biologically active sesquiterpenoid

TABLE 4. RESULTS OF ANTIMICROBIAL ASSAYS FOR *Caulerpa* METABOLITES[a]

Compound	*Lagenidium callinectes*[b]	*Vibrio leignathi*[c]	*V. phosphoreum*[c]	SK-13[c]
1	–	–	–	+
2	+	+	+	+
3	–	+	+	+
4	–	+	+	+
5a, b	–	–	–	–
6	n.t.	+	–	n.t.

[a] + = inhibition of microbial growth, zone > 2 mm; – = no inhibition; n.t. not tested. All compounds tested at 200 μg/disk.
[b] Pathogenic marine fungus.
[c] Bacterial isolates, SK-13 is an unidentified strain of gram-positive spore-forming bacteria requiring Mn for growth.

metabolites which are unique to this species. Although most species of *Caulerpa* also produce bioactive sesquiterpenoids such as caulerpenyne (**6**) and nonterpenoid metabolites such as caulerpin (**7**) (Paul, 1985; Paul and Fenical, 1987), these compounds do not show the potent activities of the *C. ashmeadii* aldehydes. Since *C. ashmeadii* is biomechanically no tougher or stronger than the more palatable species (S. Armstrong, personal communication), it seems likely that its avoidance is the result of these secondary metabolites. In addition, comparable studies on the known toxic alga *Dictyota divaricata* Lamouroux (Gerwick, 1981; Norris and Fenical, 1982), in the same habitats of Looe Key (Littler, Littler, and Lapointe, NOAA draft report), showed similar resistance to grazing. Mean loss of *D. divaricata* was 42.6% per 3 hr, which is close to the recorded 46.9% loss per 3 hr for *C. ashmeadii*.

TABLE 5. RESULTS OF ICHTHYOTOXICITY ASSAYS FOR *Caulerpa* METABOLITES[a]

Compound	Toxicity[b]	Dosage (μg/ml)	Time (hr)
1	+	10.0	1.0
2	+	2.5	1.5
3	+	2.5	1.5
4	+	5.0	1.0
5a, b	–	20.0	1.5
6	+	20.0	1.0
7	–	20.0	1.5

[a] This bioassay used the tropical damselfish *Pomacentrus coeruleus* ($N = 4$ for each compound tested). Values expressed are the lowest dosages active within 1.5 hr for all four fish.
[b] + = death within time indicated; – = no death.

Several of the *Caulerpa* species examined here have been shown to be consumed at moderate to high rates in related studies (Littler et al., 1983; Hay, 1984; Lewis, 1985). The high percentage of individuals eaten (~80% for *C. ashmeadii* in Honduras (Hay, 1984) and its comparability to *C. prolifera* in palatability contrast markedly with the results of this study (Figure 1). Hay (1984) and Lewis (1985) contrastingly, noted a low to intermediate herbivore susceptibility for *C. cupressoides* which we found to be eaten readily (Table 1). These findings raise some questions regarding variation in the secondary metabolite composition of these species collected in different habitats. Alternatively, the results may indicate the presence of different herbivores with different grazing preferences or differences in grazing intensity among reefs. Paul (1985) showed that related tropical green algae (genera *Rhipocephalus*, *Udotea*, and *Penicillus*) showed between-population variation in the production of terpenoid metabolites. Concentrations of caulerpenyne (**6**) and related terpenoids may also vary in different populations of *Caulerpa* and be related to algal susceptibility to herbivory.

The nonterpenoid, nitrogenous metabolites caulerpin (**7**) and caulerpicin have often been proposed to function as chemical defenses in *Caulerpa* species (Doty and Aguilar-Santos 1966, 1970; Lobel and Ogden, 1981; Lewis, 1985). However, the sesquiterpenoid caulerpenyne (**6**), which often exists in concentrations of 40–50% of the organic extract in some species of *Caulerpa*, possesses much greater biological activity than caulerpin in ichthyotoxicity assays (Table 5). McConnell and coworkers (1982) also showed that caulerpenyne was responsible for almost all of the feeding deterrent activity of extracts of *C. prolifera* against the sea urchin *Lytechinus variegatus*. Caulerpin caused little, if any, deterrence in these assays. Similarly, Hodgson (1984) showed that caulerpenyne was primarily responsible for the antimicrobial and antineoplastic activities found in extracts of *C. prolifera*.

The *C. ashmeadii* metabolites and other *Caulerpa* terpenoid metabolites (Paul and Fenical, 1982, 1985) also show a much higher level of activity than caulerpin. Caulerpicin is a mixture of several related sphingosine derivatives which have been isolated in very small amounts from several species of *Caulerpa* (Doty and Aguilar-Santos, 1966, 1970; Maiti et al., 1978; Vest et al., 1983). Caulerpicin was not tested in these assays because it is a very minor *Caulerpa* metabolite. The major chemical defenses in *Caulerpa* species appear to be the terpenoid metabolites which contain aldehyde or enol acetate functional groups. These compounds are produced in large concentrations but are often not readily isolated because of their unstable and reactive chemical natures.

The sesquiterpenoid esters, **5a** and **5b**, showed little biological activity in our assays (Tables 4 and 5). Structurally similar terpenoid esters have been isolated from *Caulerpa prolifera* (De Napoli et al., 1983) and also from several

nudibranchs (Cimino et al., 1981, 1982, 1983; Okuda et al., 1983). The esters in the nudibranchs were also reported to be less toxic than the sesquiterpenoid aldehyde, polygodial, which was a major metabolite. The esters were found only in the digestive tract of the nudibranchs, whereas the mantle (which is more exposed to predators) contained the toxin polygodial which is reported to function in chemical defense (Cimino et al., 1981, 1983).

The molecular structures of the sesquiterpenoid aldehydes and enol acetates from *Caulerpa ashmeadii* also support the hypothesis that these metabolites are used in chemical defense. The aldehyde and enol acetate (a masked aldehyde) functional groups can be envisioned to react with proteins in a number of ways to affect or inactivate protein or enzyme function. The bioactive *C. ashmeadii* metabolites are related to numerous insect antifeedants such as ancistrodial (Baker et al., 1978), warburgenal (Kubo et al., 1976), and the iridoid aldehydes (Cavill and Hinterberger, 1960), which could function as defensive agents by identical chemical means.

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IDENTIFICATION OF SEX PHEROMONE COMPONENTS OF DARKSIDED CUTWORM, *Euxoa messoria*, AND MODIFICATION OF SEX ATTRACTANT BLEND FOR ADULT MALES

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Abstract—Eleven "pheromone-like" compounds were identified in excised abdomen tip extracts of calling adult females of darksided cutworm, *Euxoa messoria* (Harris). The essential pheromone components were (*Z*)-7- and (*Z*)-11-hexadecenyl acetates in a ratio of 1:40, which agreed with an attractant blend developed empirically by field testing the attractancies of synthetic blends. The pheromone component, (*Z*)-11-hexadecenol, improved the attraction of darksided cutworm males whereas the components (*Z*)-9-tetradecenyl acetate and (*Z*)-7-dodecenyl acetate inhibited their attraction. The other "pheromone-like" compounds identified in the female extracts had no obvious effect on the attraction of darksided cutworm males. Three compounds that functioned as parapheromones when substituted for (*Z*)-7-hexadecenyl acetate in the two-component blend were (*Z*)-7-pentadecenyl, (*Z*)-7-tetradecenyl, and (*Z*)-7-tridecenyl acetates. (*Z*)-11-hexadecenal was not detected in the female extracts, but it had a synergistic effect on the attraction of darksided cutworm moths and inhibited the attraction of male moths of a nontarget species, *Helotropha reniformis* (Grote). As a trap bait for monitoring purposes, we recommend a four-component blend of (*Z*)-7-hexadecenyl acetate, (*Z*)-11-hexadecenyl acetate, (*Z*)-11-hexadecenol, and (*Z*)-11-hexadecenal at 12.5, 500, 1, and 10 μg/red rubber septum dispenser containing 5 μg of antioxidant 2,6-*tert*-butyl-4-methyl phenol. This blend is effective under field conditions for at least six weeks.

Key Words—Darksided cutworm, *Euxoa messoria*, Lepidoptera, Noctuidae, sex pheromone, (*Z*)-7-hexadecenyl acetate, (*Z*)-11-hexadecenyl acetate, (*Z*)-11-hexadecenol, (*Z*)-11-hexadecenal.

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INTRODUCTION

The darksided cutworm (DSC), *Euxoa messoria* (Harris), occurs across Canada and the northern United States (Hardwick, 1970; Rings et al., 1975). The larvae are a common pest of annual crops and sometimes adopt a climbing habit to cause damage of nursery stock and fruit trees. DSC is considered a major pest of tobacco in Ontario (Cheng, 1971, 1973). In western Canada, it has been considered a minor pest causing occasional damage to row crops and home gardens (Ayre et al., 1982). However, the larvae frequently occur in mixed infestations with other species, particularly redbacked cutworm, *Euxoa ochrogaster* (Guenée), and may be a more important pest in western Canada than previously recognized (Ayre et al., 1982; Beirne, 1971; Byers, unpublished data).

A synthetic sex attractant for adult DSC males was developed previously by systematically testing the attractancies of a large number of blends of synthetic chemicals (Struble et al., 1977). The attractant was a two-component blend of (Z)-7-hexadecenyl acetate and (Z)-11-hexadecenyl acetate (abbreviated as Z7-16:Ac and Z11-16:Ac) at 5 and 200 μg, respectively. It was not known whether these compounds were the same as the sex pheromone components produced by the female DSC moths. The effectiveness of the two-component blend as a trap bait for the attraction of males was comparable to two unmated female moths/trap. In a five-year test in Ontario, the blend was also much more effective than black light for the attraction of males (Cheng and Struble, 1982). It was also used to monitor DSC populations in Alberta from 1979 to 1983 (Byers and Struble, 1985) and in Manitoba from 1977 to 1979 (Ayre et al., 1982). The species specificity in these tests in Ontario, Alberta, and Manitoba was 98, 99.5, and 93%, respectively.

The behavioral responses of DSC male moths to Z7-16:AC and Z11-16:Ac have been studied in a flight tunnel and in orientation disruption experiments (Palaniswamy et al., 1984). Z11-16:Ac was found to be a long-range attractant, while Z7-16:Ac influenced the close-range flight of the moths.

In this paper, we describe the identification of the sex pheromone components in abdominal tip extracts of unmated female DSC moths and the effects of the minor pheromone components on the attraction of adult males. A more attractive and species-specific blend was developed, and it was effective for at least six weeks under monitoring conditions.

METHODS AND MATERIALS

DSC were reared on a modified pinto bean diet (Shorey and Hale, 1965). Pheromone was recovered from 4- to 10-day-old unmated adult females that exhibited calling behavior between 1 and 4 hr into the scotophase (maintained at 20°C, 80–90% relative humidity, and 16:8 light–dark). Everted abdominal

tips were removed with fine forceps and soaked (extracted) in *n*-hexane (BDH Omnisolv®, glass distilled). Extracts from 20 to 80 tips were filtered and reduced to an appropriate volume under N_2 [1.0 female equivalent (FE)/μl] for analyses without further cleanup. A total of ca. 450 females were extracted. Bioassays were done with 4- to 20-day-old adult males in a tube-type olfactometer under conditions described by Struble et al. (1980a).

The extracts were analyzed by gas chromatography (GC) using an electroantennographic detector (EAD) and a flame ionization detector (FID) simultaneously (Struble and Arn, 1984). The effluent split between detectors was ca. 1:10 (Arn et al., 1975) or 1:3 (Struble and Byers, 1985). Gas capillary columns used for the GC analyses were coated (Struble and Richards, 1983) with OV-17 (22 m × 0.35 mm ID), Silar 10C (22 and 50 m × 0.35 mm ID), Carbowax 20 M TPA (28 and 61 m × 0.32 mm ID), and SP-2340 (28 and 75 m × 0.32 mm ID). Helium carrier gas linear velocity was 30 cm/sec (at 50°C) for all analyses, and various temperature programs were used with splitless injections. GC–mass spectrometry (GC-MS) was with a Finnigan 4000 equipped with a data system and Silar 10C (22 and 50 m) capillary columns, and a Hewlett-Packard model 5985B using capillary columns of Silar 10C (22 m), Carbowax 20 M TPA (28 and 61 m) and SP-2340 (75 m).

Synthetic chemicals were either synthesized (Struble and Swailes, 1975) or purchased. All acetates were purified by argentation liquid chromatography (Houx et al., 1974) to chemical purities >99% and isomeric purity >99.8%. Purified acetates were hydrolyzed with methanolic KOH to the corresponding alcohols of similar purity. Purified alcohols were oxidized with pyridinium chlorochromate (Corey and Suggs, 1975) to yield the corresponding aldehydes which were further purified (>99% and alcohol-free) by chromatography on silica gel.

The Z11–16:Ac used throughout these tests contained <0.01% Z11–16:OH, except for the test summarized in Table 3, where the acetate was chromatographed on silica gel to remove all traces of Z11–16:OH.

Chemical blends were prepared in *n*-hexane, and red rubber septa (Arthur H. Thomas Co. catalog No. 8753-D22) were used as dispensers except for one experiment where No. 10 rubber bands (Eberhard Faber, Canada, Ltd.) were used for this purpose. An antioxidant, 2,6-*tert*-butyl-4-methyl phenol (BHT) was added (5 μg) to all blends containing aldehydes. The cone-type traps and field attractancy test procedures (1979–1984) were as previously described (Struble, 1981). Traps were spaced 20 m apart along the edge of cereal or alfalfa crops near Lethbridge, Alberta, and moth counts were recorded at least three times a week and daily in some tests. Captured moths were identified by the authors (Hardwick, 1970), with confirmation of representative specimens by Dr. J.D. Lafontaine, Biosystematics Research Institute, Agriculture Canada, Ottawa. Analyses of variance were done on transformed ($\sqrt{x + 1}$) data and treatment means were compared by Duncan's multiple-range test (SAS Institute

Inc., 1972). In all tables and text, mean catches of moths within a test (column) followed by the same letter did not differ at the 5% level ($P > 0.05$).

RESULTS AND DISCUSSION

Sex pheromone was recovered from adult females that exhibited a calling behavior which was confirmed by observing the responses that the females elicited from male moths in an olfactometer (Struble et al., 1980a). This qualitative bioassay was also used to determine the relative amount of pheromone in the female extracts.

GC and GC-MS analyses. Complete CI differential mass spectra (Struble et al., 1980b), which were comparable to those of authentic chemicals, were obtained for the following components in the female abdomen-tip extracts (relative quantities by GC-MS): 16:Ac (5.4), Z7-16:Ac (2.5), Z9-16:Ac (0.6), Z11-16:Ac (100), and Z11-16:OH (1.5). Two other components, 12:Ac and 14:Ac, were detected by their M + 1 ions in the total-ion chromatograms, but the spectra were incomplete due to the low concentrations of these components.

The total-ion chromatograms were scanned for other C_{10} to C_{18} saturated, mono-, and diunsaturated aldehydes, alcohols, and acetates. However, aldehydes and diunsaturated alcohols and acetates were not detected in the extracts.

The components detected by GC-MS all cochromatographed with authentic samples on capillary columns of Silar 10C (50 m), Carbowax 20 M TPA (61 m), SP-2340 (75 m), and OV-17 (22 m) under various temperature program conditions. Most of the positional and geometrical isomers of monounsaturated C_{10} to C_{16} acetates and alcohols with the double bond beyond carbon atom number 4 can be resolved on these liquid phases (Heath et al., 1980). The isomer assignment of Z7-16:Ac was confirmed on the OV-17 column where it eluted before 16:Ac, which was the reverse order of elution compared with the order on the more polar liquid phases. The isomer assignments of Z7-16:Ac and Z11-16:Ac were consistent with the known (Struble et al., 1977) synthetic attractant blend of these two isomers. The isomer assignments of the other pheromone components detected in the female extracts were substantiated by GC-EAD analyses and field-attractancy tests.

EAD-FID analyses. Male DSC antennae gave consistent EAD responses (Struble and Arn, 1984) to 16:Ac, Z7-16:Ac, Z11-16:Ac, and Z11-16:OH in the female extracts (Table 1). Antennal responses of comparable intensities were obtained with similar quantities of the authentic chemicals, which further supported these structural assignments. DSC male antennae did not respond to any other components in the extracts.

A special EAD technique, in which the detector antennae were from males of other species that were known to have specific responses to the DSC pheromone components, was used to further confirm the isomeric assignments of

these components (Struble and Arn, 1984). This technique was also used to prove the presence or absence of other minor "pheromone-like" components in the DSC extracts.

Antennae of male *Autographa californica*, *Loxestege sticticalis*, *Mamestra configurata*, *Euxoa tessellata*, and *Pseudaletia unipuncta* were used as detector antennae. The specific EAD responses of these antennae to DSC extract components and to the corresponding synthetic chemicals are listed in Table 1. Antennae of *E. tessellata* confirmed the presence of 16 : Ac, *Z*7–16 : Ac, *Z*9–16 : Ac, and *Z*11–16 : Ac in the female extracts, and although the antennae were highly sensitive to synthetic *Z*7–14 : Ac (0.45 mV/2 ng injected), this compound was not detected in the extracts. Antennae of *M. configurata* detected traces of 14 : Ac and *Z*9–14 : Ac and confirmed the presence of 16 : Ac and *Z*11–16 : Ac, while *P. unipuncta* antennae confirmed *Z*11–16 : Ac and *Z*11–16 : OH in the extracts. Antennae of *A. californica* detected a trace of *Z*7–12 : Ac and *L. sticticalis* detected trace quantities of 14 : Ac, *E*11–14 : Ac, and *Z*11–14 : Ac in the extracts. Antennae of *L. stricticalis* also confirmed the presence of *Z*11–16 : Ac. The components *Z*7–12 : Ac, *Z*9–14 : Ac, *E*11–14 : Ac, and *Z*11–14 : Ac were only detectable in the DSC female extracts by GC-EAD using antennae of these selected species of moths.

Based on GC (FID and EAD) and GC-MS analyses, the quantities of the pheromone components, relative to *Z*11–16 : Ac, in the DSC extracts were as follows: 12 : Ac, <0.1; *Z*7–12 : Ac, <0.1; 14 : Ac, <0.1; *Z*9–14 : Ac, <0.1; *Z*11–14 : Ac, <0.1; *E*11–14 : Ac, <0.1; 16 : Ac, 5.4; *Z*7–16 : Ac, 2.5; *Z*9–16 : Ac, 0.6; *Z*11–16 : Ac, 100; and *Z*11–16 : OH, 1.5. There was about 10 ng/FE of *Z*11–16 : Ac in the female extracts.

Attractancy Tests in the Field. The attractant developed previously by empirical testing of synthetic chemicals consisted of *Z*7–16 : Ac plus *Z*11–16 : Ac in a ratio of 1 : 20 or 1 : 40 (Struble et al., 1977). These components were identified in the female pheromone extracts by GC-MS analysis in a ratio of 1 : 40; therefore, unless otherwise stated, this ratio was used in all subsequent attractancy tests.

In the previous tests (Struble et al., 1977), rubber bands were used as dispensers; however, they deteriorated in about two weeks under field conditions and rubber septa were considered to be more suitable dispensers. To confirm this, the two-component blend was tested at a ratio of 1 : 20 (50 : 100 μg/dispenser) on (1) rubber bands and (2) rubber septa, and at a ratio of 1 : 40 (12.5 : 500 μg) on (3) rubber septa. The mean catches of DSC male moths/four replications from August 28 to September 19, 1979, were (1) 38b, (2) 119a, and (3) 137a (means followed by the same letter did not differ at the 5% level). The blends on rubber septa attracted the greatest ($P < 0.05$) numbers of moths, and the rubber septa did not deteriorate under field conditions, so they were used as dispensers throughout the remainder of these tests.

To determine the synergistic or inhibitory effects of the other components

TABLE 1. ANTENNAL RESPONSES OF ADULT MALES OF *E. messoria* AND OTHER SPECIES USED FOR EAD DETECTION OF PHEROMONE COMPONENTS IN *E. messoria* FEMALE EXTRACTS

Pheromone components	*E. messoria* male EAD responses (mV) to: Extract (1-5 FE)	*E. messoria* male EAD responses (mV) to: Synthetic (4 ng)	Other detector species (males)	EAD responses (mV) to: extract (1.0 FE)	EAD responses (mV) to: synthetic (1-2 ng)
12:Ac	ND[a]	ND	—	—	—
Z7-12:Ac	ND	0.08	*Autographa californica*	0.09	0.19
14:Ac	ND	0.08	*Loxostege sticticalis*	0.07	0.38
Z9-14:Ac	ND	0.08	*Mamestra configurata*	0.07	0.20
Z11-14:Ac	ND	0.18	*L. sticticalis*	0.13	0.23
*E*11-14:Ac	ND	0.07	*L. sticticalis*	0.51	0.77
16:Ac	0.06	0.04	*Euxoa tessellata*	0.11	0.10
Z7-16:Ac	0.04	0.07	*E. tessellata*	0.52	0.65
Z9-16:Ac	ND[a]	0.05	*E. tessellata*	0.10	0.15
Z11-16:Ac	0.51	0.38	*E. tessellata*[b]	0.46	0.15
Z11-16:OH	0.05	0.07	*Pseudaletia unipuncta*	0.06	0.15

[a] ND indicates that responses were not detectable.
[b] Responses of Z11-16:Ac in the *E. messoria* extract were also confirmed by *L. sticticalis*, *M. configurata*, and *P. unipuncta* males as detector species.

detected in the female extracts (Table 1), field-attractancy tests were done with these added singly to the two-component blend (Z7-16:Ac and Z11-16:Ac at 12.5 and 500 μg/dispenser). The components added, the quantities (μg), and the mean catches of moths for four replications from August 12 to September 3, 1980, were as follows: two-component blend, 63abc; Z11-16:OH (1 μg), 80a; Z9-16:Ac (4 μg), 56abc; 16:Ac (20 μg), 41cd; *E*11-14:Ac (2 μg), 42cd; Z11-14:Ac (2 μg), 53bc; Z9-14:Ac (0.1 and 1.0 μg), 52bc and 24d; 14:Ac (2 μg), 45bc; and Z7-12:Ac (0.1 and 1.0 μg), 23d and 7e. Three other compounds that are positional isomers of the components detected in the female extracts were also included in this test by adding them (2 μg) singly to the two-component blend. The compounds added and the mean catches of DSC males were: Z5-16:Ac, 50bc; Z9-12:Ac, 44c; and Z5-12:Ac, 41cd (throughout this test, means followed by the same letter did not differ as the 5% level). Z11-16:OH was the only component that appeared to have any synergistic effect on the attraction of DSC male moths. Both Z7-12:Ac and Z9-14:Ac were potent attractant inhibitors. The other components detected in the female extracts and their positional isomers that were included in this test tended to reduce (although

$P > 0.05$) the trap catches compared with the two-component blend, but none of these were strongly inhibitory.

The geometrical isomers of the main pheromone components, *E*7–16:Ac (2 μg) and *E*11–16:Ac (25 μg), as well as *Z*7–16:OH (10 μg) were added singly to the two-component blend in two tests (four replications) done in 1979 and 1980. These compounds had no obvious effect on the attraction of males (data are not shown). Similarly, 17 blends (four replications) containing 4–11 of the pheromone components per blend were tested during 1979, 1980, and 1981, but all of the treatments attracted fewer ($P > 0.05$) males than the two-component blend. The strong inhibitory effects of *Z*7–12:Ac and *Z*9–14:Ac were also evident whenever these components were present in the multicomponent blends.

The pheromone components *Z*7–16:Ac and *Z*11–16:Ac were essential for the attraction of DSC males, and two-component blends of *Z*11–16:Ac with each of the other components detected in the female extracts did not attract any DSC males. These results were consistent with the previous attractancy tests (Struble et al., 1977).

Two-component blends of *Z*11–16:Ac with the lower homologs of *Z*7–16:Ac, i.e., *Z*7–15:Ac, *Z*7–14:Ac, and *Z*7–13:Ac, were reported (Struble et al., 1977) to attract DSC males. These tests were repeated even though the lower homologs were not detected in the female extracts. Substitution of *Z*7–16:Ac in the two-component blend with the lower homologs resulted in the capture of smaller ($P < 0.05$) numbers of moths (Table 2). The addition of each of these compounds to the two-component blend of *Z*7–16:Ac and *Z*11–16:Ac also resulted in reduced ($P < 0.05$) catches of moths. It seems that these parapheromones must have some "fit" on the receptor for *Z*7–16:Ac, and the decrease in trap catches within this homologous series may indicate a decreasing degree of "fit." Odd-numbered carbon compounds have been reported as parapheromones for other lepidopterans (Cardé and Roelofs, 1977; Struble, 1983; Voerman et al., 1975).

In the initial attractancy tests with the minor components identified in the female extracts, addition of *Z*11–16:OH to the two-component blend of *Z*7–16:Ac and *Z*11–16:Ac appeared to enhance the attraction of DSC males. Further tests confirmed that the presence of *Z*11–16:OH at 0.1 μg resulted in a significant increase in the numbers of DSC males captured (Table 3). However, the catches of a nontarget species, *Helotropha reniformis* (Grote), also increased dramatically, as a total of 278 males were captured. In a later test, the catches of *H. reniformis* were reduced by increasing the quantity of *Z*11–16:OH to 4 or 10 μg in the three-component blend (data are not shown). These blends, however, caught large numbers of clover cutworm, *Discestra trifolii* (Hufn.), as might be expected because a blend of *Z*11–16:Ac and *Z*11–16:OH at a ratio of 9:1 is an effective attractant for this species (Struble and Swailes, 1975; Underhill et al., 1976).

TABLE 2. MEAN NUMBER OF *E. messoria* MALES ATTRACTED TO BLENDS OF Z11-16:Ac AND Z7-16:Ac OR ITS LOWER HOMOLOGS

Compounds added (μg) to Z11-16:Ac (500 μg)				Mean No. Males captured[a]
Z7-16:Ac	Z7-15:Ac	Z7-14:Ac	Z7-13:Ac	
12.5				87a
	12.5			66b
		12.5		48d
			12.5	2e
12.5	12.5			60c
12.5		12.5		62bc
12.5			12.5	47d

[a] Four replications, August 26 to September 15, 1980. Means followed by the same letter do not differ ($P > 0.05$) by Duncan's multiple-range test.

Several compounds were added singly to a three-component blend of Z7-16:Ac (12.5 μg), Z11-16:Ac (500 μg), and Z11-16:OH (1 μg) in search of an inhibitor of *H. reniformis* that would not inhibit the attraction of DSC. Z11-16:Ald met these requirements as it not only inhibited the attraction of the nontarget species, but it also had a synergistic effect on the attraction of DSC (Table 4). The addition of 1-100 μg of Z11-16:Ald to the two-component or the three-component blends resulted in the capture of the greatest ($P < 0.05$) numbers of DSC males and the capture of only a few *H. reniformis*. The blends containing 10 μg of Z11-16:Ald captured more DSC males than blends containing 1 or 100 μg (although $P > 0.05$).

TABLE 3. MEAN NUMBER OF *E. messoria* MALES ATTRACTED TO BLENDS OF Z7-16:Ac (12.5 μg) AND Z11-16:Ac (500 μg) PLUS VARIOUS QUANTITIES OF Z11-16:OH

Quantities (μg) of Z11-16:OH	Mean No. males captured[a]
—[b]	109b
0.1	138a
0.5	132ab
1.0	131ab
2.0	129ab

[a] Four replications, August 11 to September 9, 1981. Means followed by the same letter do not differ ($P > 0.05$) by Duncan's multiple-range test.
[b] Z11-16:Ac contained no detectable Z11-16:OH.

TABLE 4. MEAN NUMBER OF *E. messoria* AND TOTAL NUMBER OF *H. reniformis* MALES ATTRACTED TO BLENDS OF Z7-16:Ac (12.5 μg) AND Z11-16:Ac (500 μg) CONTAINING VARIOUS QUANTITIES OF Z11-16:OH AND Z11-16:ALD (Aug. 8-17, 1984)

Compound added (μg)		Mean No.	*Total No.*
Z11-16:OH	Z11-16:Ald	*E. messoria*[a]	*H. reniformis*
—	—	31c	10
1	—	34c	66
1	1	60ab	6
1	10	74a	2
1	100	68ab	0
—	1	64ab	0
—	10	65ab	0
—	100	47ab	0

[a]Means, of four replications, followed by the same letter do not differ ($P > 0.05$) by Duncan's multiple-range test. An antioxidant, BHT (5 μg), was added to all chemical blends.

The two-component blend or the blends containing Z11-16:OH and/or Z11-16:Ald were all excellent for the attraction of DSC males. It was important, however, to determine the relative attractancies of these blends over an extended period of at least six weeks. This was done in a test (Table 5) which consisted of three parts: a comparison of the relative attractancies of the two-component blend with blends containing Z11-16:OH and/or Z11-16:Ald; the effectiveness of blends that were preexposed for 10 days in an earlier test (August 8-17, 1984, Table 4); and the effectiveness of treatments with twice the dispenser load.

The addition of Z11-16:OH to the two-component blend increased the catches of both DSC and *H. reniformis* (Table 5), which was in agreement with the earlier data (Tables 3 and 4). The addition of Z11-16: Ald to either the two- or three-component blends significantly increased the capture of DSC males and very few *H. reniformis* were caught. Increasing the dispenser load from 500 to 1000 μg (based on Z11-16: Ac) resulted in the capture of fewer DSC with all of the blends, although the reduction was not significant for the two-component blend.

The trap catches were recorded daily throughout this test and the mean catches of DSC males per three-day period from August 18 to September 17, 1984, are illustrated in Figure 1. A freshly prepared treatment of the four-component blend was added to the test on August 29 (end of interval 4, Figure 1 and Table 5) to determine the attractancy of a freshly perpared treatment relative to equivalent treatments that had been used under field conditions since August 9 and 18. The four-component blends all captured similar numbers of DSC

TABLE 5. MEAN NUMBER OF *E. messoria* AND TOTAL NUMBER OF *H. reniformis* MALES ATTRACTED TO BLENDS OF Z7-16:Ac (12.5 μg) AND Z11-16:Ac (500 μg) CONTAINING VARIOUS QUANTITIES OF Z11-16:OH AND Z11-16:ALD (FOUR REPLICATIONS, 1984)

Components added (μg)		Mean No. *E. messoria*[a]			Total No. *H. reniformis*
Z11-16:OH	Z11-16:Ald	Aug. 18-29	Aug. 30-Sep. 18	Aug. 18-Sep. 18	Aug. 18-Sep. 18
Freshly treated dispensers					
—	—	151c	136ef	287e	17
1	—	208b	170cde	378d	70
1	10	286a	244ab	530a	0
—	10	278a	237ab	515ab	0
1	10	—	246ab	—	0
Treated dispensers preexposed August 8-17, 1984					
—	—	150c	147de	298e	23
1	10	284a	252a	536a	2
—	10	279a	206ab	486ab	0
Dispenser load, 2× the quantities of Z7-16:Ac and Z11-16:Ac (i.e., 25 and 1000 μg)					
—	—	137c	100f	236e	22
2	—	139c	103f	242e	72
2	20	234ab	191bcd	426bcd	0
—	20	218b	177cde	395cd	1

[a] Means within a column followed by the same letter do not differ ($P > 0.05$) by Duncan's multiple-range test. An antioxidant, BHT, was added to all blends at 5 μg/500 μg of Z11-16:Ac.

males from August 30 to September 18, which indicated that the general decrease in catches throughout this period was due to a decline in the moth abundance. The three-component blend of Z7-16:Ac, Z11-16:Ac, and Z11-16:Ald consistently caught fewer moths than the four-component blend, but the difference was not significant. The attractancy of the two-component blend was previously reported to be comparable to two unmated female moths/trap (Struble et al., 1977), so the new blends containing the alcohol and/or the aldehyde would be highly competitive with females.

The beneficial effect of 1 μg of Z11-16:OH in these blends is of practical importance because trace quantities of the alcohol would not need to be removed from either Z11-16:Ac or Z11-16:Ald used for preparing the DSC attractant.

There was no analytical evidence for Z11-16:Ald in the DSC female ex-

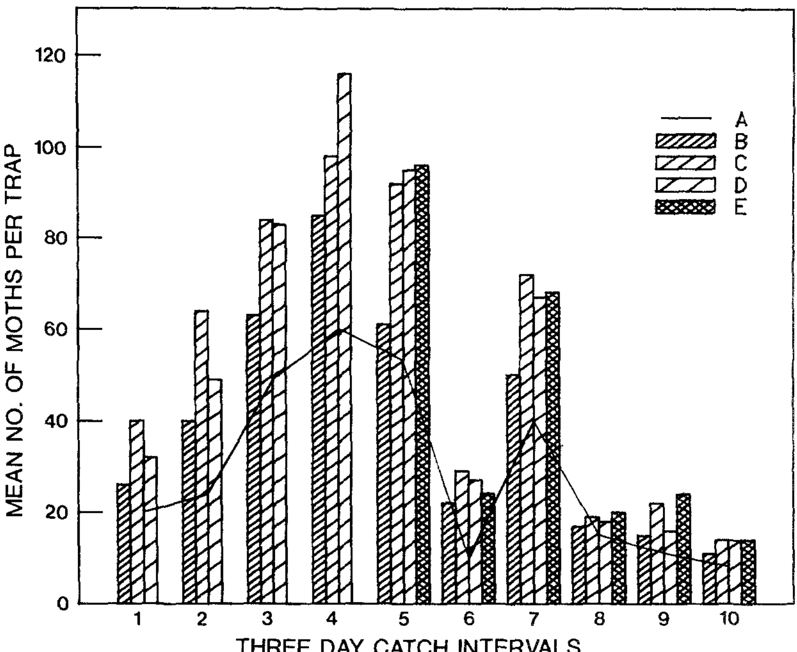

FIG. 1. Mean numbers of *E. messoria* male moths/trap/3-day intervals with four replications of five blends in an alfalfa field near Lethbridge, Alberta, from August 18 to September 17, 1984. The blends were: A, Z7-16:Ac (12.5 μg) and Z11-16:Ac (500 μg) which was the previous attractant and is represented by a line; B, blend A plus Z11-16:OH (1 μg); C, blend A plus Z11-16:OH (1 μg) and Z11-16:Ald (10 μg); D, blend A plus Z11-16:Ald (10 μg); and E, freshly prepared blend C added to the test August 29.

tracts; however, we are investigating whether it is detectable in the female effluvium.

In conclusion, for monitoring the population densities of DSC males we recommend a four-component blend of Z7-16:Ac, Z11-16:Ac, Z11-16:OH, and Z11-16:Ald at 12.5, 500, 1, and 10 μg/red rubber septum dispenser. A three-component blend without the alcohol would be nearly as attractive over at least a six-week period. Both blends contained 5 μg of BHT and are highly species-specific.

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ATTRACTION OF PREDATORILY NAIVE POSTLARVAL LOBSTERS TO EXTRACTS OF METABOLITES OF COMMON PREY: *Mytilus edulis, Mya arenaria, Cancer irroratus,* and *Asterias vulgaris*

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Abstract—Postlarval lobsters (4th–7th stage) exclusively fed frozen brine shrimp (*Artemia saline*) were assayed for food-search response to extracts and metabolites from four common prey: soft clams (*Mya arenaria*), blue mussels (*Mytilus edulis*), rock crabs (*Cancer irroratus*), and sea stars (*Asterias vulgaris*). Concentrations of soluble primary amines, protein, and ammonia in prey tissues and metabolites were determined. No significant responses were observed for any prey metabolites diluted to 1 and 10%, while only *A. vulgaris* evoked a significant response at full strength, suggesting that predatorily naive lobsters have yet to develop more pronounced chemosensory responses shown by field-collected lobsters. Removal of protein with retention of small-molecular-weight polar molecules did not appear to affect response to prey extracts. EC_{50}s, as micromoles per liter amines, computed from prey extract dose–response curves indicate differences per unit amine between prey extracts, with *A. vulgaris* extract more potent as an attractant than either bivalve extract. *C. irroratus* extract was equally attractive as the other three extracts. Ammonia levels excreted into seawater over 3 hr were similar for all prey species, while soluble primary amines and proteins were undetectable. Ammonia and protein per gram whole prey varied significantly between extracts of prey species, while primary amines were similar. Lobsters may be attracted preferentially to carrion species with higher concentrations of amines and/or higher potency of attractants per unit amine.

Key Words—*Homarus americanus*, chemoreception, chemical sensing, food attractants, prey preference, attractant quality.

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INTRODUCTION

The American lobster (*Homarus americanus*) is commonly considered an opportunist feeder, ingesting a wide variety of species (Herrick, 1895; Squires and Ennis, 1968; Squires, 1970; Ennis, 1973; Elner and Jamieson, 1979; Scarratt, 1980; Carter and Steele, 1982a). Although prey availability is an important dietary factor (Miller et al., 1971; Carter and Steele, 1982a), some prey selection may occur, especially as a function of lobster age (Scarratt, 1980; Carter and Steele, 1982a) and stage of molt (Leavitt et al., 1979; Scarratt, 1980; Carter and Steele, 1982a). Additionally, laboratory prey choice studies have demonstrated dietary preferences for particular species (Evans and Mann, 1977; Elner, 1980; Carter and Steele, 1982b).

Homarus americanus can sense living and dead prey from a distance via chemosensory structures. The ability to sense metabolites from living prey is considered weaker than the ability to sense prey tissue extracts (McLeese, 1973a; Hirtle and Mann, 1978; Carter and Steele, 1982b), because the concentration of the former is lower. Attractants from tissue extracts appear to consist of mixtures of amino acids and perhaps other small compounds (McLeese, 1970, 1973a; Derby, 1984; Daniel and Bayer, 1986a). Characterization of attractant components excreted from live prey has not been accomplished.

The sensitivity of lobsters to waterborne food chemicals has led to the suggestion that prey selection may be partially mediated by chemical sensing (Derby and Atema, 1981). Some evidence of discrimination between attractant solutions of prey species exists. Gravimetrically or volumetrically equivalent tissue extracts of crabs (*Cancer irroratus*) and bivalves (*Mytilus edulis*) elicited greater food-search behavior than sea stars (*Asterias vulgaris*) or sea urchins (*Strongylocentrotus droebachiensis*) (McLeese, 1973a; Carter and Steele, 1982b). However, the observed responses may have been a function of quantity of attractants in test solutions rather than quality of attractants, since prey tissues contained varying concentrations of amino acids (Carter and Steele, 1982b). The behavioral threshold of the food-search response appeared to decrease toward metabolites from live horse mussel (*Modiolus modiolus*) and to increase toward metabolites from live blue mussel (*Mytilus edulis*) after one month feeding exclusively on live *M. modiolus* (Derby and Atema, 1981). Opposite results were obtained if lobsters were fed exclusively live *M. edulis*. Thus, at least in the case of chemically mediated detection of live prey, response to prey species may vary as a function of attractant quality.

All studies of chemically mediated food-search behavior in lobsters have used field-collected individuals which have had some degree of feeding experience on live, naturally occurring prey (McLeese, 1970, 1973a,b; Hirtle and Mann, 1978; Derby and Atema, 1981; Carter and Steele, 1982b; Derby, 1984). Examination of food-search behavior in lobsters with no previous experience

with ingestion of natural prey may reveal whether there are initial prey preferences.

In the present study, postlarval lobsters (4th–7th stage) that were fed from hatching frozen brine shrimp (*Artemia salina*) were tested for food-search response to extracts and metabolites from four common prey: soft clams (*Mya arenaria*), blue mussels (*Mytilus edulis*), rock crabs (*Cancer irroratus*), and sea stars (*Asterias vulgaris*). Concentrations of soluble primary amines, protein, and ammonia were determined, providing a basis for comparison of attractant quantities of metabolites of live prey and extracts of prey tissues. Behavioral bioassays testing live prey metabolites provided information on chemical mediation of prey search by predatorily naive lobsters. Behavioral bioassays of extracts were performed as a function of primary amine concentration rather than extract weight or volume. Since primary amine concentration has been demonstrated to reflect attractant levels (Daniel and Bayer, 1986a), this allowed comparison of attractiveness of prey species extracts.

METHODS AND MATERIALS

Preparation of Prey Metabolites and Extracts. Metabolites and extracts were prepared from *Mytilus edulis*, *Mya arenaria*, *Cancer irroratus*, and *Asterias vulgaris*. These animals were obtained from intertidal areas near Southwest Harbor, Maine, or the University of Maine Darling Center, Walpole, Maine, and maintained in laboratory aquaria for no more than two days. Whole animals were weighed before preparation of solutions. Solutions containing metabolites were prepared by maintaining live prey for 3 hr at approximately 10–40 g wet weight whole prey per liter of "aged" filtered seawater, prepared by filtering natural seawater (32–35 ppt) to 1 μm (Microwynd filter, MWM Co., Meridan, Connecticut) and allowing it to stand for at least two weeks before use. Metabolites were vacuum filtered (G6 glass fiber) and stored at 4°C until behavioral bioassay within 5 hr.

Extracts were prepared by homogenizing whole animals (shells of bivalves were removed) in aged filtered seawater (100 g wet weight/liter seawater) in a Waring blender. Homogenates were centrifuged twice at 25,000*g* for 20 min at 4°C. Supernatants were combined and vacuum filtered to 0.45 μm using G6 glass fiber and 0.45 μm Nucleopore filters to make "raw extract." Extracts were halved and either stored at −20°C or used to prepare "Sm7 filtrate" by methanol extraction, rotary evaporation, and preparative Sm7 Biobeads chromatography in order to remove protein and peptides while leaving amines and other polar small-molecular-weight compounds intact [preparation described by Daniel and Bayer, 1986a; see also Rittschof et al., (1984) for discussion of rationale]. The eluent containing water-soluble amines was collected and frozen

as Sm7 filtrate at −20°C. Behavioral bioassays were performed with thawed fractions within 48 hr of preparation.

Ammonia and protein concentrations of metabolites, thawed raw extracts, and thawed Sm7 filtrates were determined by methods of Solorzano (1969) and Bradford (1976), while primary amine concentrations were determined by measurement of fluorescence of primary amines after *o*-phthaldialdehyde derivatization (Roth, 1971). However, derivatization reagent was prepared according to Lindroth and Mopper (1979). Bovine serum albumin and DL-alanine were used as standards for protein and primary amine determinations, respectively. The significance of interspecies differences in amines, ammonia, and soluble protein per gram whole prey was determined by analysis of variance and Tukey's (equal sample size) or Gabriel's (unequal sample size) comparison of means (Sokal and Rohlf, 1981).

Behavioral Bioassay. Postlarval lobsters (4th–7th stage) used in assay experiments were obtained from the Department of Fisheries and Oceans hatchery at St. Andrews, New Brunswick, Canada, and maintained in natural seawater which was recirculated through filters as described by Daniel and Bayer (1986a). Upon hatching, lobsters were fed ad libitum frozen brine shrimp (*Artemia salina*). The same group of lobsters was used for each prey metabolite or extract assay, although lobsters were randomly assigned to treatments tested for a given assay experiment. Presence or absence of food-search response of up to 200 lobsters was determined using a behavioral bioassay apparatus described by Daniel and Bayer (1986a). Lobsters, fasted for two days, were placed individually at excurrent ends of 36-cm × 4-cm-diam. clear acrylic chambers. Aged filtered seawater flowed at 0.8 cm/min (20 ml/min) through chambers. Stimulus release (2 ml/min) occurred through a 16.5-gauge needle placed 2–3 cm upstream. A positive food-search response consisted of components of the following behaviors: vigorous motions of the maxillipeds, periopod scooping, and lunging toward and/or grasping the stimulus needle.

Aged filtered seawater was used to dilute prey extracts to appropriate concentrations and as a control treatment for all bioassays. In addition, bioassays included a standard attractant containing 56 μM amine prepared from G6 filtrate of suspension of freeze-dried herring extract (preparation described by Daniel and Bayer, 1986a). Bioassay of prey metabolites included control, standard metabolites at full strength, and metabolites diluted to 1 and 10%. The significance of difference between responses to treatments and to control was determined by R × C log-likelihood test with Williams' correction (Sokal and Rohlf, 1981). Bioassay of raw extracts and Sm7 filtrates included control, standard, and extract solutions containing 0.2–560 μM amines. Responses to raw extracts and Sm7 filtrates were adjusted as described by Daniel and Bayer (1986a) to remove variability which may have been due to changes in behavioral threshold between experiments. Probit analysis was performed on adjusted responses to determine

EC_{50} values or effective concentration necessary to elicit a positive food-search response in 50% of the lobsters (Statistical Analysis System, SAS Institute, Inc., Cary, North Carolina).

RESULTS

Behavioral Bioassay of Metabolites. Generally, food-search responses to live prey metabolites were either at or slightly above levels for control seawater (Figure 1). No significant responses were observed for any of the prey metabolites diluted to 1 and 10%. Higher responses were observed for all full-strength preparations but only *A. vulgaris* solution elicited a significant response ($P <$

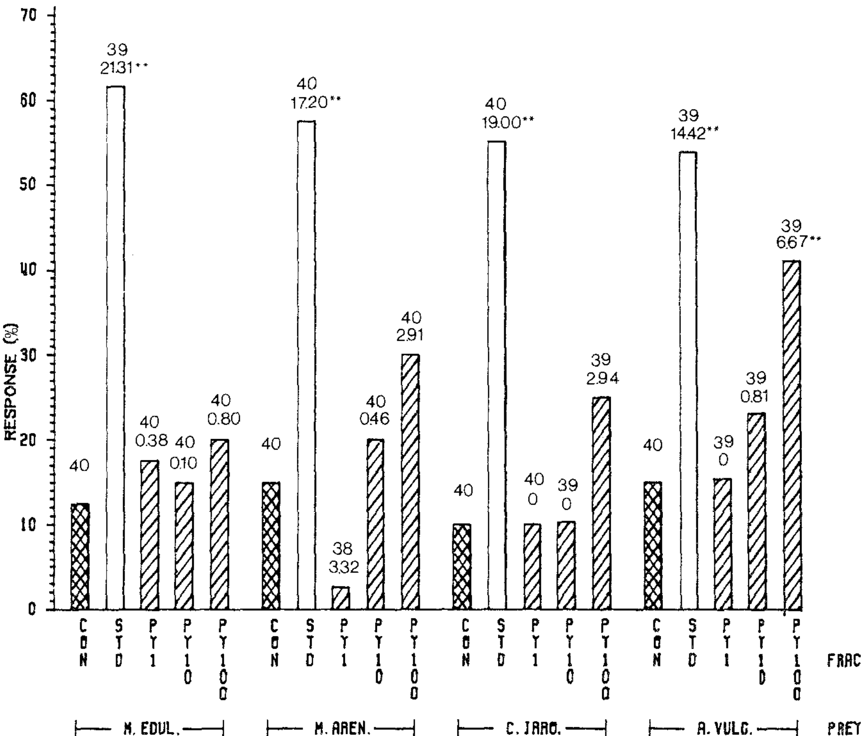


FIG. 1. Food-search response of postlarval lobsters to live prey metabolites, CON = aged, filtered seawater, STD = freeze-dried herring extract at 56 μM amines, PY1–PY100 = live prey metabolites from 1% to 100% concentration. Numbers of lobsters assayed followed by G statistic for treatment response vs. control response are given above each bar. ** = $P < 0.05$.

0.05). In contrast, response to 56 μM amine herring standard was consistently significant at 57.0 $\pm$ 1.7% ($X \pm$ SE, $N = 4$) ($P < 0.05$).

Behavioral Bioassay of Extracts. Response to filtered seawater (control) was consistent and low in extract bioassays (12.1 $\pm$ 1.45% ($X \pm$ SE), $N = 8$). In addition, responses to herring standard varied little (59.5 $\pm$ 3.57% ($X \pm$ SE), $N = 8$); thus the data transformation (Daniel and Bayer, 1986a) had little effect on probit curve estimations by affecting only slightly the y intercept. Predicted probit equations for all treatments fit these data well as determined by chi-square test for heterogeneity ($P > 0.05$).

Removal of protein with retention of most of the amines did not appear to affect the responses to prey extracts (Figure 2, Table 1). EC_{50} (as micromoles per liter amine) 95% fiducial ranges of raw extract and Sm7 filtrate of the same species showed considerable overlap. Such a comparison was not possible for *C. irroratus* since only two concentrations of Sm7 filtrate were tested; nonetheless, EC_{50} values of raw and Sm7 filtrate of *C. irroratus* were almost identical.

Some interspecific differences in attractiveness were apparent from 95% fiducial limits shown in Table 1. Responses to all raw extracts were similar, as shown by fiducial range overlap. However, EC_{50}s of Sm7 filtrates of *M. edulis* and *M. arenaria* were significantly greater than EC_{50} of *A. vulgaris* Sm7 filtrate. While the EC_{50}s for both fractions of *A. vulgaris* were quite similar, the slope of the raw extract dose–response curve was comparatively low, resulting in the largest fiducial range of any EC_{50} estimation and thus contributing to overlap with EC_{50}s for other raw extracts. Raw extracts and Sm7 filtrates of *C. irroratus* and *A. vulgaris* were probably quite similar in attractant quality, since fiducial ranges for raw extracts showed complete overlap and EC_{50}s of all preparations were very similar. Therefore, on an equimolar amine basis, *A. vulgaris* Sm7 filtrate appeared to elicit a greater food-search response than the response to bivalve Sm7 filtrates but not greater than the response to *C. irroratus* Sm7 filtrate.

Ammonia, Amine, and Protein Levels in Metabolites, Raw Extracts, and Sm7 Filtrates. Ammonia (micromoles per gram whole prey) excretion into seawater over 3 hr was similar for the four species (Table 2, ANOVA, $N = 12$, $F = 1.10$, $P = 0.4032$). Protein and primary amines were not detectable in any metabolite preparations. Protein, primary amines, and ammonia were undetectable in aged, filtered seawater. Sensitivity of assays was 50 μg/ml (bovine serum albumin equivalents) for protein, 40 μM (DL-alanine equivalents) for primary amines, and 1 μM for ammonia.

Ammonia (micromoles per gram whole prey) and protein (milligrams per gram whole prey) varied significantly between raw extracts of species (Table 2, ammonia, ANOVA, $N = 14$, $F = 14.32$, $P = 0.0006$; protein, ANOVA, $N = 14$, $F = 4.87$, $P = 0.0244$). *C. irroratus* raw extract had greater ammonia levels than all other extracts and greater protein levels than *M. arenaria* raw

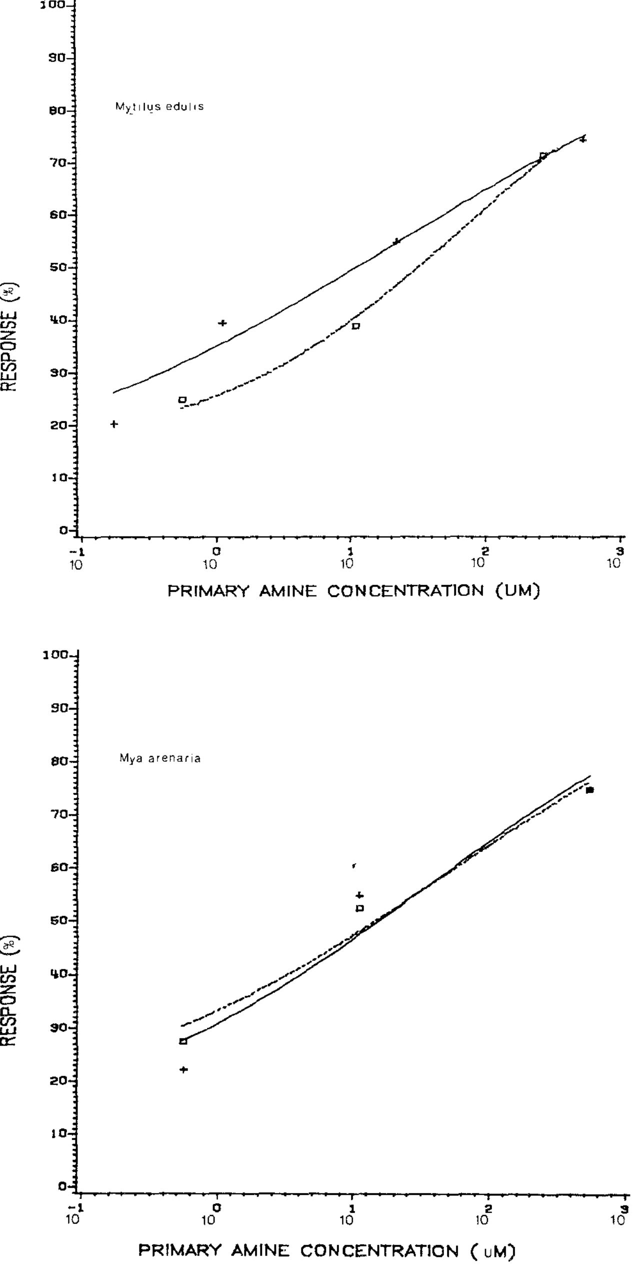
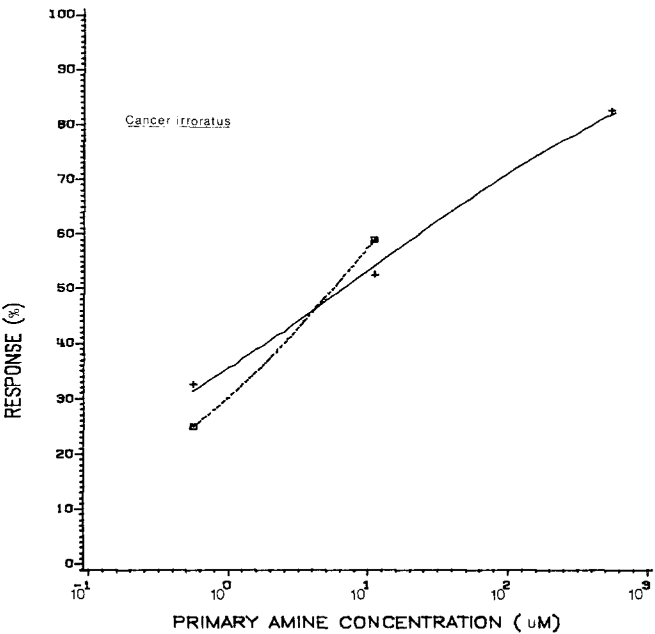


FIG. 2. Amine dose-response curves for prey extracts. Responses were normalized for variation in response to 56 μM amine freeze-dried standard. Curves generated from probit model with response to control as baseline response. Lines and data point symbols are as follows: raw extract: +, ———, Sm7 filtrate: □, - - - - - - -,.



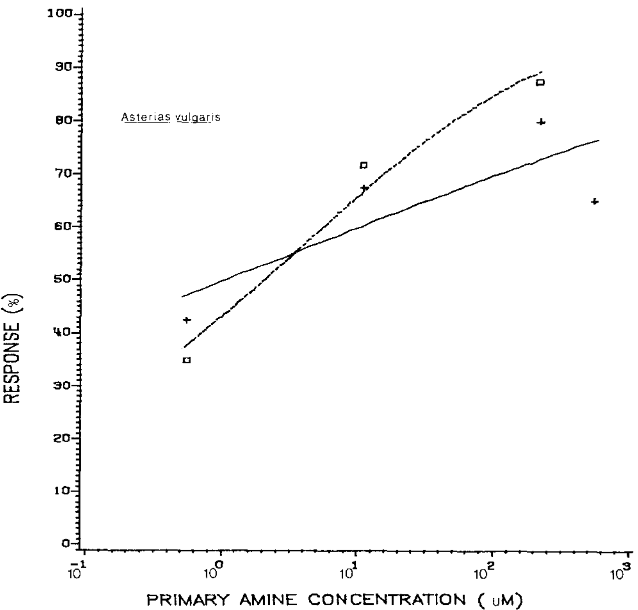


FIG. 2. Continued.

TABLE 1. PARAMETERS OF DOSE–RESPONSE CURVE FOR PREY EXTRACT PREPARATION

95% fiducial limits[a]			Probit curve parameters[b]			
Treatment	N	EC_{50}[a]	Lower	Upper	Slope	Intercept
M. edulis						
Raw	117	23.3	4.73	140	0.430	4.41
Sm7 filtrate	111	79.6	21.61	517	0.679	3.71
M. arenaria						
Raw	116	32.6	8.27	138	0.531	4.20
Sm7 filtrate	120	46.1	10.6	250	0.521	4.13
C. irroratus						
Raw	120	9.00	2.08	31.4	0.486	4.54
Sm7 filtrate	118	8.23	—	—	0.829	4.24
A. vulgaris						
Raw	120	4.33	0.0170	39.2	0.291	4.81
Sm7 filtrate	119	3.84	0.956	10.3	0.663	4.61

[a] Micromoles per liter amine.
[b] y = (probit/log conc)x + probit.

extract. *M. edulis*, *M. arenaria*, and *A. vulgaris* raw extracts had similar ammonia and protein concentrations. All four species had similar primary amine concentrations (micromoles per gram whole prey, Table 2, ANOVA, $N = 14$, $F = 1.79$, $P = 0.2129$).

Sm7 filtrates of prey extracts retained most of the primary amines with little detectable protein remaining. Retention as percent recovered in filtrate was 81.2 ± 6.10% for amines, 0.26 ± 0.11% for proteins, and 41.9 ± 5.37% for ammonia (X ± SE, $N = 13$).

TABLE 2. CONCENTRATION OF SOLUBLE NITROGENOUS COMPOUNDS IN PREY METABOLITES AND EXTRACTS PER GRAM WHOLE PREY[a]

	Metabolites		Extracts			
Prey species	N	Ammonia (μmol/g)	N	Ammonia (μmol/g)	1° amine (μmol/g)	Protein (mg/g)
Mytilus edulis	3	1.29 ± 0.0861a	4	6.13 ± 1.49a	39.8 ± 5.48a	10.0 ± 1.09ab
Mya arenaria	3	1.62 ± 0.521a	4	3.62 ± 1.81a	30.4 ± 5.43a	7.42 ± 1.45a
Cancer irroratus	3	1.35 ± 0.295a	3	13.1 ± 1.01b	34.0 ± 9.22a	24.0 ± 7.21b
Asterias vulgaris	3	2.30 ± 0.636a	3	4.82 ± 1.04a	20.1 ± 3.10a	11.3 ± 1.33ab

[a] Values are means ± SE. Values followed by the same letters represent similar concentrations according to 95% confidence limits computed from comparison of means test; Tukey's for equal sample sizes, or Gabriel's for unequal sample sizes.

DISCUSSION

The importance of low-molecular-weight polar compounds, such as amino acids, as major components of food attractants for *Homarus americanus* has been shown using extracts of *Mytilus edulis* (Derby, 1984), cod (*Gadus spp.*) (McLeese, 1973a), and *Clupea harengus* (Daniel and Bayer, 1986a). The present study extends the list to include *Mya arenaria*, *Asterias vulgaris*, and *Cancer irroratus* and reaffirms previous results with *M. edulis* extract (Derby, 1984).

Moreover, this is the first demonstration of differences in attractant potency of extracts of different prey species, independent of total primary amine concentration, for *H. americanus*. *Asterias vulgaris* protein- and peptide-free Sm7 filtrate was more attractive than *M. edulis* and *M. arenaria* Sm7 filtrates, although no differences in raw extracts were apparent by comparison of EC_{50}s expressed as micromoles per liter primary amines. This latter discrepancy appears to be a result of higher response variability in raw extracts since EC_{50}s of raw extracts and Sm7 filtrates were similar.

Chemically mediated prey preferences demonstrated by McLeese (1973a) and Carter and Steele (1982b) were probably due to the quantity of attractants in stimulus solutions using volumetrically or gravimetrically equivalent solutions rather than attractant potency as indicated in the present study. Unlike McLeese (1973a), Carter and Steele (1982b) measured amine concentrations in extracts. Ranking of percentage of lobsters showing food-search behavior of the extracts generally followed that of amine concentration for the extracts: response to *C. irroratus* extract, 92.0% (225 μM amine) > *M. edulis* extract, 80.0% (39 μM amine) > *A. vulgaris* extract, 66.7% (21 μM amine) > *S. droebachiensis* extract, 53.8% (26 μM amine). They concluded that there was an apparent preference of lobsters for *C. irroratus* and *M. edulis* over echinoderms. McLeese's (1973a) results showed similar trends, although the difference between response to echinoderm extracts and to bivalve extracts was more pronounced. Observed preferences were probably a result of variations in concentrations of attractants in the different prey extracts, partially due to differing proportions of hard parts which would not contribute to the weight or volume of extract after filtration. Indeed, primary amine concentrations as micromoles per gram whole prey including hard parts did not show significant variation between prey in the present study in comparison to much higher levels in *C. irroratus* in Carter and Steele's (1982b) study which did not include hard parts in concentration estimates.

Measurement of total amines by the o-phthaldialdehyde (OPA) derivatization method might lead to error due to variations in fluorescent intensity between individual amino acid derivatives. For the buffer and pH (boric acid, pH 9.5) used in coupling reagent, fluorescence of most individual amino acids is similar (10–20% variation) (Roth, 1971). However, proline and lysine are undetectable (Roth, 1971); both have been shown to produce feeding response

[proline: 53% (McLeese, 1970), 56.5% (Carter and Steele, 1982b); lysine: 9–70% (McLeese, 1970), 47.8% (Carter and Steele, 1982b)]. EC_{50}s as micromoles per liter amine (measured by OPA derivatization) of extracts with high amounts of proline and/or lysine might be expected to be less than EC_{50}s of extracts with low proline and/or lysine concentrations. Levels of lysine among prey extracts did not differ, although there were significantly higher levels of proline in *C. irroratus* than in *A. vulgaris*, and no difference in proline levels in *C. irroratus* and *M. edulis* (Carter and Steele, 1982b). This would suggest that only proline might bias conclusions based on EC_{50} values. However, the addition of proline concentration to total amines measured by the OPA method would serve only to increase the EC_{50} of the *C. irroratus* extract relative to other extracts. The hypothesis that there are differences in attractiveness (independent of primary amine concentration) between prey extracts, with *A. vulgaris* extract as the most potent attractant due to its low EC_{50}, would be either unaffected or strengthened by inclusion of lysine and proline in estimations of EC_{50}s.

Only Sm7 filtrates of prey extracts showed differences in attractant potency. Rittschof et al. (1985) have found that some amino acids, particularly arginine and lysine, are retained by Amberlite XAD-7 (similar to Sm7 Biobeads). Since both arginine and lysine are attractive to lobsters when presented singly (McLeese, 1970, Carter and Steele, 1982b), removal might reduce attractiveness of extracts containing these compounds as major components. This might lead to a modification of qualitative differences between prey species extracts. Relative loss of these amino acids from Sm7 filtrates is not known; however, Daniel and Bayer (1986a) did not measure significant retention to Sm7 Biobeads of a mixture of 20 radiolabeled amino acids including arginine and lysine.

Thus, lobsters may be attracted preferentially to carrion with higher concentrations of amines and higher potency of attractants per unit amine. Different mixtures of amino acids and other small compounds in extract mixtures may provide a basis for variations in preference. *Homarus americanus* showed variable food-search responses to single amino acid solutions, although responses were not consistent (McLeese, 1970; Carter and Steele, 1982b). Mixtures of amino acids may evoke greater food-search responses in *Homarus* spp. than single amino acids and the magnitude of response may vary with types of amino acids in the mixture (McLeese, 1970; Mackie, 1973).

Some chemosensory cells show specificity towards particular amino acids, while other cells respond to a variety of amino acids (Derby and Atema, 1982; Johnson and Atema, 1983). Neural integration of chemosensory response to attractant mixtures in the spiny lobster (*Panulirus argus*) can produce qualitative discrimination in some neurons as certain components elicit synergistic or suppressive effects (Derby and Ache, 1984a,b; Derby et al., 1985). Synergistic effects of mixtures of artificial attractants on whole behavior have been indi-

cated for feeding response in the shrimp, *Palaemonetes pugio* (Carr, 1978; Carr et al., 1984; Carr and Derby, 1986). Finally as the studies with *P. pugio* indicate, attractiveness may also be a function of low-molecular-weight compounds other than amino acids which do not react with OPA, resulting in an apparent lowering of EC_{50} estimations based solely on primary amines.

The food-finding behavior of another lobster, *Panulirus interruptus*, contrasts with our results for *H. americanus*. The spiny lobster, also an opportunistic feeder, appears to respond to high-molecular-weight compounds exuding from prey tissues (Zimmer-Faust et al., 1984). While individual amino acids are attractive to the spiny lobster, low-molecular-weight fractions of prey extracts may contain compounds which suppress attraction to the extract (Zimmer-Faust et al., 1984a). The higher-molecular-weight fractions contain the attractants, possibly peptides. Release of larger molecules from tissue occurs later than release of small molecules, causing a delay in attraction of the lobster to the food site (Zimmer-Faust and Case, 1982; Zimmer-Faust et al., 1984a). Zimmer-Faust et al. (1984a, b) argue that this prevents lethal encounters with predators which are attracted to the early-releasing solutes. A similar hypothesis has been proposed for delayed attraction of hermit crabs to gastropod predation sites (Rittschof, 1980). American lobsters are most attracted to polar solutes leaching from tissue within 3 hr of immersion in seawater (Daniel and Bayer, 1986b). Avoidance behavior is probably unnecessary since they are well-equipped for defense and, therefore, can safely approach a food site earlier.

Responses of postlarval lobsters to metabolites of live prey which they had never ingested and also had not experienced previous exposure to metabolites appeared to be even lower than reported previously for adult field-collected lobsters (McLeese, 1973a; Hirtle and Mann, 1978; Derby and Atema, 1981; Carter and Steele, 1982b). A significant response was obtained only to full-strength *A. vulgaris* metabolites, corresponding with the higher potency of *A. vulgaris* extract. Some of the discrepancy may be due to methodology used to prepare metabolites. Studies with older, field-collected lobsters either used greater prey weight per volume water (50–100 g/liter) or soaked prey for longer periods (10–24 hr). However, dilution of stimulus in behavioral assays was often 1000× or greater (McLeese, 1973a; Hirtle and Mann, 1978; Derby and Atema, 1981; Carter and Steele, 1982b). In the present study, dilution of stimulus was probably minimal due to shorter distances between stimulus needles and lobster shelters (2–3 cm) and lower volumes of water in assay chambers (300–400 ml).

It is more likely that lower responses to live prey metabolites reflect differences associated with either the age or the predatorily naive state of postlarval lobsters. Metabolites of live green crabs (*Carcinus maenus*), *C. irroratus*, *S. droebachiensis*, *M. edulis*, and *M. modiolus* appear to contain stimulants of feeding behavior in field-collected adult or juvenile lobsters (Hirtle and Mann, 1978; Derby and Atema, 1981; Carter and Steele, 1982b), while *C. irroratus*

and *M. edulis* metabolites did not evoke significant responses in postlarval lobsters. Field-collected lobsters have not been tested with *M. arenaria*, which did not elicit a feeding response in postlarval lobsters, or with *A. vulgaris*, which produced a significant response at full strength in postlarval lobsters. Interestingly, Carter and Steele (1982b) reported that measurable levels of amino acids (alanine, glycine, and histidine) were found only in tank water of *A. vulgaris* (which they did not test for attractiveness), reflecting relative levels in extracts, and not in tank waters containing equivalent concentrations of other prey species. This may account for the responsiveness of postlarval lobsters to the metabolites from *A. vulgaris*.

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DEVELOPMENT OF CHEMICALLY MEDIATED PREY-SEARCH RESPONSE IN POSTLARVAL LOBSTERS (*Homarus americanus*) THROUGH FEEDING EXPERIENCE

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Abstract—Postlarval lobsters were fed live amphipods (*Gammarus oceanicus*), soft clam spat (*Mya arenaria*), or frozen brine shrimp (*Artemia salina*) for five weeks in order to determine by behavioral bioassay if chemically mediated prey-search behavior is established by feeding experience. Chemosensory responses of predatorily naive lobsters to live clam and amphipod metabolites were low and erratic. After five weeks, amphipod-fed lobsters had developed strong responses towards amphipod metabolites but not clam metabolites. In contrast, clam-fed lobsters did not develop responses to either prey. Chemical fractionation of amphipod metabolites indicated that attractants were confined to the same fraction as for prey extracts, i.e., polar, low-molecular-weight compounds. Survival (80–90%) was similar for each diet group; growth was greatest for amphipod-fed lobsters (100%), followed by clam-fed lobsters (72%) and brine shrimp-fed lobsters (18%); and feeding rates increased for amphipod-fed lobsters and decreased for clam-fed lobsters. Coloration of lobsters indicated that only amphipod diet provided desirable pigments. Differences in ingestive conditioning results between clam-fed and amphipod-fed lobsters may have been related to (1) clam metabolites being qualitatively or quantitatively less attractive than amphipod metabolites or (2) differences in the predisposition of lobsters to show ingestive conditioning to different prey and their associated metabolites as a function of quality of prey as a diet.

Key Words—*Homarus americanus*, chemoreception, predatory behavior, ingestive conditioning.

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INTRODUCTION

Postlarval lobsters (*Homarus americanus*) with no previous exposure to potential prey are attracted to extracts of prey flesh but can, at best, weakly respond to metabolites exuded from living specimens of the same prey (Daniel and Bayer, 1987a). A slight but significant prey-search response was elicited only with *Asterias vulgaris* metabolites but not with *Mytilus edulis*, *Cancer irroratus*, or *Mya arenaria* metabolites. This overall lack of response to live prey metabolites by predatorily naive lobsters contrasts with stronger responses to a wide variety of prey species in field-collected juveniles and adults (Hirtle and Mann, 1978; Derby and Atema, 1981; Carter and Steele, 1982), suggesting that the establishment of sensing of metabolites for live prey may involve a mechanism dependent on age or feeding experience.

Feeding experience has been shown to be a determinant of the sensitivity of lobsters to prey metabolites (Derby and Atema, 1981). Field-collected lobsters capable of sensing metabolites of the mussels, *Modiolus modiolus* and *Mytilus edulis*, lower their threshold of detection to a given prey metabolite solution following feeding experience on that prey. This behavior, termed "ingestive conditioning" (Wood, 1968), has been observed in other invertebrates including a marine gastropod [*Urosalpinx cinerea* (Wood, 1968; Wood et al., 1981)], sea stars [*Asterias rubens* (Castilla, 1972), *Luidia clathrata* (McClintock and Lawrence, 1984), *Acanthaster planci* (Collins, 1975; Ormond et al., 1976)], a nudibranch [*Aeolidia papillosa* (Hall et al., 1982, 1984)], a terrestrial gastropod [*Achatina fulica* (Croll and Chase, 1977, 1980), and, possibly, a polyclad flatworm [*Stylochus ellipticus* (Christensen, 1972; Daniel, 1982)].

Ingestive conditioning may explain discrepancies between chemosensory responses of adult field-collected and laboratory-hatched, naive oyster drills (*U. cinerea*). Adult drills collected in areas containing only bay scallops (*Argopecten irradians*) were attracted and could ingest scallops (Ordzie and Garofalo, 1980), whereas naive drills were not attracted to scallops (Rittschof et al., 1983). Furthermore, recently hatched snails (*Achatina fulica* and *U. cinerea*) were conditioned through feeding experience to prey metabolites to which they initially showed little or no response (Croll and Chase, 1980; Wood et al., 1981). A similar hypothesis can be invoked to explain the contrast between chemosensory responses of predatorily naive, hatchery-reared versus adult, field-collected *H. americanus*.

The demonstration of the development through feeding experience of chemically mediated prey-search behavior in postlarval lobsters is obviously complicated by their small size. Natural prey species must be selected which are small enough to be handled successfully by lobsters weighing less than 500 mg. Lobsters this size have rarely been observed in nature but, according to substrate choice studies conducted in the laboratory, appear to prefer eel grass

beds (Barshaw and Atema, 1985) or rock sand substrates (Botero and Atema, 1982; Pottle and Elner, 1982; Barshaw and Atema, 1985). Small crustaceans and bivalve spat are common to these habitats. Our study used amphipods (*Gammarus oceanicus*) and soft clam spat (*Mya arenaria*) as prey items for postlarval lobsters in ingestive conditioning experiments. Live *G. oceanicus* was shown by Good et al. (1982) to constitute a suitable diet for postlarval lobsters. Initially, we selected *Mytilus edulis* spat as appropriate prey items, since Derby and Atema (1981) had fed immature lobsters adult blue mussels in their ingestive conditioning study. However, preliminary studies indicated that postlarval lobsters were incapable of opening even the smallest mussels. Soft clams were successfully opened by most of the lobsters, probably as a result of their thinner shells. Responses of prey-fed lobsters to prey metabolites were compared to responses to filtered seawater and to responses of lobsters fed frozen brine-shrimp to prey metabolites. Survival, feeding rates, and growth were monitored for determination of potential correlates between prey quality and feeding behavior.

METHODS AND MATERIALS

Source and Maintenance of Postlarval Lobsters

Postlarval lobsters were obtained from the Department of Fisheries and Oceans Hatchery at St. Andrews, New Brunswick, Canada (NB lobsters, N = 235, mean wet weight (g) = 0.065 ± 0.00078 (SE), and from the University of Maine Darling Center hatchery at Walpole, Maine (DC lobsters, N = 110, mean wet weight (g) = 0.10 ± 0.0079 (SE). NB lobsters were reared from hatching exclusively on frozen brine shrimp (*Artemia salina*) and were never exposed to any potential prey metabolites, while DC lobsters were reared through fourth stage on live brine shrimp, followed by frozen brine shrimp and filtrate from the plankton bag filtration system (240–500 μm) in the hatchery which included live barnacle larvae, bivalve larvae, amphipods, copepods, and mysid shrimp. Cannibalism probably occurred in this group as lobsters were not kept separate. Equal numbers of both groups of lobsters were randomly assigned to three tanks of natural seawater recirculated through filters and kept in individual containers (holding system described in detail by Daniel and Bayer, 1987b) for prey ingestion experiments. Group mean weights were similar at experiment initiation. However, NB lobsters in the clam-fed group were replaced after 17 days with larger DC lobsters (N = 65, mean wet weight (g) = 0.24 ± 0.015 (SE).

Source and Maintenance of Prey

Live clam spat, *Mya arenaria*, ranging from 0.0014 to 0.10 g wet weight including shell, were obtained from the Darling Center hatchery. Live amphi-

pods, *Gammarus oceanicus*, ranging from 0.0015 to 0.020 g wet weight, were collected during low tide in mean low-water intertidal pools near Bar Harbor or Pemaquid Point, Maine. Prey were maintained in a laboratory aquarium containing natural seawater and fed unicellular algae (a mixture of *Thalassiosira pseudonana* and *Isochrysis galbana* obtained weekly from Darling Center cultures, maintained in glass carboys in constant light and aeration and supplied with nutrient media) and frozen brine shrimp. Adequate populations of each prey species were kept in the aquarium to supply the requirements of the feeding regimens. Prey used for metabolite preparation were collected within one week of bioassay.

Feeding Regimes

Each group of lobsters was reared exclusively on either frozen *A. salina*, live *M. arenaria*, or live *G. oceanus* as follows:

Artemia Salina-Fed Group. Each lobster was fed ad libitum every other day. Responses of this group to prey metabolites allowed a standard of comparison with responses of prey-fed lobsters over the duration of the feeding trial.

Mya arenaria-Fed Group. Each lobster was supplied with 10 live clams every other day. Sizes of clams were generally in proportion to lobster size. Prey remaining in the container were counted and new clams were added to equal 10.

Gammarus oceanicus-Fed Group. Initially, lobsters were supplied 10 amphipods every other day. However, it became apparent that newly molted lobsters were being eaten by amphipods. After five days, further predation by amphipods was successfully prevented by reducing the daily supply of amphipods to three. Prey remaining in containers were counted and new amphipods were added to equal three. Unlike with clams, amphipods were chosen at random, independent of lobster size. Amphipod escape rates were determined by placing three amphipods in each of 10 empty containers and counting the number escaped after one day. Prey ingestion rates were corrected for escape rates (0.33 amphipods/(container $\times$ day).

Lobsters were maintained on the diets for five weeks. Two days before behavioral bioassays all prey were removed from lobster containers. Amphipods which escaped from containers into tanks were removed. Feeding regimes were reestablished immediately after completion of behavioral bioassays.

Significance of differences between the proportion of lobsters surviving after six weeks was determined for the test groups by unplanned G tests for the heterogeneity of treatments (Sokal and Rohlf, 1981). A similar test was used to determine the effect of duration of diet on frequency distribution of number of prey ingested/(lobster $\times$ day). Differences in wet weight gain of lobsters on the three diets were determined by analysis of variance and Gabriel's multiple comparison of means based on unequal sample sizes (Sokal and Rohlf, 1981).

Preparation of Live Prey Metabolites

One gram wet weight of prey per 100 ml of two weeks or older ("aged") filtered natural seawater (preparation described by Daniel and Bayer, 1987b) was lightly aerated for 10 hr for clam metabolite preparation and only 3 hr for amphipod metabolite preparation in order to eliminate mortality resulting from longer soaking time. Clams were supplied with an algae mixture at the beginning of metabolite preparation. Prey were removed and water was vacuum filtered (GF/C glass fiber) and stored at 4°C until behavioral bioassay within 4 hr.

Half of one preparation of amphipod water was passed through a Sm7 Biobeads column (bed volume = 15 ml) which was subsequently washed with seawater and HPLC-grade water. Adsorbed material, including peptides, was eluted with HPLC-grade 100% methanol. Details of chromatographic procedure were given by Daniel and Bayer (1987b). Untreated amphipod water, seawater fractions (Sm7 filtrate) and rotary evaporated methanol eluent (Me eluent) were stored at −20°C for less than 24 hr before bioassay.

Ammonia, protein, and primary amine concentrations of untreated prey metabolites were determined as described by Daniel and Bayer (1987a).

Behavioral Bioassays

Lobsters within a feeding group were randomly assigned to treatments tested for a given assay experiment. Presence or absence of prey-search response of approximately 100 lobsters was determined using a behavioral bioassay apparatus described by Daniel and Bayer (1987a, b).

Aged filtered seawater was used to dilute the stimulus solution to appropriate concentrations and as a control treatment for all bioassays. In addition, assays of unfractionated prey metabolites included a standard attractant containing 56 μM amines prepared from the G6 filtrate of a suspension of freeze-dried herring extract (preparation described by Daniel and Bayer, 1987b). Bioassay of unfractionated prey metabolites included control, standard, and prey metabolites diluted to 10% and at full strength. Bioassay of fractionated amphipod metabolites included control, untreated amphipod metabolites diluted to 10%, Sm7 filtrate and Me eluent of amphipod metabolites diluted to the same volume as 10% untreated amphipod metabolites.

Feeding groups were assayed for prey-search response as follows: (1) pretreatment test with both prey metabolites; (2) at day 15, amphipod-fed lobsters tested with amphipod metabolites, clam-fed lobsters tested with clam metabolites, and brine shrimp-fed lobsters tested with amphipod and clam metabolites; (3) at day 35, all diet groups tested with each prey metabolite solution and amphipod-fed lobsters tested with untreated and fractionated amphipod metabolites.

Significance of differences between responses to prey metabolites versus

responses to control and responses to treatments of prey-fed group versus response to treatments of brine shrimp-fed group for the same prey metabolites was determined by $R \times C$ log-likelihood test with William's correction (Sokal and Rohlf, 1981).

RESULTS

Responses of Lobsters to Prey Metabolites

Artemia salina-*Fed Lobsters.* Lobsters fed frozen brine shrimp showed some response to both prey metabolites over the five-week test period. Significant prey-search responses to 100% amphipod metabolites were observed at zero and five weeks (Figure 1). No response to 10% amphipod metabolites was apparent at any test period. A significant response to clam metabolites at both concentrations was apparent only at two weeks (Figure 2). Most of the prey-search response to amphipod metabolites at zero and two weeks was contributed by DC lobsters. Response to full-strength amphipod metabolites was 63.6% for DC lobsters ($N = 11$) and 38.9% for NB lobsters ($N = 18$) at zero days, and 50% for DC lobsters ($N = 12$) and 11.1% for NB lobsters ($N = 18$) at two weeks. A larger response of DC lobsters was not evident at five weeks with both groups having a 33.3% response ($N = 12$ for each group). In contrast to these temporal changes in responsiveness, DC and NB lobsters showed similar responses to clam metabolites independent of test period.

Mya arenaria-Fed Lobsters. No increase in response to clam metabolites occurred with feeding experience (Figure 2). Responses were not significant when compared to control for the same assay or to corresponding treatments for brine shrimp-fed lobsters. There were significant responses to 10% and full-strength amphipod metabolites compared to the control response for five-week clam-fed lobsters (Figure 1). However, responses were not significantly greater than response to corresponding treatments of five-week brine shrimp-fed lobsters.

Gammarus oceanicus-*Fed Lobsters.* There was an increase in response to amphipod metabolites with feeding experience (Figure 1). After two weeks, response to amphipod metabolites at full strength and 10% was significantly greater than control, although only the response to 10% metabolites was significantly greater than response of brine shrimp-fed lobsters at two weeks to the same metabolite solution. However, by five weeks, amphipod-fed lobsters responded as vigorously to amphipod metabolites as to herring standard. Responses were significantly greater than control and corresponding treatments of brine shrimp-fed lobsters at five weeks. After five weeks, amphipod-fed lobsters did not show a significant response to 10% or full-strength clam metabolites (Figure 2).

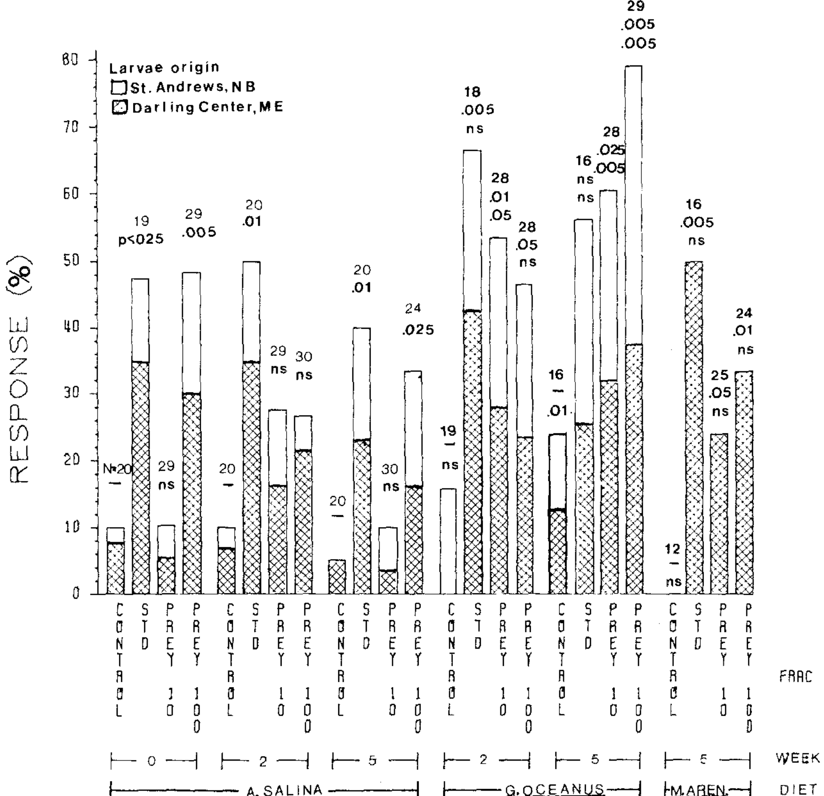

FIG. 1. Prey-search response (%) to amphipod (*G. oceanicus*) metabolites of *A. salina*-fed, *G. oceanicus*-fed, and *M. arenaria*-fed lobsters by week of feeding trial. Control = aged, filtered seawater, Std = herring freeze-dried extract at 56 μM amine; Prey 10 = 10% solution of amphipod metabolites in aged, filtered seawater; Prey 100 = full-strength solution of amphipod metabolites. Hatched areas represent proportion of response due to DC lobsters. Clear areas represent proportion of response due to NB Lobsters. Numbers above bars represent number of lobsters tested followed by (1) level of significance of log-likelihood test for stimulus vs. control and (2) level of significance of log-likelihood test for prey-fed response vs. brine shrimp-fed response to same stimulus at same feeding trial week.

Removal of peptides from amphipod metabolites did not significantly alter prey-search response (Figure 3). Responses to untreated metabolites and Sm7 filtrate were significantly greater than to the control. Response to methanol eluent was similar to control response. Response to untreated metabolites was similar to response to Sm7 filtrate but greater than response to methanol eluent.

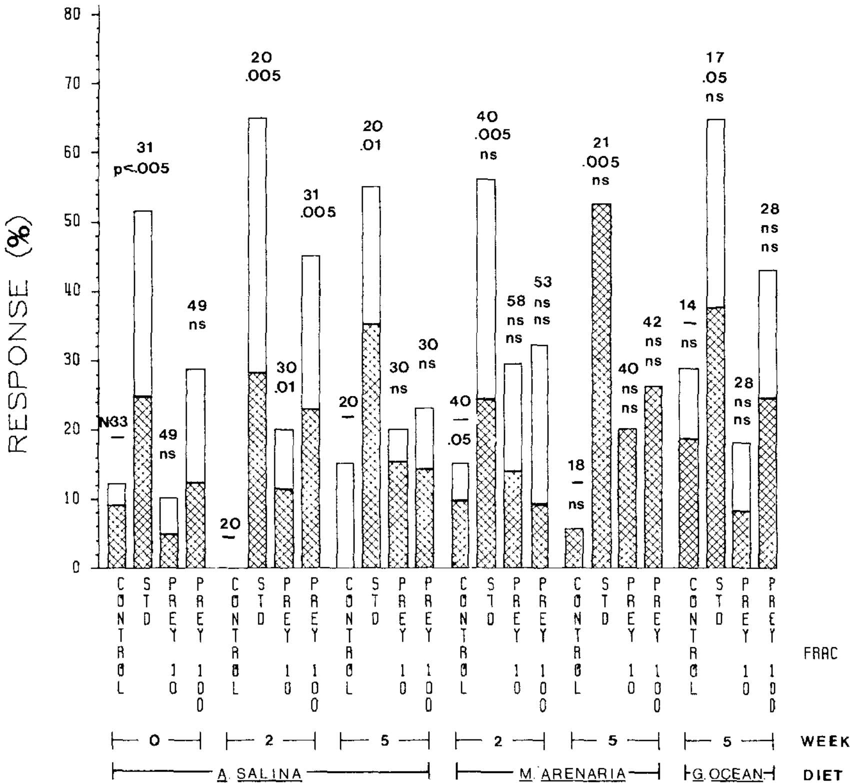

FIG. 2. Prey-search response (%) to clam (*M. arenaria*) metabolites of *A. salina*-fed, *G. oceanicus*-fed, and *M. arenaria*-fed lobsters by week of feeding trial. Control = aged, filtered seawater; Std = herring freeze-dried extract at 56 μm amine; Prey 10 = 10% solution of clam metabolites in aged, filtered seawater; Prey 100 = full-strength solution of clam metabolites. Hatched areas represent proportion of response due to DC lobsters. Clear areas represent proportion of response due to NB lobsters. Numbers above bars represent numbers of lobsters tested followed by (1) level of significance of log-likelihood test for stimulus vs. control and (2) level of significance of log-likelihood test for prey fed response vs. brine shrimp-fed response to same stimulus at same feeding trial week.

Concentration of Soluble Nitrogenous Substances in Prey Metabolites

Soluble protein and amines were undetectable in either prey metabolite preparation by the analytical methods used. Sensitivity of assays was 50 μg/ml (bovine serum albumin equivalents) for protein, 40 μM (DL-alanine equivalents) for primary amines, and 1 μM for ammonia. Ammonia concentrations of prey metabolite preparations were similar, ranging from 39 to 61 μM ($X \pm$ SE, 55 $\pm$ 8.5 μM, N = 6) for amphipod metabolites and from 46 to 80 μM ($X \pm$ SE,

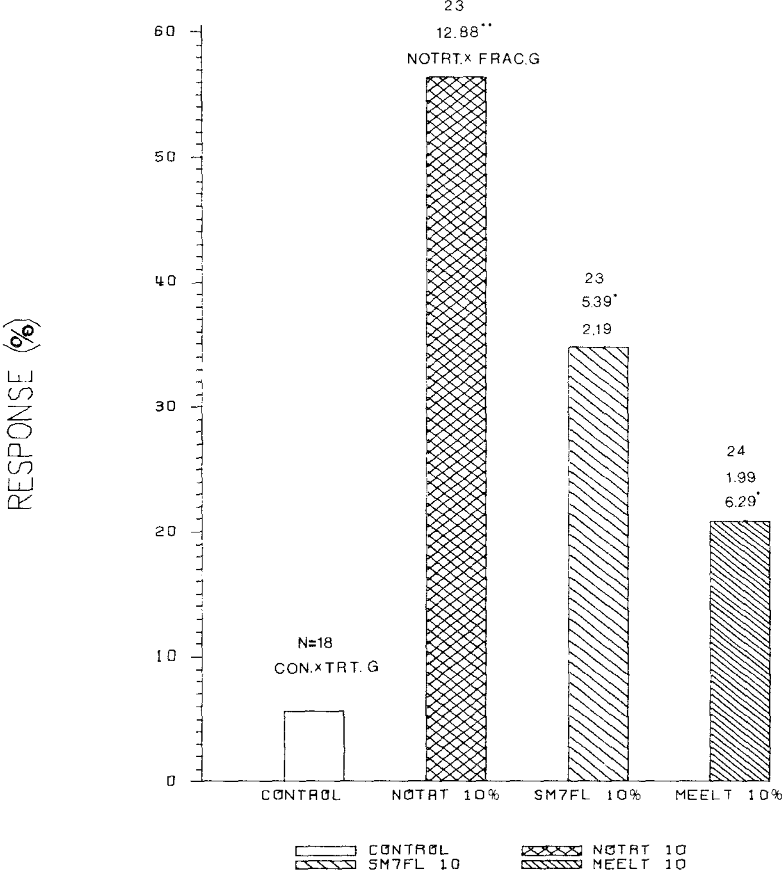

FIG. 3. Prey-search response (%) of amphipod-fed lobsters to control (aged, filtered seawater), unfractionated amphipod metabolites diluted to 10% with aged, filtered seawater, Sm7 filtrate, and Me (methanol) eluent of amphipod metabolites diluted to same volume as 10% unfractionated amphipod metabolites. Numbers above bars represent numbers of lobsters tested followed by (1) G statistic for log-likelihood test of control vs. amphipod metabolites and (2) G statistic for log-likelihood test of unfractionated vs. fractionated metabolites. $^{**}P < 0.01$, $^{*}P < 0.05$.

64 ± 8.7 μM, $N = 8$) for clam metabolites. Levels of ammonia in aged filtered seawater were undetectable.

Condition of Lobsters on Feeding Regimes

Lobsters dragged clams into shelters and broke away shell lip using chelae and maxillipeds. Lobsters under 90 mg appeared to be generally inept at open-

ing even the smallest clams while larger lobsters could open clams within a wide size range. Lobsters swam after amphipods, lunged at nearby amphipods, or tried to capture amphipods after blundering into them. Chelae were used to capture amphipods and maxillipeds were then used to improve grasp. However, even with a firm hold, amphipods were often able to squirm away. The smallest lobsters, which were unable to open clams, were capable of catching and ingesting amphipods. The most surprising observation was the disappearance of 10 of the amphipod-fed lobsters during the first week of the feeding trial. A few appendages and a recent molt shell of a lobster were often found within a large group of amphipods. Only a few molting lobsters did not disappear, leading to the conclusion that amphipods were capable of feeding on the lobsters at this vulnerable stage. By reducing prey numbers to three per day, disappearance of lobsters ceased.

Some marked color difference developed with feeding. Frozen brine shrimp-fed lobsters tended to be pale pink to blue–green, amphipod-fed lobsters were dark red, while clam-fed lobsters were pale blue to blue–green.

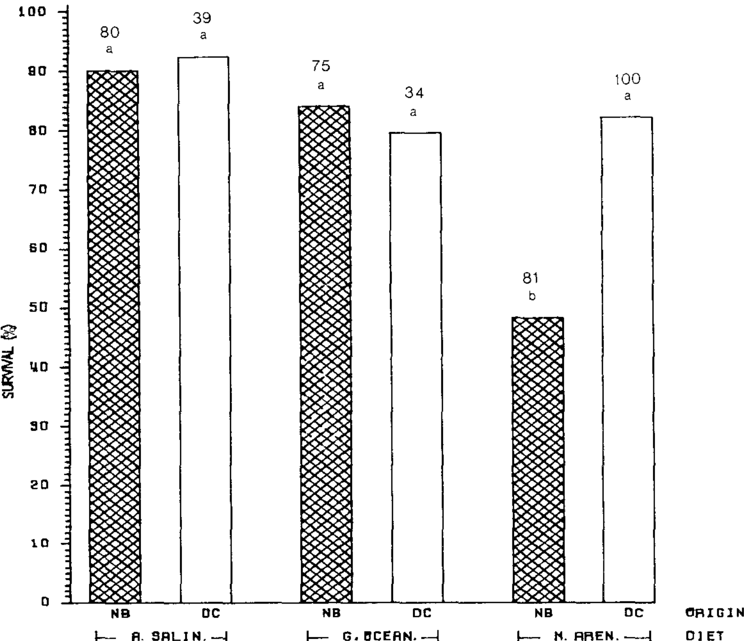

FIG. 4. Survival of *A. salina*-fed, *G. oceanicus*-fed, and *M. arenaria*-fed lobsters. NB = lobsters of New Brunswick origin. DC = lobsters of Darling Center origin. Survival is for a 35-day period with the exception of clam-fed NB lobsters which were maintained for 17 days. Numbers above bars represent numbers of animals at feeding trial initiation. Same letters above bars represent similar survival where G_h has $P > 0.05$. G test for heterogeneity ($df = 5$): all six groups = 50.9, $P < 0.005$.

Feeding rates showed that lobsters were able to capture successfully and ingest live prey over a five-week period. Survival and weight gain were similar for live prey-fed lobsters and frozen brine shrimp-fed lobsters, with the exception of clam-fed lobsters from New Brunswick which showed only 48% survival after 17 days (Figure 4). The low survival of the clam-fed NB lobsters coincided with an approximately 80% decrease in number of prey ingested/(lobster × day) after 16 days (Figure 5). In contrast, after five weeks, clam-fed lobsters

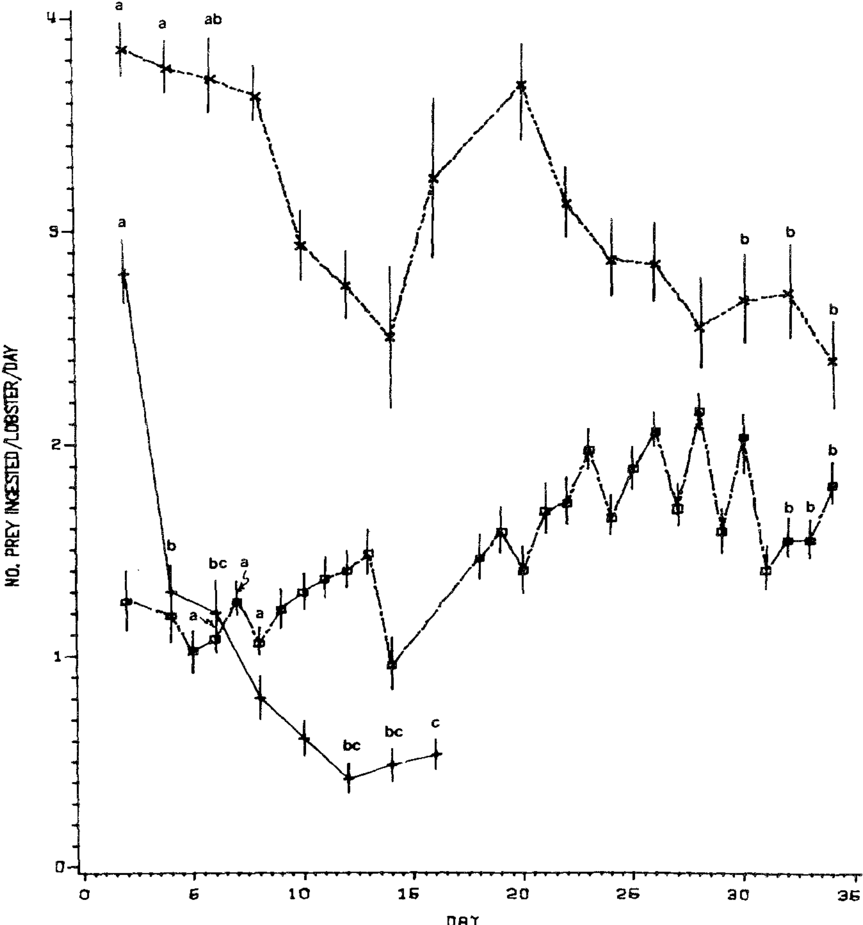

FIG. 5. Feeding rates (number of prey ingested/(lobster × day) over duration of feeding trials ($X \pm$ SE). Clam-fed NB lobsters = +, ——, N = 77–58; clam-fed DC lobsters = ×, ------, N = 100–82; amphipod-fed lobsters = □, — · — · —, N = 105–90. G tests for heterogeneity was performed on frequency distribution of first three and last three observations for each feeding group. Same letters above represent similar frequency distribution, where G_h has $P > 0.05$.

from the Darling Center showed a smaller decrease of approximately 30% and amphipod-fed lobsters appeared to increase prey ingestion rates by about 25%.

These observations were tested statistically by comparing the frequency distributions of number of prey ingested per lobster for the first three observations to that of the last three observations for each prey-fed group. After 12 days, a marked shift to lower numbers of clams ingested by NB lobsters was apparent, with no lobster consuming more than six clams in a two-day period (Figure 5, overall $G_h = 233.9$, $df = 50$, $P < 0.005$). This is a likely explanation for the high mortality observed in this group. These lobsters were smaller (0.063 ± 0.0010 g (SE), $N = 50$) than DC lobsters (0.13 ± 0.018 g (SE), $N = 35$) in the same feeding group and were placed with larger DC lobsters (0.24 ± 0.015 g (SE), $N = 65$). Still, DC lobsters showed a significant, although not as dramatic, shift toward reduced feeding rates by the end of the experiment (Figure 5, overall $G_h = 132.9$, $df = 50$, $P < 0.005$).

Since DC and NB lobsters fed amphipods showed similar ingestion rates, these groups were pooled. The first three observations could not be used for comparison with final observations since lobsters were fed initially higher numbers of amphipods. At day 6, most lobsters ingested only one amphipod per day; but, by 34 days, a majority was eating three amphipods per day, a significant increase in feeding rate (Figure 5, overall $G_h = 132.8$, $df = 50$, $P < 0.005$).

Amphipod-fed lobsters increased weight by 100% after five weeks, compared with a 72% increase for clam-fed lobsters and only an 18% increase for frozen brine shrimp-fed lobsters (Figure 6). Weight increases in amphipod-fed and clam-fed lobsters were significant, but not for lobsters fed brine shrimp. Final weights of amphipod-fed lobsters were significantly greater than final weights of brine shrimp-fed lobsters. Comparison with weights of clam-fed lobsters was inappropriate, due to the significantly higher initial weights of lobsters in that group.

DISCUSSION

These results show the development, through feeding experience, of prey-search behavior toward metabolites of live amphipods in previously naive postlarval lobsters. The results support the hypothesis that ingestive conditioning explains the discrepancies between chemosensory responses of adult, field-collected and laboratory-reared lobsters to some prey metabolites. This was demonstrated in two ways: response of amphipod-fed lobsters toward amphipod metabolites increased with feeding experience relative to response to filtered seawater and standard attractant and relative to response of brine shrimp-fed lobsters towards amphipod metabolites. Chemical fractionation of amphipod metabolites indicated that attractants were confined to the same fraction as for

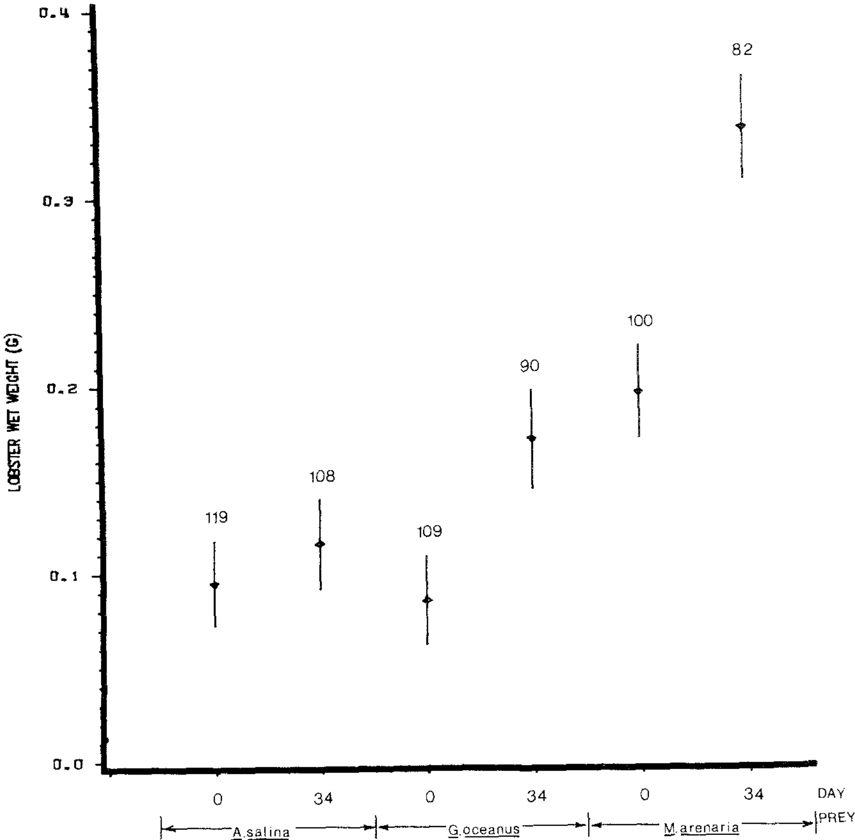

FIG. 6. Mean lobster wet weights and confidence intervals at feeding trial initiation and at termination. Numbers above bars represent numbers of animals weighed. ANOVA, $N = 608$, $F = 56.24$, $P < 0.0001$; 95% confidence intervals determined by Gabriel's multile comparison of means based on unequal sample sizes when mean square error = 0.01396, Student's maximum modulus (M) = 2.939.

food extracts, i.e., polar, low-molecular-weight compounds (Daniel and Bayer, 1987a, b). Development of response appeared to be specific for amphipod metabolites. An increase in response was not observed toward clam metabolites by amphipod-fed, clam-fed, or brine shrimp-fed groups. Responses of predatorily naive lobsters to clam and amphipod metabolites were low and inconsistent, similar to the low responses observed towards metabolites of sea stars (*Asterias vulgaris*), rock crabs (*Cancer irroratus*), soft clams (*Mya arenaria*), and blue mussels (*Mytilus edulis*) (Daniel and Bayer, 1987). Responses of lobsters which previously fed on a wide variety of prey before initiation of the

experiment (DC lobsters) appeared to have an initial response to amphipod metabolites which was lost after five weeks of feeding on frozen brine shrimp, although numbers tested were too small to be conclusive. Similar memory loss has been shown for terrestrial snails (Croll and Chase, 1977).

A confounding result was the inability of postlarval lobsters to develop responses towards clam metabolites with feeding experience. Chemical cues from other bivalve species elicit predatory behavior in older individuals; however, responses appear to be lower than towards decapod prey. Derby and Atema (1981) were able to show responses towards metabolites of two species of mussels (*Modiolus modiolus* and *Mytilus edulis*) with feeding experience. Hirtle and Mann (1978) found responses to *M. edulis* metabolites, while McLeese (1970) and Carter and Steel (1982) did not observe a significant response to *M. arenaria* metabolites. Responses to crab metabolites (*Carcinus maenus* and *Cancer irroratus*) were stronger than responses than responses to bivalve metabolites (*M. edulis*) (Hirtle and Mann, 1978; Carter and Steele, 1982).

Thus, a partial explanation of our inability to observe improved responses to clam metabolites might be that metabolites from live crustaceans are more effective attractants for lobsters than are bivalve metabolites. The difference in effectiveness of the metabolites could be due to differences in the quantity and/or quality of their components. Although the techniques used in the present study were not sufficiently sensitive to determine any such differences, this hypothesis is supported by Stephens' (1972) observations of greater excretion rates (particularly amino acids) in crustaceans relative to bivalves. However, it is clear that older lobsters can elevate their sensitivities towards bivalves with continued feeding (Derby and Atema, 1981). Differences between our results and those of Derby and Atema (1981) may be a result of the age of the lobsters, the sensitivities of behavioral assays (they counted number of times different behaviors were displayed rather than our "all-or-none" approach), or simply that lobsters are more sensitive to metabolites from mussels than from clams.

The difference in ingestive conditioning results from clam-fed and amphipod-fed lobsters might be due to differences in the genetic predisposition of lobsters to learn different chemicals. The evolution of these predispositions might be related to the food quality of prey. A central hypothesis of optimal foraging theory is that predators tend to choose prey which maximize rate of food intake while foraging (Krebs, 1978). Prey type chosen may be a function of density, size, palatability, conspicuousness, or nutrient quality. Predators may maximize intake of a preferred prey species by increasing efficiency of prey search or handling through learning (Morgan, 1972; Edwards, 1975; Lawton et al., 1974; Cunningham and Hughes, 1984; Dunkin and Hughes, 1984). For example, this might be achieved through reinforcement of a "chemical search image" which acts to focus prey-search behavior toward a particular prey (Atema et al., 1980). Animals may reinforce chemical search images

through ingestive conditioning as defined by Wood (1968). The net outcome is that feeding experience with high-quality foods tends to reinforce inherent or genetically predispositioned search images, resulting in improved detection and ultimately increased intake.

Lobsters fed amphipods showed equivalent survival, slightly greater weight gain, increased prey-ingestion rates, and more natural color than clam-fed lobsters. While both groups showed positive weight gain, amphipod-fed lobsters had an increase in prey ingestion, while clam-fed lobster ingestion rates actually decreased. Furthermore, amphipod-fed lobsters had coloration considered indicative of good health, in contrast to clam-fed lobsters. Blue color is a result of lack of suitable carotenoid pigments in diet and can be improved through supplementation of diet with crustacean tissue (Hughes and Matthiesson, 1962; D'Agostino, 1980; Good et al., 1982; D'Abramo et al., 1983). Addition of carotenoids to a formulated diet did not result in an increase in growth in *H. americanus*, but did produce coloration similar to wild lobsters (D'Abramo et al., 1983). However, greater survival was achieved with live *Artemia salina*, which, due in part to high levels of carotenoids, D'Abramo et al. (1983) argue may constitute a high-quality diet. Search image formation in lobsters may be biased genetically to those prey of higher nutritional value such as amphipods. This might result in an increase in prey-ingestion rates as predicted by optimal foraging theory. In contrast, lobsters may not be able to learn as readily, through conditioning, the chemical identity of prey of low dietary value, such as clams, resulting in low ingestion rates.

The hypothesis of a reinforcement in search image with feeding experience on a high-quality food implies a selective improvement in response toward the prey species making up the diet as demonstrated for older *H. americanus* by Derby and Atema (1981). This is in contrast to a nonselective improvement in prey-search response in which ingestion of one prey species leads to a general increase in response to a wide variety of prey species metabolites. Given the consistently subthreshold responses of postlarval lobsters to clam metabolites observed in this study, it is difficult to differentiate between selective and nonselective improvements in response to prey metabolites. Clam metabolites may be qualitatively or quantitatively less potent to preconditioned lobsters than amphipod metabolites, such that the conditioned change was still insufficient to produce an observable suprathreshold response. However, two observations support the involvement of selective improvement in some cases: the large magnitude of the increase in response observed toward amphipod metabolites after conditioning, and the lack of response to amphipod metabolites for lobsters fed clams. A convincing demonstration of one of these hypotheses would require preparation of a more potent clam metabolite solution, perhaps through concentration, or use of an alternate prey as food in which suprathreshold concentrations could be produced in preconditioned or conditioned lobsters.

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BLACKHEADED FIREWORM[1]: Laboratory and Field Studies of its Sex Pheromone

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Abstract—Electroantennogram (EAG) responses of male *Rhopobota naevana* (Hübner), the blackheaded fireworm, to all of the monoene straight-chain 12- and 14-carbon alcohols and acetates implicated (*Z*)-11-tetradecen-1-ol (*Z*11–14:OH) and its acetate (*Z*11–14:Ac) as sex pheromone components. *Z*11–14:Ac produced the strongest EAG response of all compounds tested. Gas chromatography-mass spectrometry (GC-MS) analysis of extract of female sex pheromone glands (SPG) confirmed the presence of *Z*11–14:OH (125 pg/female) and *Z*11–14:Ac (600 pg/female) (all other monoenes had different retention times). In field tests, traps baited with *Z*11–14:OH alone captured males, but traps baited with *Z*11–14:Ac alone did not. Traps baited with a combination of *Z*11–14:OH and *Z*11–14:Ac in various ratios did not produce better trap catches than *Z*11–14:OH alone. (*Z*)-9-Dodecen-1-ol acetate (*Z*9–12:Ac), reported by others to be a field attractant, did not produce trap catch in our tests, but in combination with *Z*11–14:OH (98:2 in septa corresponding to 95:5 in vapor, *Z*11–14:OH to *Z*9–12:AC) produced a sevenfold increase in catch over *Z*11–14:OH alone. If *Z*9–12:AC had been present in extract of SPG at 2–5% of *Z*11–14:OH, it would not have been detected in our GC-MS experiment.

Key Words—*Rhopobota naevana* (Hübner), *Rhopobota unipunctana* Haworth, *Rhopobota naevana naevana* (Hübner), blackheaded fireworm, Lepidoptera, Tortricidae, Olethreutinae, sex pheromone, sex attractant, (*Z*)-11-tetradecen-1-ol, (*Z*)-11-tetradecen-1-ol acetate, (*Z*)-9-dodecen-1-ol acetate.

[1]*Rhopobota naevana* (Hübner) Lepidoptera: Tortricidae: Olethreutinae.

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INTRODUCTION

The blackheaded fireworm, *Rhopobota naevana* (Hübner) (Brown, 1983), formerly *R. unipunctana* Haworth (Brown, 1980; Werner, 1982) and prior to that *R. naevana naevana* (Hübner) (Sutherland, 1978), is a worldwide pest. Throughout the United States it is a major pest of cranberry (Franklin, 1948). It is also noted as a pest of huckleberry (Breakey, 1960) and commercially grown holly (Swenson, 1958). In Europe and Asia, besides cranberry, it is a pest of cowberry and bird cherry (Shutova et al., 1981; Tibatina, 1976) and of fruit trees including apple, pear, plum, and cherry (Ivinskis and Pakal'nishkis, 1983).

No previous chemical studies of the structure of its sex pheromone have been reported, but in field screening studies, Ando et al. (1977) reported that *R naevana* males were caught in traps baited with (Z)-9-dodecen-1-ol acetate (Z9-12:Ac). We undertook a laboratory and field study of the structure of sex pheromone components in order to develop a lure which could eventually be used for population monitoring in control programs.

METHODS AND MATERIALS

Insects. Blackheaded fireworm larvae, in their protective leaf-ties, were collected from an abandoned cranberry bog located near Long Beach, Washington. Foliage was clipped a few centimeters below the leaf-ties which contained the larvae, placed in clear plastic shoe boxes, covered with plastic screen, transported to the laboratory, and then placed in a rearing room at 21°C, 55-60% relative humidity, and a 14:10 hr light-dark cycle. Periodically, the ties were examined for pupae which, upon formation, were transferred individually to 6 × 1½ cm corked, glass vials containing a strip of soft tissue paper to cushion the pupae. After adult emergence, each moth was sexed and females were held in small cages (25 × 20 × 30 cm). Adults were provided water via a vial containing a dental wick.

Collection of Pheromone. Female moths (2-5 days old) were collected 2 hr after the beginning of scotophase and placed in a refrigerator for 10 min to inactivate them. Abdominal tips were severed and steeped for 15 min in dichloromethane. Then the solution was removed with a syringe and stored at −20°C until used.

Electroantennogram (EAG). The technique was the same as previously described except that 60-μg charges were used (McDonough et al., 1980). Values for blanks were subtracted from values for test compounds, which were expressed as a percentage of a reference compound value.

Model Compounds Used in EAG and GC-MS Studies. All of the *n*-alkenyl alcohols and acetates possessing 12 and 14 continuous carbon atoms in the chain were prepared and purified by established synthetic methodology (Voerman,

1979). All compounds were at least 98% pure by gas chromatographic analysis and contained 1% or less of the geometrical isomer.

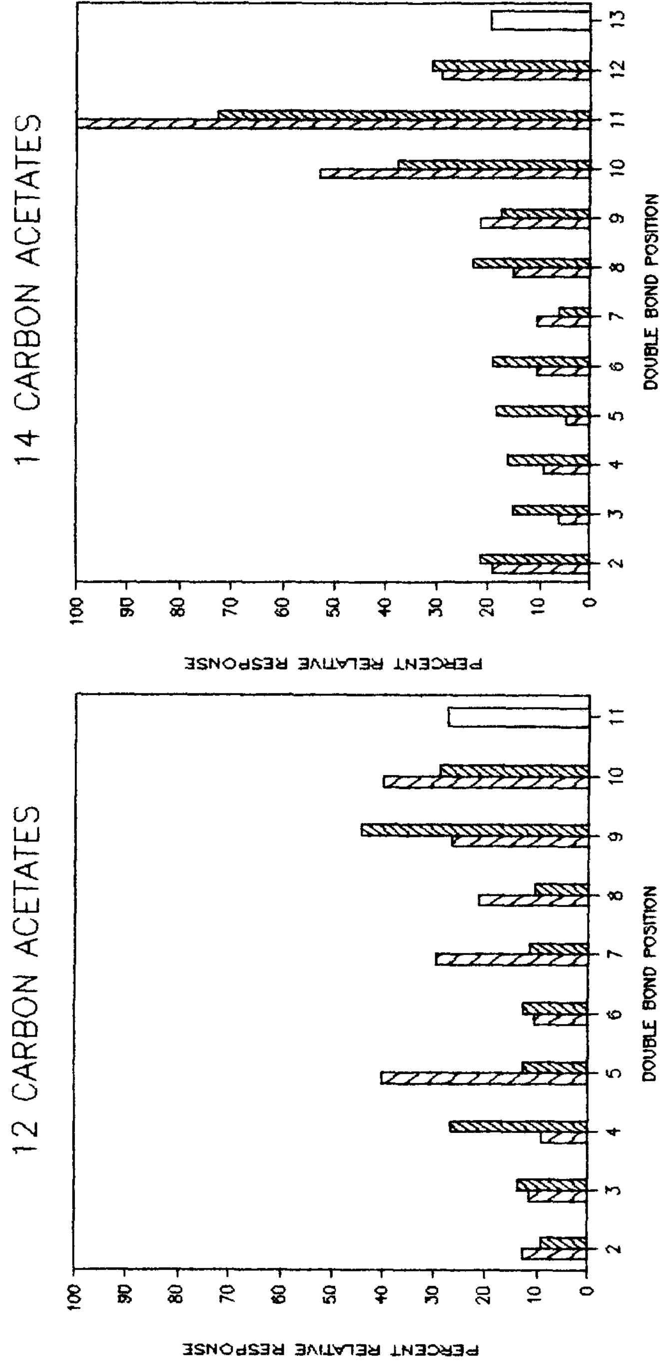
Gas Chromatograph–Mass Spectrometry (GC-MS). The following instrument was used to characterize model compounds and the components of the extract of female *R. naevana* sex pheromone glands: Hewlett-Packard, Avondale, Pennsylvania, model 5970 quadrupole mass selective detector with computerized data collection and a model 5790 gas chromatograph inlet equipped with fused silica capillary column (60 m × 0.25 mm ID) with splitless injection. The liquid phase was methyl silicone (DB-1).

Field Studies. Compounds used as candidate lures in field tests were dissolved in 100 μl of dichloromethane and impregnated into red rubber septa (size No. 1, West Co., Phoenixville, Pennsylvania). Septa used as controls contained only dichloromethane. A septum was placed on the bottom in the center of a Pherocon IC trap (Trece Corp., Palo Alto, California). In place of the usual 5-cm spacer between trap top and bottom, a 3.5-cm spacer was made by cutting the 5-cm spacer. Then the distance between top and bottom was about 0.7 cm at the widest points (the corners of the top and bottom touched). When the 5-cm spacer was used, large captures in blank traps were frequently obtained. The trap was suspended from a movable metal arm (an 18 × 23-cm shelf bracket) attached by a metal hose clamp to a 120-cm long wooden broom handle driven into the ground. This arrangement allowed the trap to be positioned just above the foliage where maximum flight activity was thought to occur. The traps were placed randomly throughout the bogs at approximately 16.5-m intervals. The trap data were transformed into $\sqrt{X + 0.5}$ and compared by Duncan's multiple-range test (DMRT) ($P = 0.05$). The compounds tested and their half-lives in rubber septa at 22°C were: *Z*11–14:Ac, 310 days; *Z*11–14:OH, 117 days; and *Z*9–12:Ac, 44.8 days (Butler and McDonough, 1979, 1981). These data were used to calculate dosages in the septa so that systematic changes of ratios in the vapor were obtained.

RESULTS

EAG Profile. A graph of male antennal responses of all of the monounsaturated 12- and 14-carbon alcohols and acetates is given in Figure 1. The strongest response to the tested compounds was obtained from *Z*11–14:Ac which produced a 5.8-mV response in the best antennal preparation. More typical values for *Z*11–14:Ac were about 3 mV. All the other values are expressed as a percentage of the value of this compound. Besides *Z*11–14:Ac, the compound which is the most obvious candidate for a pheromone component is *Z*11–14:OH. *E*9- and *Z*10–12:Ac and *Z*9–12:OH also produced fairly significant responses.

GC-MS. An extract of 20 female abdominal tips were analyzed in the single-ion monitor mode (SIM). The ions monitored were those characteristic of dodecenyl and tetradecenyl alcohols and acetates: 61, 166, 168, 194, 196. The



14 CARBON ACETATES
PERCENT RELATIVE RESPONSE
100
90
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9
10
11
12
13
DOUBLE BOND POSITION
12 CARBON ACETATES
PERCENT RELATIVE RESPONSE
100
90
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9
10
11
DOUBLE BOND POSITION

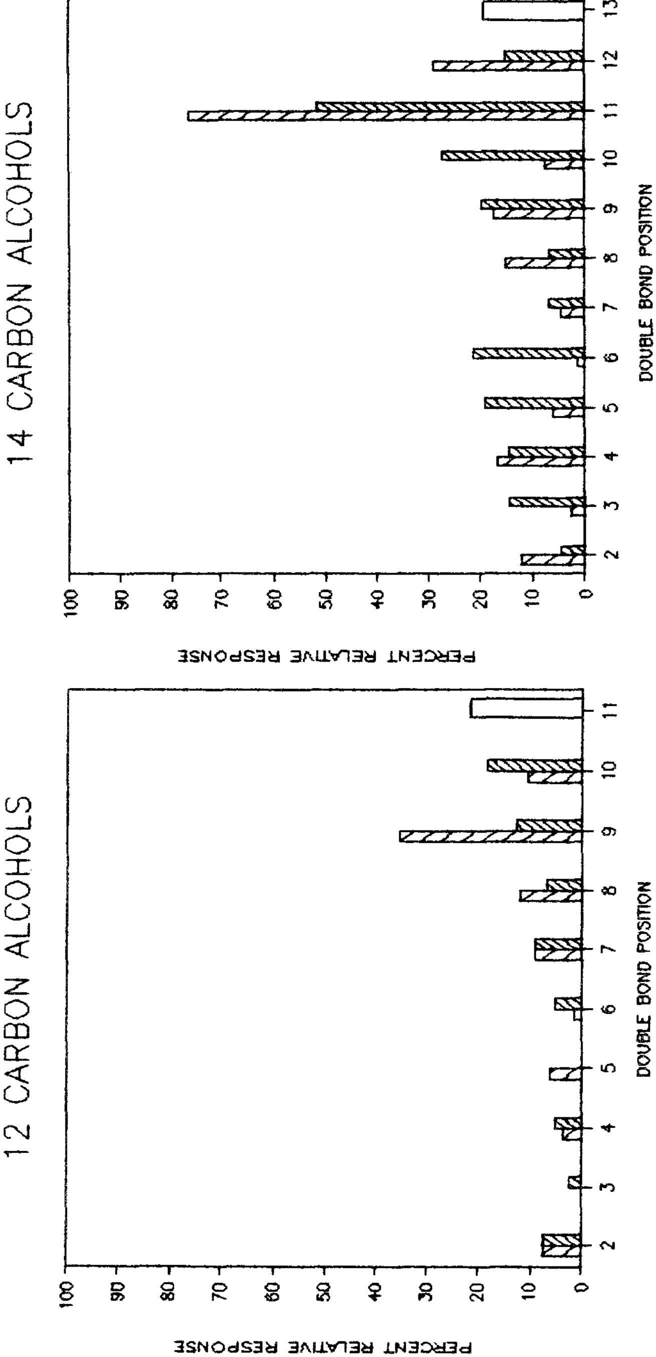


FIG. 1. Electroantennogram responses of male blackheaded fireworm (*Rhopobota naevana*) to model compounds. *Z* isomers are listed before *E*.

analysis showed the presence of Z11–14:Ac (600 pg/female) based on m/z = 194 (M^+-60), 166 (M^+-60-28), and 61 ($CH_3CO_2H_2$) and a distinctive retention time (t_r) of 23.32 min. During the standard runs, the nearest tetradecenyl acetates were E11–14:Ac with t_r = 23.04 and E12–14:Ac with t_r = 23.53. Retention times were reproducible within 0.04 min. Also 14:Ac was present (500 pg/female) based on m/z = 196, 168, and 61 and t_r = 23.42 min, identical to the standard. Z11–14:OH (125 pg/female) was also present based on m/z = 194 (M^+-18) and 166 (M^+-18-28) and a distinctive t_r = 18.20 min. The t_r of the closest standards were E11–14:OH, 17.98 min, and Z12–14:OH, 18.32 min. No evidence was found for 12-carbon alcohols or acetates. The sensitivity of this analysis was about 1 ng, so that if 12-carbon alcohols or acetates were present at levels below about 40% of Z11–14:OH, they would not have been detected.

Field Tests. Table 1 shows the 1983 field tests of Z11–14:OH and Z11–14:Ac. At a 1-mg dosage, Z11–14:OH evoked trap catch while Z11–14:Ac did not. Mixtures of these two compounds did not produce trap catch better than Z11–14:OH alone. In treatments 4, 3, and 2, trap catch decreased as the dosage of Z11–14:OH decreased. In a 1984 test, Z9–12:Ac (reported by Ando et al., 1977, to be a field attractant for *R. naevana*) was compared with Z11–14:OH and combinations of the two (Table 2). Z9–12:Ac alone (treatment 9) did not produce trap catch better than the control (treatment 10), while Z11–14:OH did (treatment 1). However, the best combinations were substantially superior to Z11–14:OH alone. The 95:5 ratio in the vapor of Z11–14:OH to Z9–12:Ac produced a 7.2-fold greater catch than Z11–14:OH alone. A plot of the data of tests 9–2 gave an excellent straight line: $n = 0.1517\,p + 1.138$ ($R^2 = 0.974$) where n = number or *R. naevana* caught per trap-day and p = percent of Z11–14:OH in the vapor.

TABLE 1. CAPTURES OF ADULT MALE *R. naevana* IN TRAPS BAITED WITH Z11–14:Ac AND Z11–14:OH IN RUBBER SEPTA, AUGUST 8–15, 1983[a]

Treatment No.	Ratio of Z11–14:Ac to Z11–14:OH: In septum	In vapor	Catch/trap-day and significance
1	100:0	100:0	0.86cd
2	96:4	90:10	1.8bc
3	73:27	50:50	2.9ab
4	23:77	10:90	4.1a
5	0:100	0:100	4.2a
6 (5 mg)	0:100	0:100	4.4a
7	0:0	0:0	0.43d

[a]Total dosage was 1.0 mg/septum except where indicated and there were 3 replicates.

TABLE 2. CAPTURES OF ADULT *R. naevana* IN TRAPS BAITED WITH Z9-12:AC AND Z11-14:OH IN RUBBER SEPTA, AUGUST 22 TO SEPTEMBER 11, 1984[a]

Treatment No.	Ratio of Z11-14:OH to Z9-12:Ac		Catch/trap-day and significance
	In septum	In vapor	
1	100:0	100:0	2.3c
2	98:2	95:5	16.6a
3	93.7:6.3	85:15	12.8a
4	85.9:14.1	70:30	10.8b
5	72.3:27.7	50:50	9.6b
6	52.8:47.2	30:70	6.4b
7	31.5:68.5	15:85	3.9c
8	12.1:87.9	5:95	1.8cd
9	0:100	0:100	0.3d
10	0:0	0:0	0.3d

[a]Total dosage was 2.0 mg/septum and there were 4 replicates.

DISCUSSION

Based on the EAG and the GC-MS studies, one would expect Z11-14:Ac to be the major sex pheromone component, that it would be required for male upwind flight, and that Z11-14:OH might be either an obligatory or synergistic component of the sex pheromone. The finding that Z11-14:Ac alone did not produce trap catch, while Z11-14:OH is a weak but significant attractant, and blends of Z11-14:OH and Z11-14:Ac were not superior to Z11-14:OH alone was surprising. The failure of Z11-14:Ac to affect trap catch in our tests does not mean it is not a sex pheromone component, but does imply that it has little effect on upwind flight. It might mediate some other aspect of male behavior.

During the summer of 1985, we became aware that Slessor et al. (1987) had also studied the sex pheromone of the blackheaded fireworm. The lure they developed used three compounds (Z11-14:Ac, Z11-14:OH, and Z9-12:Ac). It is difficult to compare our field study with theirs because the two studies used such different protocols. The dosage levels Slessor et al. (1987) used were much lower than ours. Male behavior toward different ratios of pheromone components can change substantially with dosage level (Roelofs, 1978). Clearly more extensive field tests are needed before the optimum lure can be defined with confidence.

Although the lure we developed (Z11-14:OH + Z9-12:Ac, 98:2; total dosage = 2 mg) may not be the optimum, it appears to be reasonably potent. This lure has been effective in the development of a monitoring program for cranberries in Massachusetts. A total of 2879 male *R. naevana* were captured

in 58 traps which had been placed in 58 bogs during an eight-week period in 1985. In nine of the bogs where only one male was captured, there was no subsequent evidence of infestation (Sherri L. Roberts, University of Massachusetts, personal communication).

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IMPLICATIONS OF SOLUBLE TANNIN-PROTEIN COMPLEXES FOR TANNIN ANALYSIS AND PLANT DEFENSE MECHANISMS

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Abstract—Factors which establish whether tannin and protein interact to form soluble complexes or precipitates were identified. The ratio of tannin to protein in the reaction mixture influenced solubility of the tannin-protein complexes. At protein-to-tannin ratios larger than the optimum ratio, or equivalence point, soluble tannin-protein complexes apparently formed instead of insoluble complexes. Several other factors influenced the amount of protein precipitated by tannin-containing plant extracts, including the length of the reaction time and the conditions of the tannin extraction. The analytical and ecological significances of soluble complexes were considered. A titration method which allows simultaneous determination of the equivalence point and assessment of the protein-precipitating capacity of any plant extract was developed. It was postulated that in vivo, tannin and protein may not only form insoluble complexes with antinutritional effects, but may also form soluble complexes which have unknown metabolic effects.

Key Words—Tannin, soluble tannin-protein complexes, digestibility-reducing substances, protein precipitation, phenolic anlysis.

INTRODUCTION

Tannin, a constituent of the leaves and stems of many plants, may defend plants from herbivores. One possible defensive function of tannin is precipitation of protein in the herbivore's digestive tract. Precipitated protein would presumably be less digestible than soluble protein, and the herbivore would thus obtain

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inadequate dietary protein from tannin-rich plants (Fenny,1976; Rhoades and Cates, 1976). In vitro, tannin precipitates proteins from aqueous solution at moderate pH (Hagerman and Butler, 1980a; Hagerman and Klucher, 1986; McManus et al., 1981; Goldstein and Swain, 1965), and thus tannin might be expected to form precipitates in the guts of herbivores. The ability of tannin-containing plant extracts to precipitate protein is a good predictor of protein digestibility for several large mammals (Robbins et al., 1987). Some ecological studies support the hypothesis that precipitation of proteins by tannin is important in plant defense (Schultz and Baldwin, 1982; Walters and Stafford, 1984).

However, there are several reasons to think that the potential of tannin to precipitate protein is not nutritionally significant to certain herbivores. For example, the digestive tract fluids of some insects contain surfactants and are maintained at high pH, factors which inhibit precipitation of proteins by tannin (Martin and Martin, 1984; Martin et al., 1985). Some mammals also have mechanisms for preventing detrimental protein precipitation by tannins, such as the ability to secrete tannin-binding proteins (Mehansho et al., 1983). Under some conditions, tannin increases the susceptibility of proteins to proteolytic attack (Mole and Waterman, 1985). These types of evidence have stimulated a few authors to argue that tannin is a toxic defensive compound which is absorbed rather than an antinutritional compound (Zucker, 1983; Martin and Martin, 1984).

Resolution of the controversy over the defensive role of tannin calls for more comprehensive correlative studies of the chemical characteristics of tannin and the fitness of tannin-containing plants. One chemical characteristic of tannin-containing plants which is frequently measured is the protein-precipitating capacity, expressed as the amount of protein precipitated per milligram of plant tissue. Several assays are avilable for determining the protein precipitating capacity of tannin. In the radiolabeled protein-precipitation method, the precipitation of iodine-125-labeled bovine serum albumin (BSA) is measured radiochemically (Hagerman and Butler, 1980a). In the method of Martin and Martin (1982), the amount of unprecipitated BSA is measured colorimetrically. In the dye-labeled protein precipitation method the precipitation of blue dye-labeled BSA is determined colorimetrically (Asquith and Butler, 1985). BSA is used as the test protein in all three of these methods, but the methods do not give comparable results. In one recent study, the protein-precipitating capacity of a tannin-containing extract estimated with the dye-labeled protein-precipitation assay was three- to four-fold larger than the capacity as estimated with the Martin and Marin assay (Robbins et al., 1987). The differences between the assays make it impossible to directly compare experiments from labs which routinely use different tests. Futhermore, the dependence of the protein-precipitation capacity on the assay method makes it difficult to establish whether the precipitating capacity of the plant is correlated with defensive ability.

The purpose of this study was to compare these three assays and to identify the variables which affect the results obtained. It was found that the ratio of protein to tannin in the reaction mixture significantly affected the amount of protein precipitated. At ratios of protein to tannin larger than the optimum ratio, soluble tannin–protein complexes apparently formed instead of insoluble complexes. Based on the results of these studies, a recommended protocol for determining the protein-precipitating capacity of tannin-containing plant extracts was established. The tendency of tannin to form soluble complexes as well as insoluble complexes with protein supports the hypothesis that tannin may not act exclusively as a protein-precipitating agent in vivo.

METHODS AND MATERIALS

Tannin and Plant Extracts. Condensed tannin was purified from *Sorghum bicolor* grain (Hagerman and Butler, 1980b), and hydrolyzable tannin was purified from commercial tannic acid (Mallinckrodt, Inc., St. Louis, Missouri) (Hagerman and Klucher, 1986).

The plant extracts were prepared from air-dried forage samples of various high-tannin plants (Robbins et al., 1987). For most experiments, 1.5 g of the dry forage was extracted with 50 ml of 50% (v/v) aqueous methanol while sonicating for 15 min; the extract was separated from the tissue by vacuum filtration. The tissue was then extracted with 50 ml of 50% methanol at 90–95°C for 8 min. This extraction was repeated twice more with fresh 50-ml aliquots of 50% methanol. Each extraction was followed by vacuum filtration to separate the extract from the tissue. The four extracts were then combined to give a final extract volume of approximately 200 ml. Each milliliter of the extract was equivalent to 7.5 mg forage.

For a few experiments, smaller amounts of tissues were extracted following the same protocol. The four sequential extractions were applied to 100-mg samples of dry tissue, using 2.0 ml of 50% methanol for each of the four steps. The extracts were separated from the tissue by centrifugation, and the four combined to yield a final volume of eight ml. Each milliliter of the extract was equivalent to 12.5 mg forage.

Precipitation Assays. Similar procedures were used for all three of the precipitation assays. The sample volume was adjusted to a constant volume with 50% methanol in each experiment. For all experiments except the kinetics experiment, purified tannin samples were allowed to react with the protein for 15 min at room temperature and the crude plant extracts were allowed to react with the protein for 24 hr at 4°C before separating the precipitate from the supernatant. The precipitates were always separated from the supernatant by centrifugation for 15 min at 2000 *g*.

The colorimetric assay for protein-precipitable phenolics was performed as described by Hagerman and Butler (1978). The sample was added to 2.0 ml buffer containing 2 mg BSA or iodine-125-labeled BSA. After centrifugation, the tannin–protein precipitate was dissolved and the color was developed (Hagerman and Butler, 1978). The absorbance at 510 nm (A_{510}) was determined.

The radiolabeled protein-precipitation assay was performed as described by Hagerman and Butler (1980a). The sample was added to 2.0 ml buffer containing up to 12 mg iodine-125-labeled BSA. After centrifugation, the precipitate was counted in a gamma counter and the amount of protein precipitated was calculated from a standard curve relating counts per minute to milligrams BSA. If desired, the isolated precipitate could then be dissolved and the precipitated phenolic determined colorimetrically as described above.

The dye-labeled protein precipitation assay was performed as described by Asquith and Butler (1985). The sample was added to 4.0 ml buffer containing about 8 mg of the blue dye-labeled BSA. After separation of the precipitate from the supernatant, the precipitate was redissolved in 3.0 ml of sodium dodecyl sulfate–isopropanol–triethanolamine reagent (Asquith and Butler, 1985), and the absorbance at 590 nm (A_{590}) was determined. The protein concentration of the blue dye-labeled BSA was determined using the Lowry assay (Peterson, 1983). The A_{590} values were converted to milligrams protein with the calibration line obtained by diluting aliquots of the blue dye-labeled BSA solution to 3.0 ml with the sodium dodecyl sulfate–isopropanol–triethanolamine solution. Each new batch of blue dye-labeled BSA was restandardized; a typical calibration line had the equation $A_{590} = 0.292$ (mg BSA) $+ 0.035$.

The protein precipitation assay of Martin and Martin (1982), was performed essentially as described by the authors. The sample was added to 1.4 ml buffer containing 1.47 mg of BSA. After separating the supernatant and precipitate, the supernatant was applied to a gel filtration column. Either commercially prepared columns (Pharmacia PD-10 columns; Pharmacia Inc., Piscataway, New Jersey), or equivalent columns made from 10-ml disposable pipets packed with Bio-Gel P-6DG (Bio-Rad Laboratories, Richmond, California) were used; columns could not be reused. The unprecipitated protein was separated from unprecipitated tannin on the column; all the protein was eluted in the first 6 ml of eluent. The protein in the eluate was determined with a dye-binding assay (Bradford, 1976), and the amount of protein precipitated was calculated by difference. If desired, the protein-precipitable phenolics in the precipitate could be determined colorimetrically as described above.

The competitive binding assay for determining the affinity of the dye-labeled protein for tannin was performed as described by Hagerman and Butler (1981). Various amounts of blue dye-labeled BSA were mixed with the radiolabeled protein, and purified tannic acid was then added. The amount of iodine-125-labeled protein precipitated was determined by counting the precipitate with a gamma counter.

RESULTS

The formation of tannin–protein precipitates is time- and temperature-dependent. The crude extracts from tannin-containing plants formed more precipitate after 24 hr of reaction with protein at 4°C than after only 15 min of reaction at room temperature (Figure 1). More precipitate formed after 24 hr of reaction at 4°C than after 24 hr of reaction at room temperature (data not shown). The difference in precipitation at the two times depended on the tannin source; the extract of alder formed 79% more precipitate after 24 hr than after 15 min, while the extract of fireweed flower increased only 20% between 15 min and 24 hr (Figure 1). For all extracts, the precipitation reaction was complete after 24 hr at 4°C.

The data shown in Figure 1 are from an experiment in which protein precipitable phenolics were determined after the reaction between protein and tannin. Similar data were obtained if, instead of measuring precipitated phenolics, precipitated protein was measured using either the radiolabeled BSA protein-

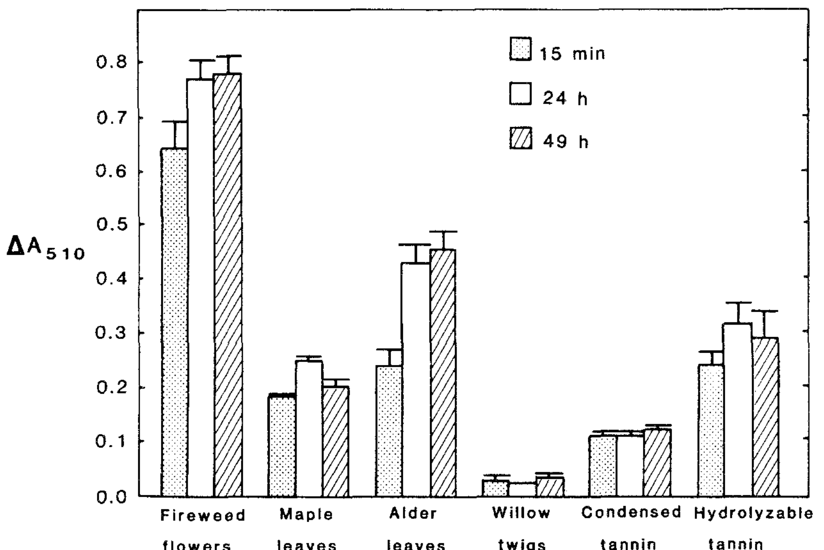


FIG. 1. Dependence of formation of tannin–protein precipitates on time. A 0.25-ml aliquot of the tannin-containing solution was mixed with 2.0 ml buffer containing 6.77 mg ^{125}I-labeled BSA. The mixtures were allowed to incubate at room temperature for 15 min (▨) or at 4°C for 24 hr (□) or 49 hr (▨). The mixtures were then centrifuged and the amount of precipitated tannin was determined colorimetrically as described in the text. The plant extracts were prepared from fireweed flowers (*Epilobium anguistfolium*), maple leaves (*Acer spicatum*), alder leaves (*Alnus rubra*), or great willow twigs (*Salix* spp.). Purified condensed and hydrolyzable tannins were prepared as described in the text.

precipitation assay, the dye-labeled protein-precipitation assay, or the Martin and Martin precipitation assay.

The precipitation of purified condensed tannin by protein was complete after 15 min, and longer reaction time or lower reaction temperature did not increase the amount of precipitate formed (Figure 1). This result is similar to the results reported in the literature, in which a 15-min reaction at room temperature was always found to be adequate for complete precipitation of protein by condensed tannin (Hagerman and Butler, 1978, 1980a; Martin and Martin, 1982; Asquith and Butler, 1985). The precipitation of purified hydrolyzable tannin by protein was significantly greater after 24 hr of reaction at 4°C than after 15 min at room temperature (Figure 1). Although 15 min of reaction at room temperature is adequate for studies of purified condensed tanning, crude tannin-containing extracts should be allowed to react with the protein for 24 hr at 4°C, before determining precipitated protein, to ensure complete precipitation.

The radiolabeled protein-precipitation assay, the dye-labeled protein-precipitation assay, and the Martin and Martin precipitation assay were directly compared by determining the amount of protein precipitated by various amounts of tannin-containing leaf extracts. Results obtained with a dogwood leaf extract are shown in Figure 2. The regions of linearity were different for each of the assays and for each of the leaf extracts tested. The protein-precipitating capacity is usually calculated from the slope of the linear portion of the results. For the Martin and Martin assay, the amount of protein precipitated was linearly related to the amount of dogwood extract added for amounts of extract corresponding to 1–2 mg forage. The slope of the linear region was 0.49 mg BSA/mg forage. For the radiolabeled protein-precipitation assay, the region of linearity corresponded to 2–3 mg dogwood leaves and the slope of the linear region was 3.6 mg BSA/mg forage. For the dye-labeled protein-precipitation assay, the region of linearity corresponded to 3–5 mg dogwood leaves, and the slope of the linear region was 1.9 mg BSA/mg forage.

Since an exhaustive extraction procedure was used to extract tannin from the tissue, the ratio of tissue to solvent used for the extraction did not affect the amount of tannin extracted. For example, extraction of 100 mg of dogwood leaves yielded extracts equivalent to those obtained from the extraction of 1.5 g tissue (Figure 2).

Each of the assays was characterized by a nonzero x intercept (Figure 2), suggesting that a threshold amount of dogwood extract was required to precipitate protein. The threshold value was different for each of the assays. Zero intercepts were reported for each of these assays in the literature (Hagerman and Butler, 1980a; Martin and Martin, 1982; Asquith and Butler, 1985). The nonzero intercepts obtained with the dogwood extract and with the other plant extracts tested made it impossible to express the protein-precipitating capacity

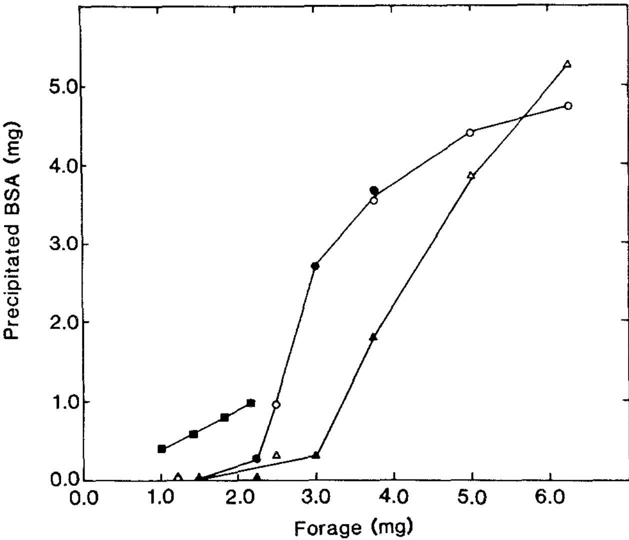


FIG. 2. Protein precipitated by an extract of dogwood leaves using three different assays. The amount of protein precipitaed by the extract of dogwood leaves was determined with the Martin and Martin assay (■); with the radiolabeled protein-precipitation assay (●, ○); with the dye-labeled protein-precipitation assay (▲, △). Each assay was performed as described in the text. In the Martin and Martin assay, the reaction mixture contained 1.47 mg BSA in a total volume of 2.0 ml; in the radiolabled protein-precipitation assay, the reaction mixture contained 6.5 mg iodine-125-labeled BSA in a total volume of 2.5 ml; in the dye-labeled protein-precipitation assay, the reaction mixture contained 7.64 mg dye-labeled BSA in a total volume of 4.5 ml. The extracts were prepared from dried samples of dogwood leaves (*Cornus stolonifera*) by sequential sonication and heating. Either 1.5 g dry tissue was extracted with 200 ml solvent (■, ●, ▲) or 0.10 g dry tissue extracted with 8.0 ml solvent (○, △).

simply as the amount of protein precipitated per gram of tissue. An accurate expression for precipitating capacity from an assay with nonzero intercepts would include not only the slope of the line but also the intercept of the line.

In an attempt to identify the sources of difference between the assays, the conditions of each assay were compared. All three assays were run at pH 4.9, the optimum pH for precipitation of BSA by condensed or hydrolyzable tannin (Hagerman and Butler, 1978; Hagerman and Klucher, 1986). Although the dye-labeled protein-precipitation assay was run at lower ionic strength than the other assays, previous work showed that the precipitation of condensed tannin by BSA was independent of ionic strength over a range of ionic strengths from 0.05 M to 5M (Hagerman and Butler, 1978). The amount of organic solvent in the assay mixture was slightly different for each of the assays, ranging from

6.5% (v/v) methanol in the final reaction mixture for the dye-labeled protein-precipitation assay to 15% methanol in the final reaction mixture for the Martin and Martin assay. In earlier studies of the effects of solvents on tannin–protein interactions, it was found that concentrations of alcohol ranging from 10% to 25% had no effect on the precipitation reaction, while lower amounts of alcohol (4.4%) suppressed precipitation slightly (Hagerman and Butler, 1980a). The low concentration of alcohol in the dye-labeled protein-precipitation assay could be partially responsible for the diminished precipitation observed with this assay. The dye-labeled protein-precipitation assay and the radiolabeled protein-precipitation assay were compared using 5.9% methanol in the reaction mixture for each (Figure 3); the dye-labeled protein was not precipitated as efficiently as was the radiolabeled BSA even when alcohol concentrations were identical. Thus, neither pH, ionic strength, nor organic solvent concentration adequately explained the differences observed for the three precipitation assays.

One significant difference between the three assays in the final concentration of protein in the assay mixture, since the recommended amount of protein and the volume of the reaction mixture are different for each method. The formation of insoluble complexes of gelatin and tannic acid is dependent on protein concentration (Van Buren and Robinson, 1969). The reaction volume used in the Martin and Martin assay was 2.0 ml, and approximately 1.5 mg BSA was used, producing a final protein concentration of about 0.75 mg/ml. The radiolabeled protein-precipitation assay was developed for a 2.5-ml reaction mixture containing about 6.5 mg iodine-125-labeled BSA, giving a final protein con-

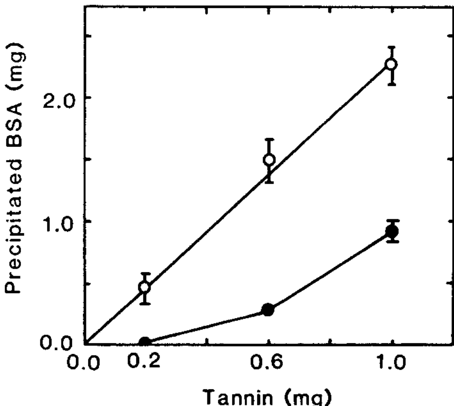


FIG. 3. Amount of iodine-125-labeled BSA and of blue dye-labeled BSA precipitated by purified tannin. An aliquot (0.25 ml) of 50% methanol containing purified condensed tannin was added to 4.0 ml buffer containing 7.64 mg blue dye-labeled BSA (●) or 7.64 mg iodine-125-labeled BSA (○). The amount of dye-labeled BSA or of radiolabeled BSA precipitated was determined as described in the text.

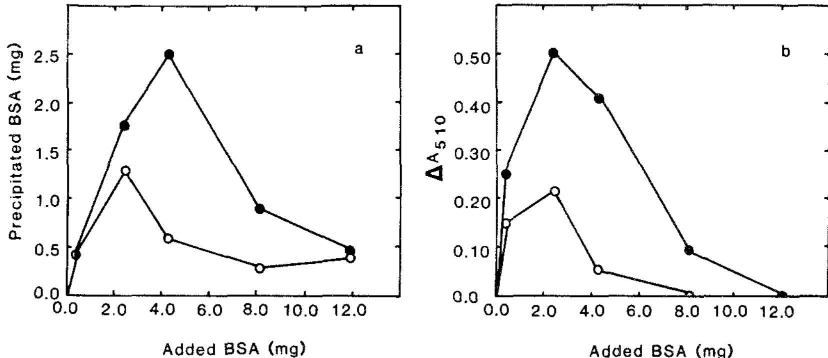
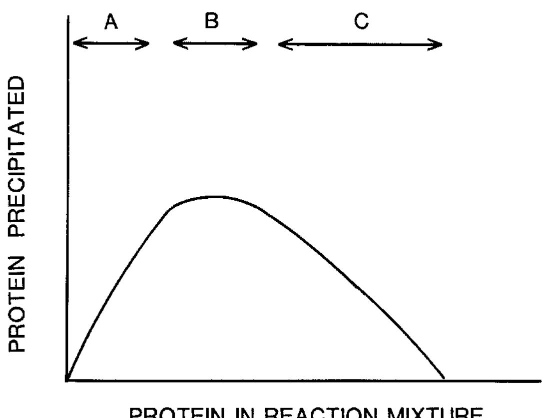


FIG. 4. Precipitation of protein and tannin by extracts of alder leaves in reaction mixtures containing various amounts of protein. An aliquot of the alder (*Alnus ruba*) extract equivalent to 2.25 mg forage (○) or to 3.75 mg forage (●) was added to 2.0 ml buffer containing between 0 and 12 mg iodine-125-labeled BSA. The amount of protein precipitated (a) and the amount of tannin precipitated (b) were determined as described in the text. The protein-precipitating capacity for alder calculated from this data is 1.31 mg BSA/2.25 mg forage = 0.58 mg BSA/mg forage (○); 2.5 mg BSA/3.75 mg forage = 0.67 mg BSA/mg forage (●).

centration of around 2.6 mg/ml. In the dye-labeled protein-precipitation assay, the reaction volume was 4.5 ml, containing approximately 8 mg dye-labeled BSA, or a final protein concentration of about 1.8 mg/ml.

The effects of protein concentration on protein precipitation assays were determined by experiments with the radiolabeled precipitation assay and crude tannin-containing plant extracts. The amount of precipitable complex formed by a constant amount of tannin-containing alder extract was significantly influenced by the amount of protein in the reaction mixture (Figure 4a and b). Curves of similar shape were obtained with extracts of other plants. Each curve could be divided into three regions (Scheme 1). When very little protein was present, there was excess tannin in the reaction mixture and only a small amount of precipitate formed (region A in Scheme 1). When a moderate amount of protein was present, the optimal ratio between tannin and protein, or equivalence point, was achieved, and precipitation was maximized (region B in Scheme 1). If a large excess of protein was added, smaller amounts of precipitate again formed (region C in Scheme 1). When a large excess of protein was present, no protein-precipitable phenolics were detected (Figure 4b), but a small amount of protein was precipitated (Figure 4a). The absence of precipitated tannin suggests that insoluble tannin–protein complexes did not form when protein was present in large excess. The protein precipitated under these conditions was perhaps denatured by exposure to tannin (Mole and Waterman, 1985). Curves of similar overall shape were obtained using the dye-labeled protein-precipitation assay



SCHEME 1. Titration of tannin-containing plant extract with protein: region A, excess tannin; region B, equivalence point; region C, excess protein.

instead of the radiolabeled-BSA assay, although the maximum precipitation was obtained with different amounts of the dye-labeled BSA than of the radiolabeled BSA.

The characteristics of the curve obtained when tannin is titrated with protein may depend on the tendency of tannin from a particular species to form soluble or insoluble complexes. The amount of protein required for maximum precipitation was different for each plant extract. It was not possible to predict the concentration of protein required for maximum precipitation of an uncharacterized tannin sample. The curves obtained with some plant extracts were sharp, with narrow regions of equivalence, and the curves obtained with other extracts were broad, with wide regions of equivalence. The equivalence point was narrower when crude plant extracts were used (Figure 4a and b) than when purified tannins were used (Van Buren and Robinson, 1969; Hagerman and Butler, 1980a). With purified condensed tannin, the optimal region was extremely flat and broad (Hagerman and Butler 1980a). At values exceeding the point of limiting tannin, precipitation of protein by purified condensed tannin was independent of protein concentration. However, precipitation by crude plant extracts was always dependent on protein concentration.

The determination of protein-precipitating capacity of a plant sample was affected by the amount of protein used in the precipitation assay, as is shown in Figure 5 for a maple leaf extract. When 1.90 mg of BSA was used in the radiolabeled protein-precipitation assay, the apparent protein-precipitating capacity of maple leaf extract was 0.50 mg BSA/mg forage. If the amount of protein used in the assay was increased to 4.76 mg, the apparent precipitating capacity was 1.1 mg BSA/mg maple leaves, while with 10.9 mg of BSA in the

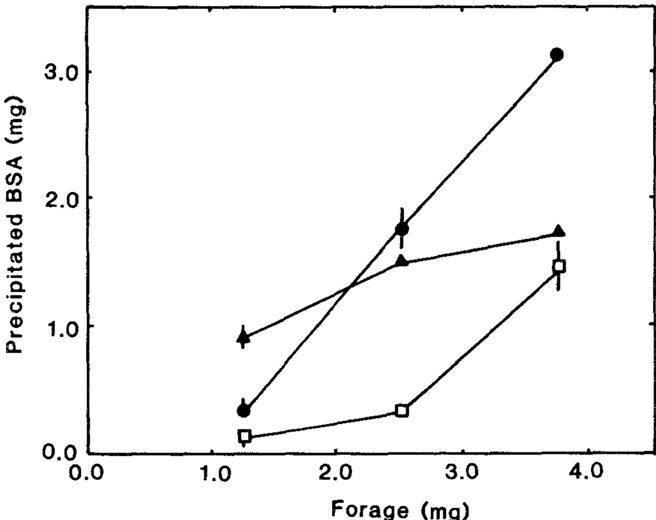


FIG. 5. Precipitation of protein by various amounts of maple extract. Aliquots of the maple (*Acer spicatum*) extract were added to 2.0 ml buffer containing 1.90 mg iodine-125-labeled BSA (▲), 4.76 mg iodine-125-labeled BSA (●), or 10.9 mg iodine-125-labeled BSA (□). The amount of protein precipitated was determined as described in the text.

assay, the apparent precipitating capacity was 0.90 mg BSA/mg maple leaves. The maximum precipitating capacity was obtained when the assay was run near the equivalence point, where neither protein nor tannin was in excess. The amount of protein precipitated per unit of tannin-containing plant tissue was diminished either in the region of excess tannin or in the region of excess protein. Thus the Martin and Martin assay, the radiolabeled protein-precipitation assay, and the dye-labeled protein-precipitation assay, which were run with different protein concentrations, gave different estimates of protein-precipitating capacity (Figure 2).

The amount of protein used in the assay also influenced the intercepts (Figure 5), giving rise to threshold effects like those observed with all of the precipitation assays (Figure 2). Zero intercepts were obtained when small amounts of protein were used in the assay, since under those conditions precipitation was favored even when only small amounts of tannin were added. With larger amounts of protein, precipitation was unfavorable for small aliquots of tannin-containing extract, and so a threshold level of tannin was necessary for precipitation to occur (Figure 5).

Apparently one source of differences among the precipitation assays is the variable protein concentration used in the methods. To further test this hypoth-

esis, the radiolabeled protein-precipitation assay and the Martin and Martin assay were set up under identical conditions, and the amount of protein precipitated was determined. It was found that the amount of protein precipitated was identical for these two assays if the same ratio of protein to tannin was used in the assays. A similar comparison was set up for the dye-labeled protein-precipitation assay and the radiolabeled protein-precipitation assay. It was found that even when identical protein-to-tannin ratios were used in these two assays, the amount of dye-labeled protein precipitated was significantly less than the amount of radiolabeled protein precipitated (Figure 3).

The competitive binding assay (Hagerman and Butler, 1981) was used to determine the affinity of dye-labeled BSA for tannic acid. The reaction conditions were set so that in the absence of competitor, 0.60 mg of the 1.0 mg of radiolabeled BSA present was precipitated by the tannic acid. The amount of competitor required to reduce precipitation of the radiolabeled protein by 50% was then determined. For blue dye-labeled BSA, 0.57 mg was required for 50% inhibition. For unlabeled BSA, 1.0 mg was required for 50% inhibition. Thus the affinity of dye-labeled BSA for tannic acid is slightly higher than the affinity of unlabeled or radiolabeled BSA for tannic acid. However, the blue dye-labeled BSA apparently forms soluble complexes with the tannin under conditions which promote the formation of insoluble complexes with the radiolabeled or unlabeled BSA (Figure 3). Insoluble complexes of dye-labeled BSA and tannin only form when the concentration of tannin is very high, so the dye-labeled protein-precipitation assay is rather insensitive.

Even after minimizing the differences between experiments or research groups by selecting common parameters for precipitation assays, problems of variability may arise. One source of such problems is the use of a variety of extraction conditions for tannin-containing tissues. Exhaustive extraction procedures like those described here are most desirable since the recovery of tannin is maximized. If only a single brief extraction period is to be used, then aqueous acetone extracts tannin more efficiently than aqueous alcohol (Bate-Smith, 1975; Fletcher et al., 1977; Hagerman, unpublished data). Using less efficient solvents for tannin extraction can significantly affect the estimate of tannin content in a plant sample (Hagerman, unpublished data). The solvent used to extract the tannin can also affect the precipitation assay. For example, even small amounts of acetone inhibit the formation of tannin–protein precipitates (Figure 6). To avoid such inhibition effects, all acetone must be removed from acetone-containing extracts before the assay is attempted, or an assay insensitive to acetone must be selected (Hagerman, 1987).

DISCUSSION

Determination of Protein-Precipitating Capacity. When attempting to determine the protein precipitating capacity of a tannin-containing plant extract,

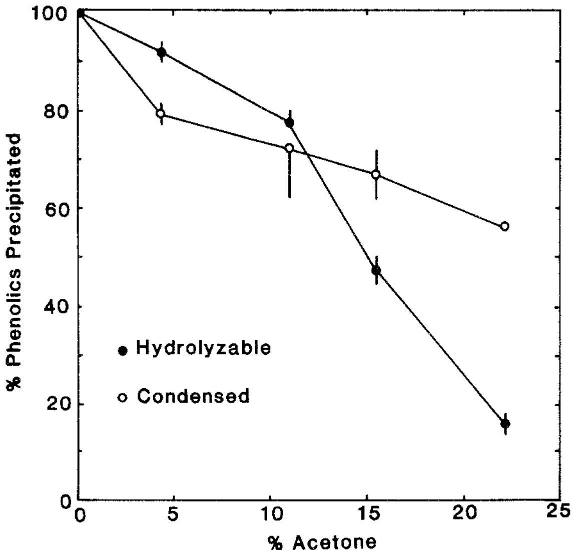


FIG. 6. Acetone inhibition of tannin–protein preciptation. Aliquots (0.25 ml) of tannin-containing solution were added to 2.0 ml reaction mixtures containing 2.0 mg BSA in buffer and up to 22.2% (v/v) acetone. After 15 min, the mixtures were centrifuged and the amount of tannin precipitated was determined colorimetrically as described in the text. The precipitated phenolics were expressed as a percent of phenolics precipitated in the absence of acetone. All of the mixtures contained 5.6% (v/v) methanol.

the conditions for precipitation and the method of analysis must be chosen with care. The effects of solvent on extractibility of the tannin from the tissue and on the precipitation reaction must be considered. Acetone is the most efficient solvent for extracting tannin, but acetone inhibits precipitation reactions except under certain conditions (Hagerman, 1987). Sufficient time must be allowed for the precipitation reaction to come to completion. Although reaction at room temperature for 15 min or less is recommended for many assays (Hagerman and Butler 1978, 1980a; Martin and Martin, 1982; Asquith and Butler, 1985), a 24-hr reaction at 4°C is required if crude plant extracts are being analyzed.

The choice of assay is dependent on the facilities available and the number of samples to be run. The Martin and Martin assay and the radiolabeled protein-precipitation assay give identical results if the precipitation reactions are run under identical conditions. With either of these assays, precipitated protein and precipitated tannin can be determined simultaneously. The radiolabeled protein-precipitation assay is simple to run, although the use of radioisotopes is required. The method is accessible to most labs since iodine-125-labeled BSA is now commercially available (ICN Radiochemicals, Irvine, California 92713). The Martin and Martin assay is technically difficult to run and would be inap-

propriate for studies involving more than a few samples. A recent modification of that assay is technically easier (Martin et al., 1985). The dye-labeled protein-precipitation assay has the advantage of being simple and not requiring any special techniques or reagents. However, precipitated tannin and protein cannot be determined simultaneously with this assay. In addition, the dye-labeled protein-precipitation assay is less sensitive than either of the other methods since the blue dye-labeled BSA preferentially forms soluble rather than insoluble complexes.

A new approach is needed for determining protein-precipitating capacity for tannin-containing samples. The capacity is presently determined as the slope of the line obtained when precipitated protein is measured as a function of the amount of tissue analyzed. The method yields variable results, depending on the amount of protein used in the precipitation assay. For each sample, the optimum ratio of protein to tannin is different and cannot be predicted. A better method for determining the protein-precipitation capacity is to titrate a single amount of plant extract with protein. The equivalence point (region B in Scheme 1) is determined by graphing the amount of protein precipitated as a function of the amount of protein added. The protein-precipitating capacity of the tannin sample is defined as the amount of protein precipitated at the equivalence point divided by the amount of tissue analyzed.

The method is illustrated graphically in Figure 4. As is seen in that example, different amounts of tannin-containing extract can be used in the assay. The equivalence point corresponds to a different protein concentration for each amount of extract. The protein-precipitating capacity, calculated as the amount of BSA precipitated at the equivalence point per milligram tissue analyzed, is approximately the same for different amounts of extract assayed (Figure 4a). The amount of tannin precipitated at the optimum ratio can also be determined using the colorimetric assay for protein-precipitable phenolics (Figure 4b). The same approach can be used with the dye-labeled protein-precipitation assay, although different values for protein precipitating capacity would be obtained since the dye-labeled protein behaves differently from BSA.

Ecological Implications. The results obtained in this study suggest that soluble tannin-protein complexes exist when a mixture of tannin and protein contains excess protein. The nutritional effects of soluble tannin–protein complexes may be relevant to the function of tannin as a defense metabolite. Formation of insoluble tannin–protein complexes is probably of little consequence to herbivores which have developed defenses against protein precipitation (Mehansho et al., 1983; Martin and Martin, 1984; Martin et al., 1985). We postulate that soluble tannin–protein complexes may form in the gut whenever protein-to-tannin ratios are favorable, despite herbivore adaptations which prevent formation of insoluble complexes. Since concentrations of tannin and protein in herbivore guts have not been measured, the frequency of occurence of soluble

complexes cannot be evaluated. However, if such soluble complexes do form, protein digestibility may be enhanced (Mole and Waterman, 1985) or absorption and metabolism of tannin may be increased (Potter and Fuller, 1968; Butler et al., 1986).

A model similar to that proposed for antigen–antibody interactions has been formulated to explain the formation of soluble tannin–protein complexes. Precipitation curves like those obtained with tannin (Figure 4) are obtained if an antibody is titrated with antigen (Kabat, 1980). At concentrations corresponding to excess antibody or excess antigen, soluble antibody–antigen complexes form rather than precipitable complexes. When tannin is present in excess, almost all of the available protein is precipitated, and no soluble complexes form. When protein is present in excess, there is not enough tannin available to form a significant number of cross-links between the protein molecules. Thus, at concentrations corresponding to excess protein, soluble tannin–protein complexes are present. At protein-to-tannin ratios corresponding to the equivalence point, crosslinking is favorable and a maximum amount of precipitable complex is formed. Thermodynamic studies of protein–phenol interactions suggest a similar model (McManus et al., 1981).

Although the characteristics of insoluble tannin–protein complexes have been extensively studied (Hagerman and Butler, 1978), 1980a, 1981; Fletcher et al., 1977; Goldstein and Swain, 1965; Van Buren and Robinson, 1969), soluble complexes have been neglected. Soluble complexes of tannin and gelatin have been detected (Calderon et al., 1968). Soluble complexes of tannin with seed proteins have been isolated (Hagerman and Butler, 1980b; Fishman and Neucere, 1980), and soluble complexes were recently detected in the aqueous extracts of pine needles (Nyman, 1985). However, little is known about features of the soluble complexes such as their size or their composition. Some proteins, such as blue dye-labeled BSA, preferentially form soluble complexes with tannin, but the general characteristics of proteins which promote formation of soluble complexes have not been identified.

There is not only a need to characterize the chemistry of soluble tannin–protein complexes, but also to study the formation of soluble complexes in vivo. Complementary nutritional and ecological studes will be necessary to elucidate the importance of soluble tannin-protein complexes in plant defense against herbivores.

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RESPONSES TO *n*-DIPROPYL DISULFIDE BY OVIPOSITING ONION FLIES: Effects of Concentration and Site of Release[1,2]

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Abstract—Onion fly females, *Delia antiqua* (Diptera: Anthomyiidae) laid the most eggs on ovipositional dishes having *n*-dipropyl disulfide (Pr_2S_2) release rates of 1–6 ng/sec from polyethylene capsules placed beneath a sand substrate. When dipropyl disulfide was released from the wax coating of surrogate foliage rather than from the substrate, ovipositing females again responded differentially to various concentrations, laying more eggs around stems containing 0.075 and 0.089 mg/stem. Factorial combinations of several concentrations released from surrogate foliage and substrate showed that releases from surrogate foliage stimulated four times more egg-laying than releases from the substrate. Females tended to lay more eggs around surrogate stems having Pr_2S_2 at the base rather than on the upper half of foliage. Observations of individual females performing preovipositional examining behaviors on Pr_2S_2-treated surrogate stems indicated that females tended to land on the upper portions of the foliage, but after landing, spent most of their time examining areas of soil and surrogate within 1 cm of the soil–surrogate foliage interface. Surrogate stems provide a realistic context for investigating effects of plant chemicals on host-acceptance behaviors.

Key Words—Onion fly, onion maggot, *Delia antiqua, Hylemya antiqua*, Diptera, Anthomyiidae, host selection, oviposition, dipropyl disulfide, behavior, herbivore, plant–insect interactions.

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INTRODUCTION

Like many other herbivore specialists, the onion fly, *Delia antiqua* (Meigen), has a narrow host range delimited by preferences of egg-laying females. Newly eclosed larvae have limited mobility and are subject to desiccation in open areas and dry soils; therefore, eggs must be placed close to or on proper hosts (Workman, 1958).

The question of what stimuli signal "host" to ovipositing *D. antiqua* has been investigated in some detail. Because females oviposit only on a small number of species in the genus *Allium*, initial studies focused on compounds unique to this plant genus. Secondary chemicals are formed in alliums when cells are disrupted, and the normally compartmentalized cysteine sulfoxides contact the enzyme alliinase (Whitaker, 1976). The labile thiosulfinates produced by this reaction then react further at room temperatures to form thiosulfonates and other sulfur-containing compounds such as dipropyl disulfide (Pr_2S_2) and propanethiol. Secondary chemicals are also formed from intact onions when propenyl cysteine sulfoxides are exuded by roots into the soil and converted into sulfides by soil bacteria (Coley-Smith and King, 1969; Hough et al., 1981; Ikeshoji, 1984).

Host-finding and acceptance in the onion fly are stimulated by the end-products of *Allium* defensive reactions, the stable mono-, di-, and trisulfides. Increased numbers of flies are caught in traps baited with Pr_2S_2, propanethiol (Matsumoto, 1970), methyl propyl and propenyl propyl disulfide, and several other mono- and disulfides (Vernon et al., 1981). Trap catches in response to Pr_2S_2 are significant at release rates of 60 μg/hr (Dindonis and Miller, 1981). Final acceptance of oviposition sites is also influenced by onion chemicals. Propanethiol and Pr_2S_2 stimulate females to probe with their ovipositors and lay eggs (Matsumoto and Thorsteinson, 1968), as do numerous other compounds containing a sulfur atom having two unshared pairs of electrons and bonded to a saturated hydrocarbon chain three to five carbons long (Vernon et al., 1978; Ishikawa et al., 1978).

Nonchemical plant stimuli also play an important role in host recognition. Addition of surrogate foliage to oviposition dishes synergizes responses to onion slices buried beneath moist sand (Harris and Miller, 1982). Color (Harris and Miller, 1983), shape, and size (Harris and Miller, 1984) of surrogates have major effects on acceptance of oviposition sites. These foliar stimuli are sensed by females primarily during extended runs over the surface of surrogates.

While observing females engaged in these preovipositional examining behaviors, we wondered whether runs over the surface of onion foliage might expose flies to plant chemicals in addition to color, size, and shape stimuli. The importance of chemicals released from foliage was dismissed by Vernon et al. (1977), who reported that extracts from onion bulbs were more stimulatory than

extracts of foliage; however, recent work on the carrot fly, which performs similar runs over host-plant foliage, has shown that a complex mixture of chemicals in surface waxes of foliage is critical to host recognition (Städler and Buser, 1984).

Our approach to the question of whether *D. antiqua* females obtain chemical information from onion foliage was to quantify effects of releasing a range of doses of the ovipositional stimulant, Pr_2S_2, from the substrate as well as from foliar surrogates. Locations of various preovipositional behaviors were also recorded to clarify whether females examine certain areas of the foliage and substrate more thoroughly than others before committing their eggs to a host.

METHODS AND MATERIALS

Insect Rearing. Delia antiqua pupae were collected (autumn of 1981 and 1983) from onions left in harvested fields in Grant, Michigan. Adults emerging from these pupae were placed in 60 × 60 × 80-cm screened cages housed in environmental chambers (21 ± 1°C and 35 ± 5% relative humidity) illuminated by Verilux (Greenwich, Connecticut) fluorescent bulbs with a 16:8 light–dark cycle. Cages were provisioned with water, honey, dry artificial diet, and the ovipositional dishes described by Harris and Miller (1982).

Foliar Surrogates. Foliar surrogates were of two types. Surrogates used in the wind-tunnel experiment were identical to those described in Harris and Miller (1983) and consisted of sealed Pyrex tubes (8 mm diam × 9 cm long) which contained strips of yellow silk-screened paper. In all other experiments, surrogates consisted of 4-mm-diam Pyrex tubing cut into 12-cm lengths and heat sealed at one end. Color stimuli were provided by oil pigments (see Harris and Miller, 1984) mixed to match (as detected by the human eye) the green of onion foliage. Painted surrogates were allowed to dry three weeks before use in experiments, then coated with a thin layer of wax. Surrogates were placed vertically in the centers of a 4-cm-diam × 4-cm-deep plastic ovipositional dishes filled with moist white silica sand. All but 3 cm of the "foliage" stood above the substrate surface.

"Choice" Tests. Flies used in experiments were two to five generations removed from the original field-collected populations and were 7–21 days old. Flies were transferred (ca. 100 females per cage) when 7 days old to food- and male-provisioned screened cages (165 × 65 × 85 cm). Treatments (1 replicate/cage) were spaced evenly (ca. 10 cm apart) within cages and surrounded by wire-screen barriers (16 cm high × 9 cm diameter) in all experiments except those in the wind tunnel. Experimental design was randomized complete block. Eggs were separated from sand by flotation and were recorded as numbers of eggs laid per ovipositional dish per cage per sampling period. Experiments com-

paring oviposition on only two treatments were analyzed by two-way ANOVA at $P < 0.05$. All other data required transformations (log or square root) before being subjected to analysis of variance. Means were then separated by the least significant difference test at $P < 0.01$. A probability of <0.01 was used rather than $P < 0.05$ to minimize experiment-wise error rate.

Dose-Response Curves for Pr_2S_2 Released from Substrate. Six Pr_2S_2 release rates from the substrate of oviposition cups were generated by inserting size 3 BEEM polyethylene embedding capsules (Pelco Electron Microscopy Supplies, Ted Pella Co., Tustin, CA) containing 100 μl of either neat Pr_2S_2 (Eastman Kodak, Rochester, New York, 98% pure by GLC), pure peanut oil, or a mixture (range 0.05–0.9 M) of Pr_2S_2 and peanut oil (PVO International, Inc.). These capsules, which allowed outward diffusion of the Pr_2S_2 but not of the peanut oil, were set, lid-side up, in ovipositional dishes containing 2 cm of moist silica sand, then covered with 2 cm of additional sand. Color and structural stimuli were provided by the aforementioned colored glass tubing. Twelve hours after assembly, ovipositional dishes containing different concentrations of Pr_2S_2 were placed in cages. A similar set of Pr_2S_2 dosages was also tested in a cage ($60 \times 60 \times 80$ cm) placed in the wind tunnel described by Cardé and Hagaman (1979). The arrangement of ovipositional dishes in the tunnel prevented overlap of chemical plumes of different concentrations. Further details on experimental procedures are described by Harris (1986).

Because preliminary experiments showed that Pr_2S_2 is not adsorbed by sand, we estimated release rates from single ovipositional dishes by measuring the amount of Pr_2S_2 in capsules at the beginning and at the end of the experimental period (six replicates of each). Thus, one complete set of treatments was analyzed 12 hr after assembly (initiation of bioassay) and one 24 hr after assembly (termination of bioassay). Both initial and final concentrations of capsules with neat chemical were obtained gravimetrically. All other capsules were opened and placed in 8-ml vials containing 1 ml of 10 : 1 methanol–water. Concentrations in peanut oil were analyzed by injecting 1 μl of the methanol layer of the extract onto a 10% Carbowax 20 M GLC column programmed for an initial 2 min hold at 70°C, followed by temperature increases of 10°C/min and a final temperature of 160°C. Diallyl disulfide was used as an internal standard because of its similar solubility in methanol and similar flame ionization detector sensitivity. Differences in Pr_2S_2 content between the two sets of treatments (analyzed at 12 and 24 hr) were expressed as nanograms released per second, with the approximation that release rate over the 12-hr experimental period was constant.

Dose-Response Curves for Pr_2S_2 in Wax Layer on Surrogate. Various concentrations of Pr_2S_2 were formulated on the surface of surrogate foliage by adding different amounts (0.5, 1, 5, 10, 50, 100, 500, 1000, and 2000 μl) of Pr_2S_2 to 100 ml of melted paraffin wax (Cullen Industries, Huntingdon Valley, Pennsylvania). Green glass tubing was dipped in melted Pr_2S_2-containing wax so

that a 0.5-mm-thick layer was retained on the surrogate surface. Loading rates per stem were estimated to be 0.002, 0.003, 0.017, 0.033, 0.166, 0.332, 1.66, 3.32, and 6.64 mg Pr_2S_2 per stem. Temperature of the melted wax when adding Pr_2S_2 (65°C) and when coating stems (68°C) was identical for all replicates. Stems were allowed to equilibrate for 24 hr before placement in ovipositional dishes. Dishes were placed in cages for 24 hr in this and all remaining experiments. No Pr_2S_2, the optimal dose (50 μl Pr_2S_2/100 ml wax), and the highest dose (2000 μl Pr_2S_2/100 ml wax) were also compared using the same procedures used in the previous dose–response experiment.

Concentrations of Pr_2S_2 in wax layers at the initiation of choice experiments were estimated by coating glass (unpainted) surrogates with Pr_2S_2-containing wax (five replicates of each), allowing them to age for 24 hr, and then scraping the Pr_2S_2-containing wax from the surrogates directly into the vials containing 2 ml of distilled pentane. One microliter of Pr_2S_2-containing-wax in pentane (replicated three times) was then injected onto a DB-5 GLC capillary column (column temperature 130°C). Concentrations in wax were calculated by comparing peak areas with those of Pr_2S_2 (in pentane) standards containing comparable amounts of pure wax.

Interaction between Pr_2S_2 Released from "Foliage" and Substrate. Five substrate dosages (peanut oil control, 0.15, 0.5, 0.7, and 1.0 M) and five surrogate foliage dosages (untreated wax, 5, 10, 50, and 2000 μl Pr_2S_2/100 ml wax) were combined factorially to investigate possible interactions between Pr_2S_2 released from the two sites. Formulations were identical to those described previously. Another experiment with fewer treatments was also run to test again for possible interactions; stems containing optimal doses of Pr_2S_2-treated wax (50 μl/100 ml wax) were placed in dishes with capsules containing various concentrations of Pr_2S_2 in peanut oil (0.05, 0.15, 0.5, 0.7, 0.8, 0.9, and 1.0 M).

Effectiveness of Pr_2S_2-treated Stems Over Time. Onion foliar surrogates coated with an optimal dosage of Pr_2S_2 (50 μl/100 ml wax) and prepared 15, 10, 5, 3, 2, and 1 day(s) before treatments were bioassayed. Surrogates coated with wax containing no Pr_2S_2 served as controls and were also prepared 15 days before use. A final set of Pr_2S_2-stems was prepared immediately before treatments were placed in cages.

Pr_2S_2 Placement on Surrogate Stems. Effects of placing Pr_2S_2 at different locations on surrogate foliage were examined by presenting flies with surrogates coated with Pr_2S_2-treated wax (50 μl/100 ml wax) in seven different locations: on the upper 1 cm, on the upper 4.5 cm, on the upper 9 cm, on the lower 3 cm, on the lower 4 cm, on the lower 7.5 cm, and on the entire 12-cm length (see Figure 5). Areas not coated with Pr_2S_2-treated wax were coated with unadulterated wax. A surrogate covered with unadulterated wax served as a control. Surrogates were placed in plastic cups filled with moist silica sand so that 9 cm of the 12-cm surrogate stood above the sand surface.

Location of Preovipositional Examining Behaviors. Groups of 5–15 newly emerged female flies were placed along with equal numbers of males in clear plastic cages (15 cm diam × 30 cm height) provisioned with water and food. For 2 hr every day at various times between 3 and 8 PM, flies were exposed, starting at age 7 days, to an optimal surrogate stem standing upright in the center of a pot (15 cm diam × 20 cm deep) containing organic "muck" soil. This period (3–8 PM) was chosen for behavioral observations because ca. 78% of egg production occurs during this interval (Havukkala and Miller, 1987).

During each observational period, location and duration of the following stances and behaviors of individual females were noted: no movement (still), grooming, running, running with the proboscis repeatedly brought in contact with plant or soil surface (proboscis extended), running with abdomen bent and tip of abdomen (and sometimes the proboscis) touching plant or soil surface (ovipositor probing), and stationary probing with ovipositor fully extended and wedged into soil crevices (insertion and oviposition). Foliar surrogate and substrate were divided into five zones: foliage 0–1 cm from surrogate–substrate interface, foliage 1–3 cm, folige 3–9 cm, substrate 0–1 cm from stem base, and substrate 1–4 cm from stem base. Observations began when individuals entered the 1-cm-diameter zone around the base of the surrogate or flew to the surrogate. Observations of individuals were terminated after females left the surrogate area either by flying away or by walking more than 3 cm from the surrogate stem base. Behavioral observations of individuals (identified by acrylic-paint color codes on the thorax) were recorded on a Radioshack TRS 80, model 100 using a program developed by T. Bierbaum, Department of Zoology, Michigan State University.

Surrogate Onions vs. Real Onions. The best surrogate (50 μl Pr_2S_2/100 ml wax, 4-mm-diam. green cylinder) was compared to three types of onions in pairwise choice tests: (1) onion plants (grown from sets) having four leaves, a height of 14 cm, and a basal diameter of 4–5 mm; (2) onions grown from seed (Downing Yellow Globe, 6 weeks old) having three leaves, a height of 180–240 mm, and a basal diameter of 3.0–3.5 mm; and (3) a single onion leaf ca. 120 mm long cut from the foliage of onion sets (4–5 mm diam.) and positioned upright in moist sand.

Comparisons of oviposition on surrogates and onion seedlings were also made using a no-choice bioassay. Individual 7-day-old females (N = 16) were placed in cages (10 cm diam. × 30 cm height) with food, water, two males, and the surrogate or onions grown from seed. Oviposition dishes were replaced and eggs counted every 24 hr on each of the next seven days.

RESULTS

Dipropyl Disulfide Dose–Response Effects. When *n*-dipropyl disulfide was released from the substrate, females responded to a fairly wide range of release

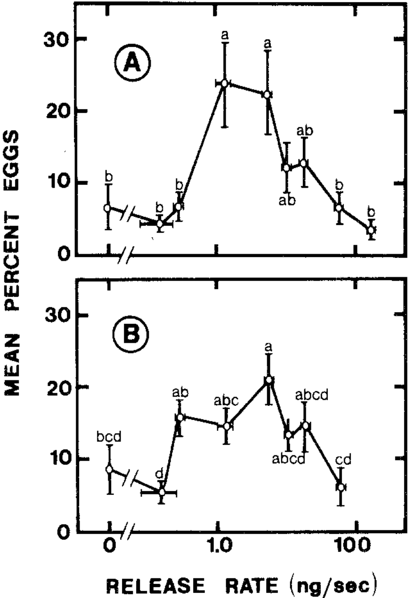

FIG. 1. Semilogarithmic plot of *Delia antiqua* ovipositional responses (means and standard error bars) to *n*-dipropyl disulfide released from the substrate using cages of flies placed in a wind tunnel (A) or in normal environmental chambers (B). Treatments accompanied by the same letter in each figure are not significantly different at $P < 0.01$ (ANOVA followed by LSD test for separation of means). Total number of eggs for 12 replicates for (A) = 22,428, for 15 replicates for (B) = 1517 eggs.

rates (Figure 1). In the wind tunnel (Figure 1A), increasing release rate from 0.32 to 1.38 ng/sec caused a significant increase in oviposition. When this same range of concentrations was tested in a cage having no rapid turnover of air (Figure 1B), significant increases in oviposition occurred at slightly lower release rates (0.32 ng/sec). A release rate of 60.8 ng/sec depressed ovipositional responses in both bioassays.

When Pr_2S_2 was released from the wax coating of surrogate foliage rather than from the substrate (Figure 2), ovipositing females again responded differentially to various concentrations. Maximal responses occurred around surrogates loaded with 0.17 and 0.33 mg/stem and dropped off sharply with the next lowest loading rate of 0.033 mg/stem. The highest loading rate of 6.64 mg/stem appeared to depress oviposition responses relative to the plain wax control. An experiment consisting of a subset of the original treatments [including the wax control, the optimal loading rate (0.17 mg/stem) and the highest rate] suggested that inhibition did occur at the highest loading rate: the high rate received a mean of three eggs vs. 16 and 55 eggs laid on the wax stem control and the optimal loading rate, respectively [eight replicates, ANOVA $F = 24.06$, sig-

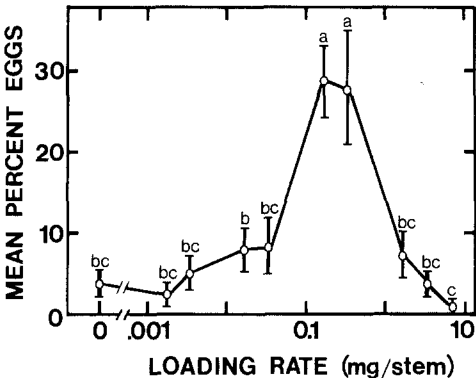

FIG. 2. Semilogarithmic plot of *Delia antiqua* ovipositional responses to *n*-dipropyl disulfide released from surrogate foliage. Treatments accompanied by the same letter are not significantly different at $P < 0.01$ (ANOVA followed by LSD test for separation of means). Total number of eggs for seven replicates = 676.

nificant at $P < 0.001$, all three treatments significantly different from each other at $P < 0.01$, least significant difference (LSD) test].

GLC analysis of actual amounts of Pr_2S_2 in wax at the beginning of the bioassay (as opposed to initial loading rates) revealed that stems with optimal loading rates of 0.17 and 0.33 mg/stem contained 0.075 and 0.089 mg/stem at the beginning of the experimental period. The next lowest dose (loading rate of 0.033 mg/stem), which stimulated no more oviposition than the wax control, contained only 0.014 mg/stem, whereas stems inhibiting oviposition (initial loading rate of 3.32 mg/stem) contained 1.83 mg/stem. The loss of Pr_2S_2 from wax from time of loading to initiation of bioassays probably occurred when Pr_2S_2 was added to heated wax during formulation.

Interactions between Pr_2S_2 Released from "Foliage" and Substrate. Females laid few eggs (Figure 3) when Pr_2S_2 was released only from the substrate. The previously determined optimal substrate dose (Figure 1B) received only 4% of the total eggs laid, hardly twice as many eggs as were received by the wax stem–peanut oil control. Flies were far more responsive to changes in concentration of Pr_2S_2 in the wax coating of surrogates. Loading rates of 0.033 and 0.17 mg/stem caused two- and ninefold increases in oviposition, respectively; these increases were similar to those seen in Figure 2.

Dose–response effects were significant both for Pr_2S_2 released from the substrate (ANOVA $F = 18.2$, significant at $P < 0.001$) and from the stem (ANOVA $F = 14.3$, significant at $P < 0.001$). A significant interaction occurred between Pr_2S_2 released from the two areas (ANOVA $F = 3.07$, significant at $P < 0.001$). Whether this interaction consisted of both synergy and interference could not be determined; however, a subsequent experiment in which a range of Pr_2S_2 substrate concentrations was presented with optimal

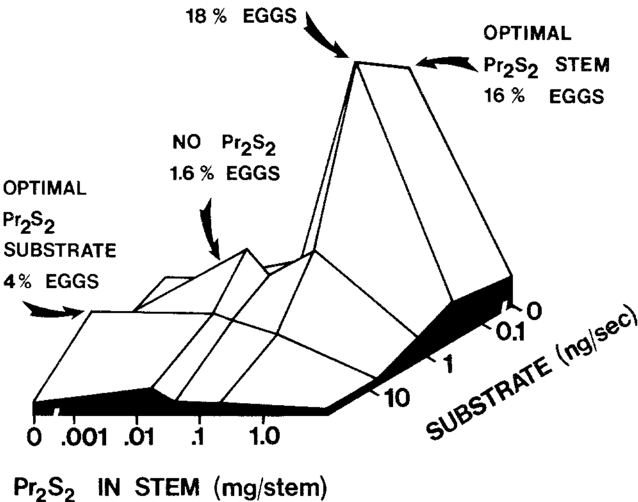

FIG. 3. Response surface generated by presenting gravid onion flies with doses of *n*-dipropyl disulfide released from substrate and surrogate foliage combined factorially (discontinuous log-log plot). Mean number of eggs laid on each of the 25 treatments is indicated by height on the vertical axis. Total number of eggs for seven replicates = 4157.

Pr_2S_2-treated wax stems (14 replicates) indicated that small concentrations of Pr_2S_2 released from the substrate did not synergize optimal concentrations of Pr_2S_2 on stems. On the other hand, higher release rates from the substrate did interfere with optimal release from the stem; release rates of 5.83 ng/sec from the substrate significantly inhibited egg-laying relative to a Pr_2S_2-treated wax control (LSD test, $P < 0.01$).

Effectiveness of Pr_2S_2-treated Stems over Time. Optimal Pr_2S_2-treated stems (loading rate 0.17 mg/stem) retained their stimulatory effects on oviposition for up to five days after being formulated (Figure 4). Stems formulated more than 10 days before exposure to females received significantly fewer eggs.

Pr_2S_2 Placement on Surrogate Stems. Stems covered both above and below the substrate with optimal doses of Pr_2S_2-treated wax, and stems with Pr_2S_2 above the substrate or on all but the upper 4.5 cm of the stem elicited the most oviposition (Figure 5). Stems having Pr_2S_2-treated wax placed on the section beneath the substrate and on the lower 1.0 cm of the stem received fewer eggs, but more eggs than the wax control or treatments having Pr_2S_2-treated wax only beneath the substrate or on the upper half of the stem. An experiment including the four top ranking treatments confirmed no significant differences in ovipositional responses (six replicates, $P < 0.01$).

Observation of Preovipositional Examining Behaviors. Encounters with Pr_2S_2-treated wax surrogates began when individual females ($N = 24$) either

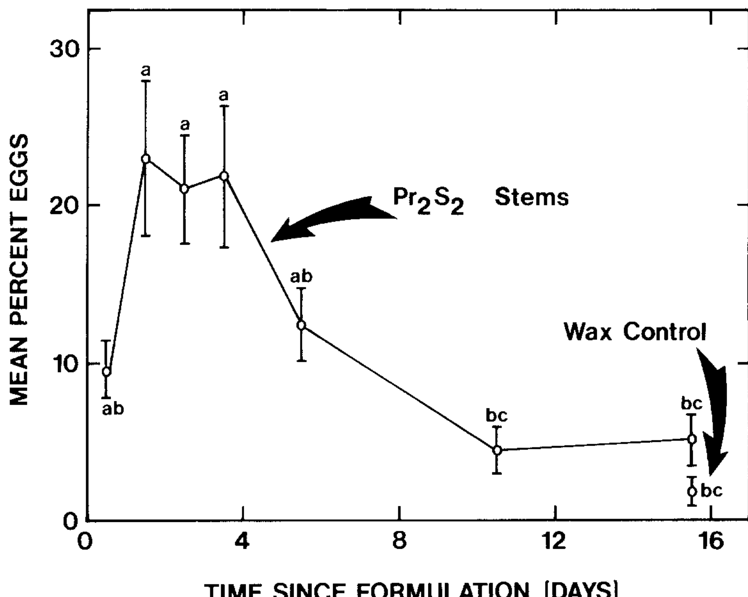

FIG. 4. Ovipositional responses of onion flies to *n*-dipropyl disulfide-treated surrogate foliage formulated 0–15 days before being used in ovipositional bioassays. Treatments accompanied by the same letter are not significantly different at $P < 0.01$ (ANOVA followed by LSD test for separation of means). Total number of eggs for 10 replicates = 2518.

flew to the surrogate ($N = 12$) or walked up to the surrogate via the substrate ($N = 12$). Females not ovipositing during these encounters ($N = 12$) stayed on the surrogate or in the immediate area of the surrogate for only 167 sec on average and spent 67% of their time grooming or sitting immobile on the surrogate. The remainder of their time was spent running over the surrogate surface and examining the surrogate with proboscis while running. These nonovipositing visitors did not proceed beyond extension of the proboscis; ovipositor probing and insertion were never observed.

Females that eventually oviposited during encounters ($N = 12$) were on the average 8.1 days old and laid 13.6 eggs (SE = 2.4). Encounters lasted ca. 17 min, 41 sec. Ovipositor insertion and oviposition accounted for the largest proportion of this time (mean ± SE = 65 ± 2.1%), with the remainder spent sitting still and grooming (3%) or examining the surrogate and substrate (32%).

Examining behaviors were primarily confined to areas of soil and surrogate within 1 cm of the soil/surrogate stem interface (mean ± SE tenure time = 253 ± 50.8 and 98 ± 16.8 sec, respectively, for substrate and surrogate, see Figure 6). Females spent significantly less time (mean ± SE tenure time = 6.2

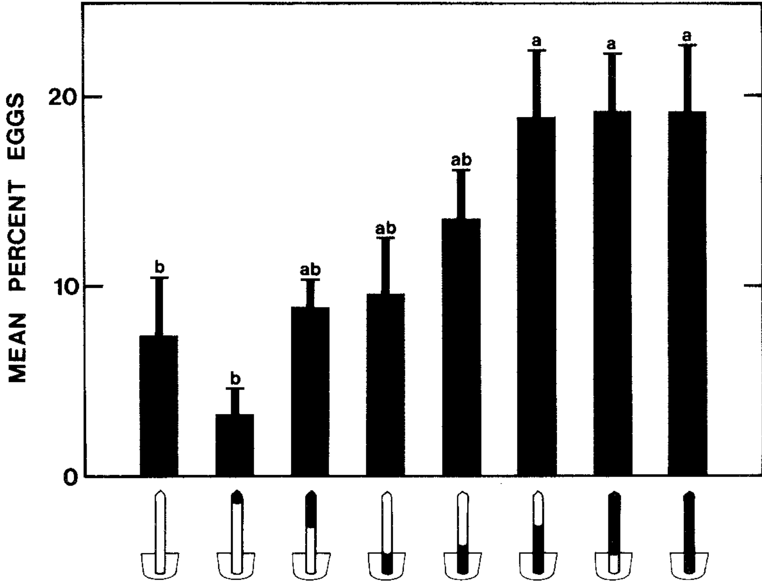

FIG. 5. *Delia antiqua* ovipositional responses to surrogate foliage with *n*-dipropyl disulfide-treated wax placed in different locations. Dark portions of surrogate foliage indicate treated areas, white areas were covered with plain wax. Treatments accompanied by the same letter are not significantly different at $P < 0.01$ (ANOVA followed by LSD test for separation of means). Total number of eggs for 10 replicates = 4312.

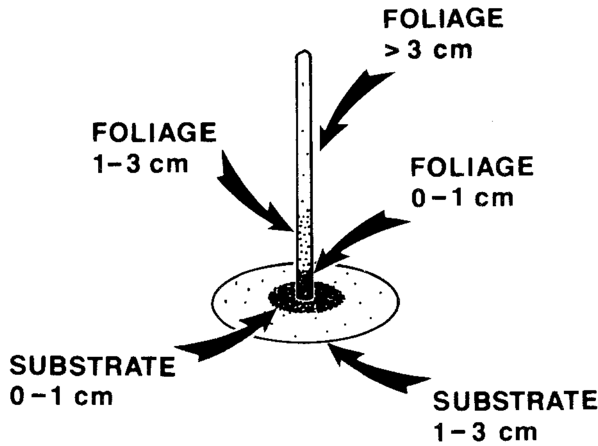

FIG. 6. Location and relative duration of *Delia antiqua* preovipositional examining behaviors on *n*-dipropyl disulfide-treated surrogate foliage. Density of shading indicates how much time is spent examining each zone.

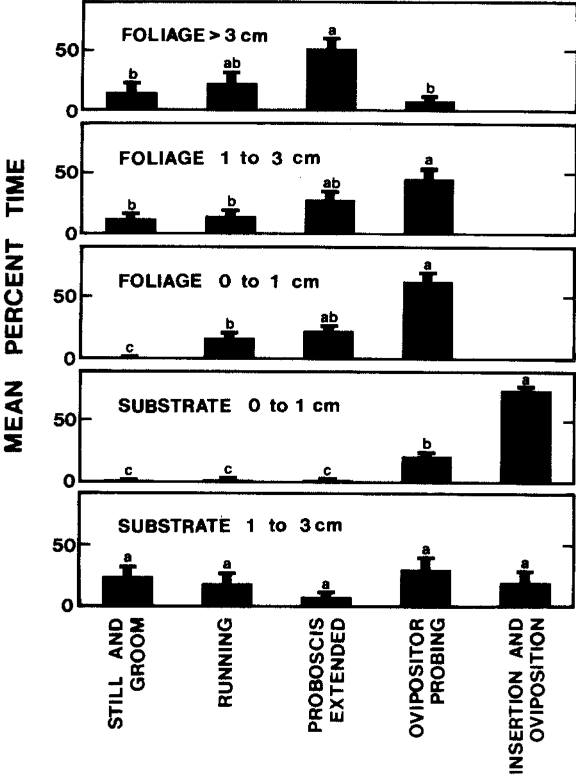

FIG. 7. Time allocated by ovipositing *Delia antiqua* females to five different behaviors in five different zones comprising oviposition sites. Values for percent time spent in behaviors within a single zone accompanied by the same letter are not significantly different at $P < 0.01$ (ANOVA followed by LSD test for mean separation).

$\pm$ 1.3 sec) examining the upper portion (foliage > 3 cm) of the surrogate. When flies did examine this area (Figure 7), it was by running over the surface or by extending the proboscis; probing with the ovipositor at this height on the surrogate occurred infrequently (mean $\pm$ SE = 0.8 $\pm$ 0.05 sec). Below 3 cm and above 1 cm (mean $\pm$ SE tenure time = 41 $\pm$ 8.7 sec), females continued to use the proboscis to examine the surrogate but were also more likely to engage the tip of the ovipositor to probe the surface. This latter form of examining was even more common at the base of the surrogate (foliage 0–1 cm).

Behaviors on the substrate differed somewhat from those seen on the surrogate itself (Figure 7). In the area immediately surrounding the base of the surrogate (foliage 0–1 cm), females spent most of their time with their ovipositors inserted into crevices in the soil. This latter behavior differed from ovi-

positor probing in that it was done in a stationary position, involved a full extension of the ovipositor, and placed the abdomen deeply within crevices in the soil surface. Ovipositor insertion was highly correlated with egg-laying; time spent in this behavior corresponded closely with the numbers of eggs laid by individual females ($r^2 = 0.90$, F for regression = 91.84, $P < 0.001$). As females moved farther away from the stem base (substrate 1–4 cm) they were less apt to insert the ovipositor into crevices and oviposit, and more apt to probe the soil with their ovipositors, sit still, or groom.

Surrogate Onions vs. Real Onions. When presented with the optimal surrogate (0.17 mg Pr_2S_2 per stem, 4 mm basal diam. × 12 cm height) and a 6-week-old onion seedling (3.5 mm diam. × 20 cm height) in a choice bioassay, females laid as many eggs around the surrogate (mean = 43 eggs) as around the onion plants (mean = 30 eggs, difference not significant at $P < 0.05$, two-way ANOVA). Significantly more eggs were laid around surrogates in no-choice bioassays (mean total eggs per female per day = 6.7 vs. 3.2 for onion seedling, significantly different at $P < 0.01$, two-way ANOVA) in which females were given access to either surrogates or onion seedlings for the first eight days of their reproductive lives. The larger number of eggs laid around surrogates was in part due to the fact that more females accepted the surrogate as an oviposition site during the eight-day test period (44 vs. 22% ovipositing per day, significantly different at $P < 0.01$).

However, when surrogates were placed in cages with 2-week-old onions grown from sets (4 mm diam. × 18 cm height), they received significantly fewer eggs (mean = 56) than did the real onions (mean = 194, $P < 0.05$, two-way ANOVA). Foliage removed from these same sets (4 mm diam. × 9 cm height) and placed upright in moist sand received fewer eggs (mean = 12) than did surrogates (mean = 47, not significantly different at $P < 0.05$, two-way ANOVA).

DISCUSSION

Because extracts of onion bulbs contain more of the stimulatory thiopropyl chemicals than extracts of onion foliage (Vernon et al., 1977, Ishikawa et al., 1978), it has been assumed that, when ovipositing into soil adjacent to onion plants, female onion flies are stimulated by volatile compounds that originate in the bulb. These assumptions have led to the design of bioassays that test effects of chemicals administered in a similar fashion; volatiles are presented either in conjunction with small holes and moistened filter paper (Vernon et al., 1977, 1978; Pierce et al., 1978) or are adsorbed onto charcoal granules and covered with moistened glass beads (Matsumoto and Thorsteinson, 1968; Ishikawa et al., 1978; Ikeshoji et al., 1980). Chemicals presented in this way never elicited more than 50% of the oviposition stimulated by onion slices.

Onion fly females, however, do not perceive onion plants solely through the chemical senses. Even when chemical stimuli are separated spatially from surrogate foliage by placing onion slices beneath the substrate, the addition of foliar surrogates synergizes ovipositional responses to odors, causing females to lay 18 times more eggs (Harris and Miller, 1982). Females respond strongly to changes in surrogate color, shape, and size and lay more eggs around yellow or green cylinders 4–8 mm in diameter and greater than 2 cm in height (Harris and Miller, 1983; 1984). These stimuli are sensed, at least in part, during runs up and down surrogate foliage.

Evidence presented in this paper indicates that acceptance of ovipositional sites may occur more readily if females perceive host-plant chemical cues in addition to foliar structural cues during runs over the plant surface. Optimal doses of Pr_2S_2 released from surrogate foliage stimulated four times as much oviposition as optimal doses released from the substrate. Placement of dipropyl disulfide on the surrogate itself also influenced oviposition. Females tended to lay more eggs around surrogates with disulfide at the base rather than on the upper half of "foliage." Observations of individual females performing pre-ovipositional examining behaviors suggested a reason for this trend: females tended to land on the upper portions of the foliage but, after landing, spent very little time in this area. The lower portions of the surrogate and areas of substrate immediately adjacent to the surrogate base were extensively examined (Figure 7) during runs with or without the proboscis and/or the tip of the ovipositor repeatedly contacting the wax surface.

During these examining behaviors, Pr_2S_2 formulated in wax could be detected by contact or olfactory receptors on the tarsi, antennae, proboscis, or ovipositor. Indeed, running over the surface of artificial or natural onion foliage may be akin to drumming in Lepidoptera (Ilse, 1937) and palpation in grasshoppers (Blaney and Chapman, 1970). Abrasion of foliage by spines or hairs located on the tarsi, proboscis, and ovipositor may increase concentrations of host-plant chemical stimuli to which the female is exposed. Antennae of *D. antiqua* are covered with trichoid, basiconic, grooved, and clavate sensilla (Honda et al., 1983) and give EAG responses to Pr_2S_2 and numerous other compounds that have stimulatory or neutral effects on oviposition (Ikeshoji et al., 1981; Guerin and Stadler, 1982). Preliminary experiments on tarsal hairs (done in the laboratory of Dr. Frank Hanson) indicate that contact chemoreceptors also respond to Pr_2S_2. Other herbivorous flies, whose examining behaviors closely resemble those of *D. antiqua*, sense host chemicals via chemoreceptors located on the antennae, tarsi, and maxillary palps (Städler, 1977; 1978; Guerin and Städler, 1982).

Since we do not know how accurately our present optimal surrogate approximates the stimuli presented by onion foliage, in-depth analyses of leaf surface chemistry and bioassays must be done to ascertain what compounds are

present and whether compounds function as stimulants or deterrents for ovipositing onion flies. For example, stimulatory chemicals such as Pr_2S_2 may be accompanied by other *Allium* secondary chemicals such as diallyl disulfide. Although this chemical does not appear to inhibit or stimulate oviposition when presented singly (Harris, 1982), it does inhibit responses when added to dipropyl disulfide (Harris and Miller, in preparation). Dimethyl disulfide may function in the same way. Both of these non-propyl-containing disulfides are more toxic than dipropyl disulfide to onion fly adults and larvae (Powell and Miller, in preparation) and are present at higher concentrations in *Allium* species (Freeman and Whenham, 1975) that are less preferred by ovipositing females and less suitable for larval development (Loosjes, 1976). On the other hand, sulfides may not be the only chemicals used in host recognition. The leek moth, *Acrolepiopsis assectella*, which also specializes on *Allium* species, responds positively to thiosulfinates in olfactometers and to thiosulfonates when ovipositing (Thibout et al., 1982). Methanol extracts of leek leaves are also highly stimulatory to ovipositing leek moths but do not contain sulfur compounds (Auger and Thibout, 1983).

Foliar surrogates and wax formulations of plant chemicals provide excellent tools for both applied and basic researchers studying insect–plant interactions. Chemicals present in resistant breeding lines can be formulated in wax coatings of surrogates and tested in lab bioassays before carrying out expensive and time-consuming field trials. The production of identical surrogates also provides realistic oviposition sites that could be used as a positive control to standardize responses to breeding lines tested at different times or in different countries. And finally, manipulation of surrogates, involving alterations of one or more plant character(s), will provide needed information on whether host acceptance is triggered by a few "key" stimuli or by interactions among a larger set of stimuli sensed by multiple sensory modalities (Miller and Harris, 1985).

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KINETIC RESOLUTION OF SECONDARY ALCOHOLS WITH COMMERCIAL LIPASES: Application to Rootworm Sex Pheromone Synthesis

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Abstract—The relative rates of enzyme-catalyzed esterification of the enantiomers of 2-octanol with various acids were determined for several commercial lipases. Interesterifications and hydrolyses of racemic 2-octanol esters catalyzed by these enzymes were also examined. Novo's *Mucor miehei* lipase exhibited considerable enantioselectivity and was therefore employed to prepare 8-methyl-2-decanols with high configurational purity at the carbinol carbon. Esters of these alcohols had been previously identified as sexually attractive to several rootworm (*Diabrotica*) species, and the stereochemistry of those esters had been shown to be critical to the attraction. The enzymatic resolution provides a convenient method to obtain such esters in a desired state of configurational purity.

Key Words—*Diabrotica* sp., kinetic resolution, lipase, methyl carbinol, *Mucor miehei*, pheromone, western corn rootworm, Coleoptera, Chrysomelidae.

INTRODUCTION

The relationship of insect pheromone stereostructure to insect behavior has received considerable attention (Brand et al., 1979). Successful completion of a pheromone identification and evaluation of methods by which to control the behavior of an insect pest often are dependent on the ability to prepare pure stereoisomers. Therefore there is a continuing interest in the development of novel, or improved, syntheses of stereocenters that can be incorporated into structures that are commonly found in pheromone blends. Additionally, such new methodology often serves the broader interest of organic synthesis generally. Among the approaches commonly used for asymmetric synthesis, enzy-

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matic resolution can perform quite well providing (1) a preparation is commercially available or can be reproduced easily in a laboratory, and (2) the precursor of the target stereocenter mimics the enzyme's normal substrate adequately to provide reasonable reaction rates. Enzymatic conversions that have been targeted for chiral insect sex pheromone structures include *N*-deacylation of racemic acetamido acids with an *Aspergillus acylase* (Mori and Iwasawa, 1980; Sugai and Mori, 1984), selective hydrolysis of alkynol esters by cultures of *Bacillus subtilis* var. *Niger* (Mori and Akao, 1980), reduction of β-ketoesters and ketones with Baker's yeast (*Saccharomyces cerevisiae*) (Mori, 1981; Mori et al., 1985), and the reduction of α-methyl-α, β-unsaturated aldehydes to chiral α-methylcarbinols also with Baker's yeast (Gramatica et al., 1985). In all of these cases an enzyme preparation of previously documented properties was examined with a selected group of substrates. We report here a study of a number of commercial lipases in which stereoselective hydrolysis, esterification, and transesterification of 2-octanol as a typical 2-*n*-alkanol were evaluated. The information was then used to synthesize 8-methyl-2-decanol samples in which the carbinol carbon was $\geq$97% (94% *ee*) configurationally pure. Esters of such alcohols are pheromones of various *Diabrotica* species (Guss et al., 1985).

METHODS AND MATERIALS

Gas–liquid chromatography (GLC) was performed with a Shimadzu GC-Mini-2 instrument using an SPB-1 column (0.25 mm ID $\times$ 30 m) operated at temperatures indicated below with a 50:1 split ratio and He carrier gas. Infrared (IR) spectra were obtained on a Perkin-Elmer 1310 Spectrophotometer (3% solutions in CCl_4 or $CHCl_3$). Mass spectra (MS) were recorded with a Hewlett-Packard HP-5995 GC-MS system employing an OV-1 capillary column (0.25 mm ID $\times$ 30 m). Nuclear magnetic resonance (NMR) spectra were obtained in $CDCl_3$ using a JEOL JNM-GX 400 FT NMR spectrometer. Free fatty acid titrations were performed with a Radiometer pH instrument comprised of an ABU-80 Autoburette module operated in "end point" or "pH stat" modes as appropriate. Lipases were obtained from commercial sources and were employed directly without any purification. Specific lipases and manufacturers are described in text and tables; additional information follows: Gist-Brocades USA, Inc., Charlotte, North Carolina 28224; Amano International Enzyme Co., Inc., P.O. Box 1000, Troy, Virginia 22974; Novo Laboratories, Inc., P.O. Box D, Wilton, Connecticut 06897-0820. The α-methylbenzyl isocyanates, both *R* and *S*, were synthesized from the corresponding amines that had been purchased from Hexcel Corp., using the method of Pirkle and Hauske (1977). The α-methylbenzylamines were 98.7% *R* and 99.4% *S*, but the analyses reported here were not corrected. The actual ratios were in fact slightly higher than those reported in this paper.

Rate ratios are calculated from the expression:

$$\frac{k_A}{k_B} = \frac{\ln (1 - C)(1 - ee)}{\ln (1 - C)(1 + ee)}$$

wherein C is fraction conversion and ee is enantiomeric excess of starting material expressed as a fraction (Martin et al., 1981). For hydrolysis the stereobias was evaluated in the product and,

$$ee_a = \frac{C(ee_b)}{1 - C}$$

wherein a and b represent starting material and product, respectively.

The 2-octanol was purchased from Aldrich Chemical Co.; other reagents and solvents were of commercial origin and employed directly except for esters of 2-octanol and 8-methyl-2-decanol. The esters were synthesized in the usual manner from the alcohols using acid chlorides and pyridine solvent. They were distilled and characterized by IR, MS, and GLC.

Initial Rate Assay. Weighed amounts of commercial lipase were allowed to react in an emulsion created by brief sonication of 10% (w/w) purified olive oil and 10% (w/w) gum arabic. Five milliliters of emulsion, 0.2 ml of 2.85 M NaCl, and 30–100 μl of aqueous enzyme were brought together at ambient temp. Free fatty acid release was measured by titration with 0.100 N NaOH using "pH stat" mode at pH 7.3 or other indicated pH (Table 1). Enzyme

TABLE 1. INITIAL RATE ASSAYS OF COMMERCIAL LIPASES ON OLIVE OIL[a]

Lipase	Manufacturer code	Rate (μmol/min/mg)	Position selectivity[b]	Fatty acid selectivity[c]
Aspergillus niger, 1	Amano-AP	0.154	1,3	18 (*cis*-Δ9)
Aspergillus niger, 2	Amano-K	11.2	1,2,3	10,12
Candida rugosa	Enzeco	9.82	1,2,3	18 (*cis*-Δ9)
	Sigma	9.70	1,2,3	18 (*cis*-Δ9)
Mucor miehei	Amano-MAP	2.67	1,3	<12
	Gist Brocades-S	40.0	1,3	<12
	Novo (powder)[d]	5.33	1,3	<12
	Nova 3A (resin)	0.095	1,3	<12
Porcine pancreatic[e]	Sigma	15.6	1,3	4
Rhizopus arrhizus[f]	Gist Brocades	33.4	1,3	8,10

[a]pH maintained at 7.3 unless otherwise indicated.
[b]Position selectivity with respect to triglyceride hydrolysis.
[c]Fatty acid selectivity (Brockerhoff and Jensen, 1974; Borgstrom and Brockman, 1984); the preferences are slight in most cases.
[d]The powder form is not currently available, although the lipase is now formulated on both an ion exchange resin sold as "3A" and as a solution as "225".
[e]pH 8.0 with 0.2 ml of 0.3 M $CaCl_2$ to 5 ml emulsion (Methods and Materials).
[f]0.2 ml of 0.3 M $CaCl_2$ to 5 ml emulsion.

solutions were prepared in distilled water with additives as indicated. The conditions were chosen to optimize activity of the lipase on the olive oil. Initial rate assays provide reproducible data by which comparisons of enzymatic activity on a given substrate may be made.

α-Methylbenzyl Carbamates. The alcohols, and reaction mixtures that were being analyzed for enantiomeric alcohol content, were derivatized by reaction with excess (*R*)- or (*S*)-α-methylbenzyl isocyanate (for example, 10 μl of neat racemic alcohol plus 20 μl of isocyanate) at 50–60°C for 0.5 hr in a screw-cap vial. The excess isocyanate was discharged by adding a few drops of methanol and warming at 50–60°C for 0.25 hr. The resulting mixture was diluted with acetone and filtered through Na_2SO_4 in a disposable pipet, if necessary, for GLC analysis. Spectral and chromatographic data for the carbamates follow: (1) 2-octanol *N*-α-methylbenzyl carbamate: IR (CCl_4) 3460, 1715 cm^{-1}; EI-MS (*m/e*) 277 $(M)^+$, 262 $(M\text{-}CH_3)^+$, 164,150; GLC (220°C) k′ 3.58 (*R*S**), 3.77 (*R*R**), $\alpha = 1.054$; (2) 8-methyl-2-decanol *N*-α-methylbenzyl carbamate: IR (CCl_4) 3460, 1715 cm^{-1}; EI-MS (*m/e*) 319 $(M)^+$, 164,150; [^{13}C]NMR ($CDCl_3$): 11.4, 19.2, 20.3, 22.6, 22.7, 27.0, 29.5, 29.9, 34.4, 36.3, 36.5, 50.5, 71.6, 116.6, 125.9, 127.2, 128.6, 155.7 ppm. GLC (240°C) k′ 3.73 (*R*S**), 4.00 (*R*R**), $\alpha = 1.072$.

Lipase Screening for Kinetic Resolution. Racemic 2-octanol (0.33 g, 2.5 mmol) was dissolved in 10 ml of nanograde hexane. The organic acid (2.5 mmol) was added as was 1.0 g of the lipase powder (2.0 g of porcine pancreatic lipase powder). The mixture (heterogeneous) was stirred for a period of time in an incubator at a specified temperature (Tables 2–5), diluted with ethanol, and immediately titrated to pH 9.5 with 0.1 N NaOH. Blanks were obtained for each lipase employed. The titrated mixture was partitioned with hexane, and

TABLE 2. ESTERIFICATION OF (±)-2-OCTANOL WITH *Candida rugosa* LIPASE[a]

Acid	Time (hr)	C^b	ee^c	k_R/k_S
Acetic	20	0	—	—
Propanoic[d]	20	76.0	<3	ca. 1
Octanoic	20	84.8	12.8	1.14
Dodecanoic[d]	3.5	17.2	<4	<1.5
	6.5	43.2	6.5	1.14
	20	85.8	16.6	1.19
	(20°C) 22	61.7	20.4	1.53
	(4°C) 22	29.9	13.0	2.12

[a]Enzeco's lipase suspended in hexane at 40°C unless otherwise indicated.
[b]Percent conversion.
[c]Enantiomeric excess of residual 2-octanol.
[d]Results with Sigma's lipase virtually identical.

TABLE 3. ESTERIFICATION OF (±)-2-OCTANOL WITH PORCINE PANCREATIC LIPASE[a]

Acid	Time (hr)	C^b	ee^c	k_R/k_S
Propanoic	70	17.2	6	1.9
Octanoic	48	51.8	56	5.4
2-Ethylhexanoic	70	0.9	—	—
Dodecanoic	48	52.7	66	7.6
Tetradecanoic	70	53.8	66	7.0
Hexadecanoic	70	50.4	52	5.1
Octadecanoic	70	46.2	54	7.4
9-Octadecenoic[d]	70	55.0	78	10.4
Methoxyacetic	70	0	—	—
2-Chloropropanoic	70	0	—	—

[a] Sigma's lipase suspended in hexane at 40°C.
[b] Percent conversion.
[c] Enantiomeric excess of residual 2-octanol.
[d] Cis-isomer.

TABLE 4. ESTERIFICATION OF (±)-2-OCTANOL WITH LIPASES OF *Rhizopus arrhizus* AND *Mucor miehei*[a]

Acid	Time (hr)	C^b	ee^c	k_R/k_S
R. arrhizus				
Propanoic	70	63.8	ca. 13	ca. 1.3
Octanoic	70	14.6	12	6.4
Dodecanoic	70	8.5	10	> 10[d]
9-Octadecenoic (*cis*)	70	19.6	16	5.6
M. miehei				
Propanoic	70	15.8	9.5	3.4
Octanoic	70	39.1	66	> 100
Dodecanoic	70	43.5	66	25.7
Propanoic[e]	70	18.2	14	5.0
Octanoic[e]	70	49.7	74	15.3
Dodecanoic[e]	70	46.1	67	16.4

[a] *R. arrhizus* lipase from Gist Brocades; *M. miehei* lipase powder from Novo; 40°C in hexane.
[b] Percent conversion.
[c] Enantiomeric excess of residual 2-octanol.
[d] Value of *C* too low to make ratio calculation accurate.
[e] Small amount of water added initially, namely 1 μl of pH 6.0 phosphate buffer, which is equivalent to ca. 2% of that which would be generated by complete conversion.

TABLE 5. ESTERIFICATION OF (±)-2-OCTANOL WITH OCTANOIC ACID USING OTHER LIPASES[a]

Lipase	Time (hr)	C^b	ee^c	k_R/k_S
A. niger, 1[d]	120	4.8	—	—
A. niger, 2[e]	120	59.6	83.6	9.1
M. miehei[f]	46	7.5	—	—
M. miehei[g]	70	42.4	26.8	2.8
M. miehei[h]	90	40.8	72.6	>100

[a] Reactions were conducted in hexane at 40°C. Two additional lipases produced very low conversions (*R. niveus*: Amano-N, *R. oryzae*: Amano-FAP).
[b] Percent conversion.
[c] Enantiomeric excess of residual alcohol.
[d] Amano-AP.
[e] Amano-K.
[f] Amano-MAP.
[g] Gist Brocades-S.
[h] Novo-3A.

the hexane layer was washed 2× with equal volumes of water. The organic phase was dried ($MgSO_4$) and concentrated; the resulting product mixture was then derivatized for GLC analysis of residual alcohol. The esters present were also observed sometimes but did not interfere. Hydrolyses and transesterifications were conducted and analyzed in a similar manner and are reported in Tables 6 and 7. Larger amounts of enzyme were required to effect hydrolysis. The following procedures are representative.

Kinetic Resolution by Hydrolysis. (*R*)-2-octanol from racemic 2-octyl octanoate, the ester (5.12 g, 20.0 mmol) was vigorously stirred in 20 ml of distilled water at room temperature containing 1.0 g of Novo's *Mucor miehei* lipase powder. The pH was maintained at 7.0 with 2 N NaOH. After 2 days, 1.10 ml of base had been consumed (11% conversion). Ethanol (50 ml) was added, and the mixture was extracted with 3 × 50 ml of hexane. The extract was washed with 2 × 30 ml of water, dried ($MgSO_4$), and concentrated. Distillation of the residue produced 0.27 g of 2-octanol that was 98.0% *ee* (*R*): bp 90°C/30 mm. Further hydrolysis (another 12.5% conversion) gave an additional 0.1 g that was 97% (*R*).

For (*R*,*S*)-8-Methyl-(*R*)-2-decanol from racemic octanoate ester, the ester (5.95 g, 20 mmol) was stirred in 20 ml of Novo-225 after brief sonication. Novo-225 is a solution of glycerol-stabilized *M. miehei* lipase. The mixture was maintained at room temperature and at pH 7.0 until 25–30% conversion had occurred (24 hr). The product was worked up as above to give 0.67 g of 8-methyl-2-decanol that was 97.6% *ee* at the carbinol carbon: bp 54–70°C/0.2 mm.

TABLE 6. HYDROLYSIS OF ESTERS OF (±)-2-OCTANOL[a]

	C^b	ee^c	Time (hr)	k_R/k_S
C. rugosa (Enzeco)				
Propanoate	30	4		
Octanoate	30	8		
Dodecanoate	21	0		
M. miehei (Novo powder)				
Propanoate	22.7	92	72	31[d]
Octanoate	18.7	97.8	20	>100
Dodecanoate	8.0	95	22	47
(±)-8-Methyl-2-decanol				
Propanoate	8.8	96.8	18	60[e]
Octanoate	15	98.7	90	>100[e]

[a] Reactions were conducted at ambient temp at pH 7.0, no additives.
[b] Percent conversion.
[c] Enantiomeric excess of product alcohol.
[d] The rate was 59 for this reaction using the liquid form of the lipase, Novo-225.
[e] Novo-225.

Kinetic Resolution by Esterification. For 8-methyl-(R and S)-2-decanols, the racemic alcohol (4.51 g, 26.2 mmol) was dissolved in 45 ml of hexane containing 9.43 g (65.5 mmol) of octanoic acid. Novo's lipase 3A (5.0 g) was added, and the heterogeneous mixture was sitrred at 30°C for six weeks. The progress of the reaction was followed by GLC monitoring of the residual alcohol enantiomer content, and the reaction was worked up by filtration to recover enzyme. The filtered solution was washed with aqueous base and then water. After the organic phase had been dried, the solvent was removed and the alcohol distilled [bp 65–72°C/0.1 mm, 1.83 g of 94.6% 2(R)]. Distillation was continued until the head temperature was 120°C. This produced ca. 0.2 g that was discarded. The pot residue was saponified with 33 ml of 6 N KOH and 50 ml of MeOH under reflux overnight. The saponified material was extracted into hexane and processed as above. Distillation produced 1.88 g of 93.2% 2-(S)-alcohol.

Recovered lipase was exposed competitively with fresh Novo-3A in an esterification of 2-octanol with octanoic acid. The recovered enzyme preparation had retained ca. 85% of its activity.

RESULTS AND DISCUSSION

Lipases are a class of enzymes that hydrolyze natural triglyceride mixtures (animal fats and vegetable oils) to diglycerides, monoglycerides, glycerine, and fatty acids. Excellent reviews have been provided of their purification, char-

TABLE 7. INTERESTERIFICATIONS

Lipase and ester	Time (hr)	Temperature (°C)	C^a	ee^b	k_R/k_S
C. rugosa					
Tributyrin	24	25	30–40	6	ca. 1
Tributyrin[c]	24	25	ca. 5	4	ca. 1
Tributyrin[c]	48	25	10–15	4	ca. 1
Methyl methoxyacetate	24	40	0	—	—
Methyl α-chloropropanoate	24	40	>50	<4	1
Olive oil	20	40	100	0	—
Olive oil	4	40	58	0	1.0
Porcine pancreas					
Tributyrin	70	40	40	38	5
Olive oil	20	40	54	39	2.8
M. miehei (Novo powder)					
Tributyrin	70	40	50	72	13
Tributyrin[d]	20	30	29	26	5.8
Trioctanoin[d]	24	30	28	6	1.4
Olive oil[d]	24	30	47	28	2.5
Tallow[d]	24	30	47	26	2.5
Tributyrin[e]	20	30	32	50	>100
Tributyrin[f]	20	30	16	24	>100
Tripropionin[f]	168	40	50	83	28
Tallow[f]	72	40	60	71	6

[a] Percent conversion.
[b] Enantiomeric excess of residual alcohol.
[c] Chromosorb 101 (Cambou and Klibanov, 1984a).
[d] Hexane solvent.
[e] One μl of water.
[f] Hexane solvent plus 1 μl of water.

acterization, and catalytic action (Brockerhoff and Jensen, 1974; Borgstrom and Brockman, 1984). In addition to pork pancreatic lipase that is available as a by-product of the swine industry, a number of fungal lipases have been made available to benefit the dairy and cheese industries. Considerable interest exists in enzymatic restructuring of natural triglycerides and in enzymatic cleavages on an industrial scale. The outlook for lipase availability as catalysts for general organic synthesis therefore seems positive, although their potential has not yet been fully explored.

Triglyceride lipases are uniformly lacking in stereoselectivity in hydrolysis of triglycerides (Brockerhoff and Jensen, 1974, pp. 56–58). Nevertheless, stereobiases of varying degree are exhibited by these enzymes in esterification, transesterification, and hydrolysis of other substrates. Lipases of *Candida rugosa* (Cambou and Klibanov, 1984a,b; Langrand et al., 1985; Laumen and

Schneider, 1984; Satoru et al., 1985), porcine pancreas (Lavayre et al., 1982; Ladner and Whitesides, 1984; Wang et al., 1984), *Pseudomonas aeruginosa* (Hamaguchi et al., 1984, 1985), and *Rhizopus* sp. (Laumen and Schneider, 1984) have been variously employed to effect kinetic resolutions or to selectively esterify diols that contain stereoisotopic alcohol groups. In addition there is an indication that lipases may exhibit a stereobias in transesterification, therefore likely esterification as well, with alcohols that closely resemble diglycerides (Sonnet et al., 1986).

With this information in mind we wished to screen available lipases for possible utility in preparing 8-methyl-2-(*R* and *S*)-decanol, the parent alcohol of a pheromone structure that is common to a number of diabroticites (Guss et al., 1985). Several of these species are serious pests of corn, and the ability to monitor selectively may depend critically on the stereochemistry of the bait. Current knowledge indicates that the configuration of the methyl-bearing hydrocarbon center must be *R* for all species; the *S* configuration is inactive. Hence this center may be racemic in chemicals intended to attract with no disadvantage beyond dilution. The carbinol center, however, is species differentiating. Moreover, evidence has accrued that indicates that several percent of antipodal configuration of a pheromone can inhibit response (Brand et al., 1979). Since racemic 8-methyl-2-decanol can be readily prepared in several ways (Sonnet et al., 1985), including a procedure developed for the USDA by Zoecon Corporation,[1] an efficient enzymatic resolution of the racemic carbinol center might be a useful accessory reaction.

Initially 2-octanol and its esters were exposed to various reaction conditions using commercial lipases. The enzymes were calibrated for activity on olive oil (ca. 85% triolein) using an "initial rate assay" (Methods and Materials). The results are given in Table 1. Considerable differences exist in the lipase activity of these materials. In part this is attributable to the manner and degree of purification of each commercial preparation as well as to potential intrinsic differences. The lipase of *Candida rugosa* (previously named *C. cylindracea*) offered by Sigma is, we learned later, supplied by Enzeco, and the initial rate measurement of activity and subsequent evaluation of stereobias in resolutions were consistent with this. However, great differences in activities toward olive oil were exhibited by the several commercially available lipases of *Mucor miehei*. Moreover, the degrees of stereobias exhibited (see below) were also different. Because of the heterogeneous character of these preparations, only a more probing study of the protein content and knowledge of the (proprietary) preparation of the lipase could provide a definitive answer to questions concerning the enzymes themselves. We have opted therefore to treat these materials simply as undefined but, if reproducibly prepared, potentially useful catalysts for organic synthesis.

[1]Specific Cooperative Research Agreement No. 58-7B30-1-176, August 14, 1981.

Esterification. The alcohol, 2-octanol, was allowed to react in hexane with an organic acid at a selected temperature for a period of time in the presence of the lipases. Residual free fatty acid was determined by titration and the enantiomeric composition of the residual alcohol was determined by conversion to carbamates with (*R*)- or (*S*)-α-methylbenzyl isocyanate (Methods and Materials) (Pirkle and Hauske, 1977). Although a number of chiral derivatizing agents for secondary alcohols have been employed (Doolittle and Heath, 1985), the carbamates could be formed directly from crude reaction products, and separations of the diastereomers of 2-octanol and 8-methyl-2-decanol were excellent (Figures 1 and 2). The rate ratio, k_R/k_S could then be calculated (Methods and Materials). The results employing *C. rugosa* lipase (Table 2) were disappointing; the rate ratios were very low although they could be improved by lowering the reaction temperature. Porcine pancreatic lipase (Table 3) proved to be more selective and demonstrated the validity of varying the fatty acid as a means to optimize the selectivity. The lipase of *Mucor miehei* (Novo powder) showed only low selectivity with propanoic acid, but very high selectivity with octanoic acid (Table 4). *Rhizopus arrhizus* lipase was rather unreactive with acids of medium and long chain length as well as only modestly selective (Table 4). Other lipases were evaluated with octanoic acid only, and the results are given in Table 5.

Summarizing these results, *C. rugosa* lipase catalyzed relatively fast esterifications of 2-octanol but with low selectivity. Porcine pancreatic lipase acted more slowly (lauric acid fastest) with greater discrimination. *R. arrhizus* lipase acted slowly also (propanoic acid fastest). *M. miehei* lipase (Novo only) provided greatest discrimination between the enantiomeric 2-octanols. Temperature affects both the overall rate and the relative rate; and α-branching of the acid (see Table 2) dramatically lowers reactivity. Initial presence or absence of small amounts of water affects results as well (Table 4). Since esterification produces water, the observed results starting "dry" must be a composite. Fastest reactions may occur with shorter or longer acid chains, but stereobias (invariably $R > S$) was always poorest with shorter acid chains.

Hydrolysis. The propanoate, octanoate, and dodecanoate esters of 2-octanol were arbitrarily chosen for exposure to lipases. The pH was automatically maintained at 7.0 and the titration thereby indicated instantaneously the degree of conversion. As with the esterifications, the crude reaction mixture was derivatized and analyzed for enantiomeric excess of the alcohol, which was this time the product. Rate ratios were calculated again and the results for *C. rugosa*, *R. arrhizus*, and *M. miehei* lipases are given in Table 6. All of the lipases reacted very slowly and, although emulsifying agents were not specifically added, Novo-225, (*M. miehei* lipase intended for direct lipolysis of triglycerides) reacted no more readily with 2-octanol octanoate than did the Novo powder. This implies that the enzymes are inherently slow in their reactions with esters 2-octanol. Nevertheless, the Novo lipase (both powder and Novo-225)

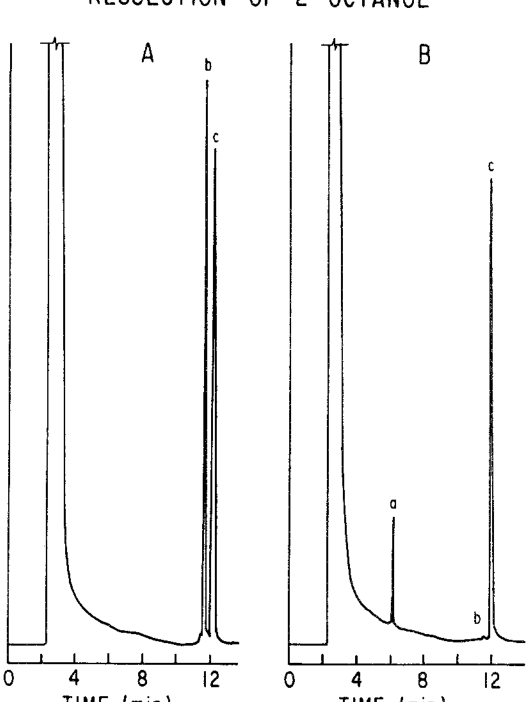
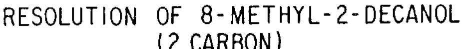


FIG. 1. (A) Analysis of α-methylbenzyl carbamates found by the reaction of (±)-2-octanol with (*R*)-α-methylbenzyl isocyanate by GLC using an SPB-1 capillary column (220°C). Peak b is the *RS* diastereomer; peak c is the *RR* diastereomer. (B) Analysis of 2-octanol obtained by hydrolysis of the racemic octanoate ester using *M. miehei* lipase (Novo, powder). Peak a is contamination by the ester, peaks b and c are the diastereomeric derivatives as in A. The major enantiomeric alcohol formed has the *R* configuration.

did show strong stereoselection, particularly for the octanoate ester; the *R*-esters reacted preferentially whenever bias was noted.

Transesterification. This process appears to be receiving considerable attention in the fats and oils industries. There the application is intended to produce new triglyceride mixtures by displacing existing acid residues with new ones to generate materials with more desirable physical properties. Stereochemistry probably plays an insignificant role in determining gross physical properties compared to positional isomerism on the glycerol moiety. However, one might use a fat or oil as a very cheap source of acid residues for kinetic resolutions in which acid residues are being transferred from glycerol to another (racemic) alcohol.

Reactions of 2-octanol with a selection of esters including triglycerides as



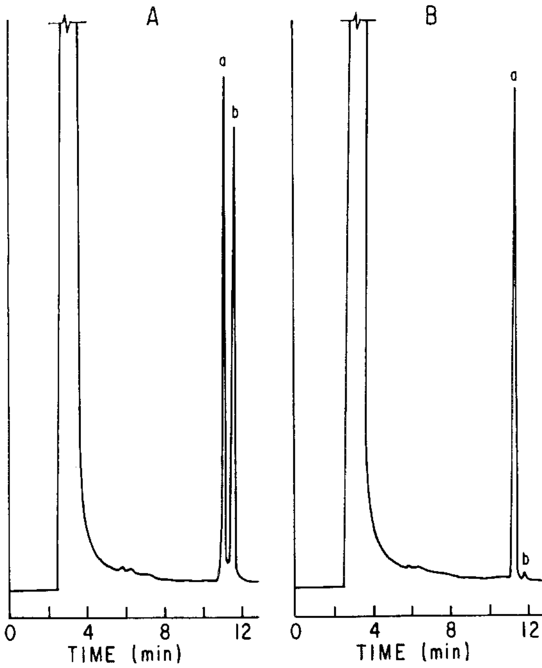


FIG. 2. (A) Analysis of α-methylbenzyl carbamates of $(\pm)$-8-methyl-2-decanol formed by reaction with *S*-α-methylbenzyl isocyanate (SPB-1, 240°C). Peak a is the *RS* diastereomer, and b is the *SS* diastereomer. (B) Analysis of 8-methyl-2-decanol obtained by hydrolysis of the racemic octanoate ester using *M. miehei* lipase (Novo-225). Peak a is the *RS* diastereomer, and the major product of lipolytic hydrolysis has the *R* configuration

sources of acid residues were conducted neat (Methods and Materials). The degree of conversion was estimated by gas chromatography and distillation to recover alcohol. Results are presented in Table 7; only the lipases of *C. rugosa* and *M. miehei* gave reasonable conversions. The most striking observation was that the addition of a small amount of water to reaction mixtures containing the Novo *M. miehei* lipase, but not the other lipases, produced a profound increase in stereoselection. It may be that in a nonaqueous medium the enzyme is deprived of water at the catalytic site, water that if present may be held in such manner that the enzyme operates stereoselectively.

Application. The transesterifications using olive oil and tallow had not shown good selectivity, even with Novo's *M. miehei* lipase. Therefore preparative reactions were limited to hydrolysis and esterification. The octanoate ester of 2-octanol was allowed to react in water at pH 7.0 to 11% conversion

using Novo *M. miehei*. The 2-octanol recovered by distillation, 10% yield (91% of theory based on conversion), was 99% *R*. Similarly, the octanoate ester of 8-methyl-2-decanol was allowed to react to 15% conversion and yielded (10%) 98.7% 2-*R* isomer. The rate ratio for this ester is therefore ca. 95. Employing the currently available liquid form of this lipase, Novo-225, a 26% yield of 98.8% 2-*R* isomer was obtained implying a somewhat improved stereobias. These reactions had been allowed to proceed at room temperature for 24–40 hr.

Esterification of racemic 8-methyl-2-decanol in hexane with octanoic acid was conducted with Novo's lipase 3A, which is derived from *M. miehei* and is formulated on an ion exchange resin. Because that rate ratio is again fairly high (ca. 100), and the resin-borne enzyme is very stable, it is possible to produce both *R* and *S* alcohols with high configurational purities and in good yields. Reactions were allowed to proceed to nearly 50% conversion and the recovered ester was saponified to *R* alcohol. Adjustments (reduction) in percent conversion can be made to provide a purer *R* ester; a higher conversion would result in an alcohol richer in the *S* configuration. In a particular experiment, 41–42% yields of each alcohol were obtained: 94.6% *R* and 93.2% *S*; in another, similar yields were obtained: 95.4% *R* and 91% *S*.

These processes represent resolutions of aliphatic alcohols that are among the more difficult structural types to resolve. Simple secondary alcohols frequently find use in organic syntheses and occasionally have important biological activity. Since the *M. miehei* lipase is commercially available, and the procedures employed are quite simple, these results should prove quite useful.

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INTRA- AND INTERSPECIFIC CHEMOATTRACTION STUDIES ON *Schistosoma mansoni*, *S. japonicum*, AND *S. haematobium* IN THE ABSENCE OF BARRIERS

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Abstract—Heterosexual intra- and interspecific chemoattraction studies were done in polycarbonate chambers without barriers using 8- to 10-week-old *Schistosoma mansoni* from mice, 10-week-old *Schistosoma japonicum* from hamsters, and 14-week-old *Schistosoma haematobium* from hamsters. Experiments were done for up to 4 hr at 37°C in a humidified incubator. Single worms were not attracted to decoys or heat-killed worms. Heterosexual intraspecific and interspecific attraction was significant at all time points with all combinations. No clear differences were apparent between intra- and interspecific attraction. Differences between same and different *S. japonicum* or *S. haematobium* pairs were not significant at any time point.

Key Words—Trematode, digenetic, *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma haematobium*, chemoattraction, sexual attraction, interspecific, fluke, blood.

INTRODUCTION

Using a bioassay system described by Imperia et al. (1980) to study the effects of *Schistosoma mansoni* excretory–secretory products in vitro, Eveland et al. (1982) demonstrated greater chemoattraction responses between male and female worms which were initially paired with each other, than those which were

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paired with other males or females. Eveland et al. (1983) also showed that homosexual, but not heterosexual, attraction between *S. mansoni* adults was reduced in the presence of mechanical barriers constructed from dialysis tubing with a molecular weight cutoff of approximately 12,000.

A number of investigators, including Vogel (1941), Armstrong (1965), Taylor et al. (1973), and Southgate et al. (1976, 1982) have reported cross-mating of schistosomes in vivo, but similar in vitro studies have not been done.

We report here results of studies to compare intra- and interspecific chemoattraction in our bioassay system, using *S. mansoni*, *S. japonicum*, and *S. haematobium*.

METHODS AND MATERIALS

Experimental Animals and Media. Adult 8- to 10-week-old PR-1 strain *Schistosoma mansoni* (Richards and Merritt, 1972; Fletcher et al., 1981) from CD-1 mice (Charles River, Wilmington, Massachusetts) and the albino M line of *Biomphalaria glabrata* (Newton, 1955) were used. Also used were a Chinese strain of 10-week-old *S. japonicum* from hamsters and 14-week-old *S. haematobium* from hamsters. The *S. mansoni* had been routinely maintained in our laboratory for several years and through many passages. The *S. japonicum* and *S. haematobium* were supplied by the University of Lowell (Lowell, Massachusetts) under an NIAID supply contract. Worms were recovered by perfusion of the portal and mesenteric veins and maintained until use at 4°C in Earle's balanced salt solution (EBSS) containing 0.1% glucose and 0.5% lactalbumin hydrolysate (Clegg, 1965; Imperia et al., 1980). All experiments were done using this incubation medium. The time between perfusion and the beginning of an experiment was always less than 1 hr. Distances between worms were measured in millimeters at 0.5, 1.0, 2.0, 3.0, and 4.0 hr. From 13 to 18 trials were carried out for each experimental design.

Bioassay Design. Polycarbonate chambers with 14 linear channels, each channel 3 cm long, 1 cm wide and 1.5 cm high, were used. Each channel was filled to a height of 0.85 cm with 1% agar in phosphate-buffered saline (pH 7.2). The agar was overlaid with EBSS. Experiments were carried out using nonbarrier designs (Eveland et al., 1982) at 37°C in a humidified incubator. Worms were washed in EBSS then pipetted into the chamber 15 mm apart in separate channels. This distance allowed each worm to move either toward the other, which was initially 15 mm away, or 7.5 mm in the other direction, toward the end of the channel. Distances were measured between worm cetners at each time period (i.e., 0.5, 1.0, 2.0, 3.0, and 4 hr). At each time period, the distance between a worm couple was measured, and the percent attraction determined by the formula: $(15 - D/15) \times 100$, where D = the actual distance between a worm couple at a particular time point.

Observations were based on the mean ±SE values from the summation of 13–18 individual trials. Thus, by this formula 0% attraction would indicate that worms remained at their original distance, and 100% attraction would indicate that all the worms were in contact. Attraction between *S. japonicum* or *S. haematobium* male and female worms from the same or different male–female worm pairs was tested. Also, *S. mansoni* male and female worms of opposite sex but from different pairs were compared with *S. japonicum* or *S. haematobium* worms of opposite sex for their attraction to *S. mansoni*. Tests for the significance of difference between means were performed using Student's t test, and $P < 0.05$ was considered significant.

RESULTS

All worms were alive and active after 4.0 hr in the bioassay. Results are shown in Figures 1–3. Attraction between *S. japonicum* or *S. haematobium* males and females from the same or different worm pairs was tested (Figure 1). At 0.5 hr, *S. japonicum* from the same pairs were 64% attracted, compared to 45% attraction between worms from different pairs, and at 1.0 hr, *S. japonicum* from the same pairs were 71% attracted to each compared to 53% attraction between worms from different pairs. However, the differences were not significant ($P > 0.05$). Likewise, differences between the same and different *S. haematobium* pairs were not significant at any time point.

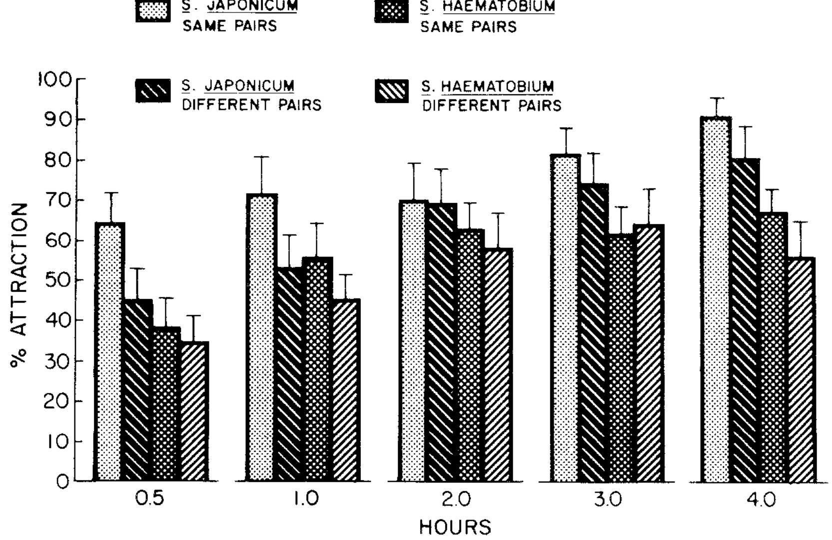

FIG. 1.

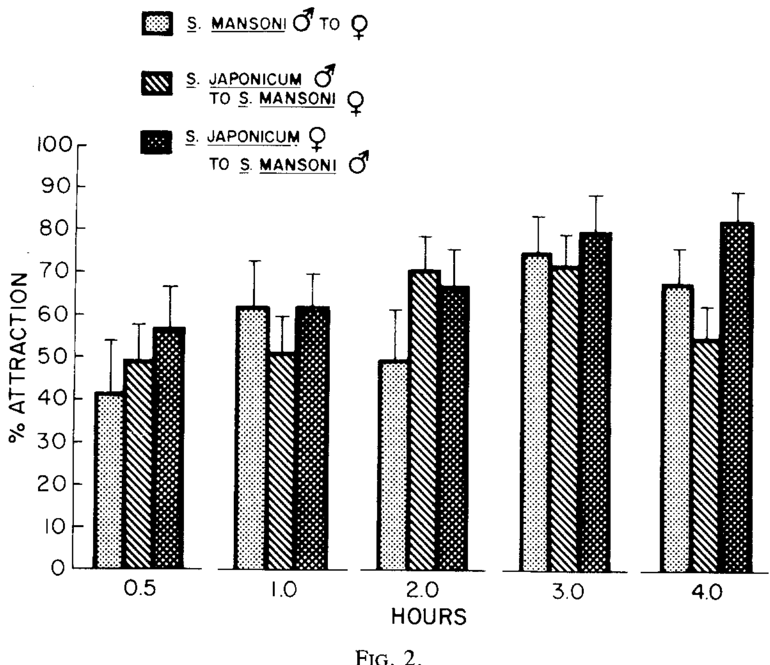

FIG. 2.

Results comparing heterosexual attraction of *S. mansoni* to *S. mansoni*, and *S. mansoni* to *S. japonicum* are shown in Figure 2. Attraction always occurred, and interspecific attraction was as great as intraspecific attraction. At some time points interspecific attraction appeared stronger than intraspecific attraction, but significant differences were not observed at any time point. In both interspecific and intraspecific attraction, 10–15% of the males and females were actually in contact at all observed time points.

Results comparing *S. mansoni* with *S. haematobium* are shown in Figure 3. Attraction always occurred, and interspecific attraction was roughly comparable to intraspecific attraction. In both interspecific and intraspecific attraction, approximately 10–15% of the males and females were actually in contact at all observed time points.

When single worms of either sex were placed at an origin in our bioassay system, they rarely migrated from that spot, although minor migrations were occasionally observed toward both sides of the origin. Likewise, net movement of single worms toward worms killed by heating at 60°C for 1-2 min or toward decoys (filter paper of approximate worm size) was zero.

DISCUSSION

In our bioassay system heterosexual attraction always occurred in the absence of barriers, and studies using *Schistosoma mansoni*, *S. japonicum*, and

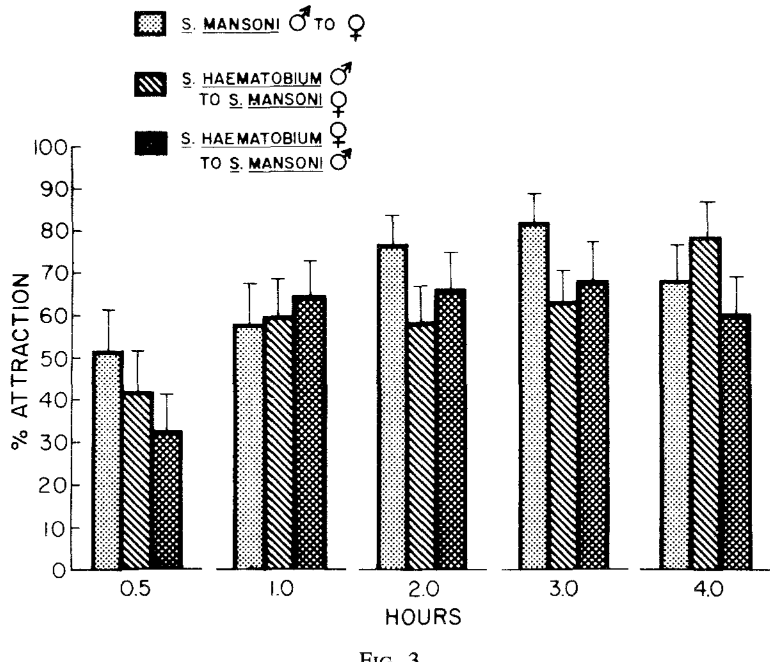

FIG. 3.

S. haematobium demonstrated no clear differences between intra- and interspecific attraction. In *S. japonicum*, slightly more heterosexual attraction between the same than different pairs may have occurred for up to 1 hr, although the differences were not significant ($P > 0.05$). This result was in general agreement with that previously reported for *S. mansoni* (Eveland et al., 1982), although in *S. mansoni* the effect was statistically significant and increased after 1 hr. Differences between worms from the same and different pairs were not observed in *S. haematobium*. The results of our studies, along with Shirazian and Schiller (1982), show that opposite sexes were attracted to each other in vitro and that, at least in *S. mansoni*, worms prefer to couple with their original partners.

In nonbarrier design experiments which we reported previously, homosexual attraction was as strong as heterosexual attraction (Eveland et al., 1982), but when barrier designs were used homosexual attraction was much weaker (Eveland et al., 1983), suggesting that more than one type of chemoattractant may be operative. It is possible that the attractants responsible for interspecific and homosexual effects were similar and differed from those responsible for the more specific effects seen in the presence of barriers. Studies are in progress to test interspecific attraction using barrier designs. In any case, these studies show that at least some schistosome chemoattractants cross species lines, and this suggests that if blocking agents can be developed against schistosome chemoattractants, they may be broadly applied.

Although the exact nature of the chemoattractants is not known, excretory-secretory products and at least their lipophilic fraction are involved (Eveland and Haseeb, 1986; Haseeb and Eveland, 1986).

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TECHNIQUE FOR INJECTING INTACT GLANDS FOR ANALYSIS OF SEX PHEROMONES OF LEPIDOPTERA BY CAPILLARY GAS CHROMATOGRAPHY: Reinvestigation of Pheromone Complex of *Mamestra brassicae*[1]

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Abstract—The structure elucidation of sex pheromones of Lepidoptera by a solid-sample injection technique in conjunction with capillary gas chromatography is described. The applicability of this method in GC and GC-MS modes was demonstrated by reanalyzing the sex attractants of females of *Ostrinia nubilalis* and *Bombyx mori*. The pheromone complex of *Mamestra brassicae* was reinvestigated and (Z)-9-hexadecenyl acetate and (Z)-11-hexadecenol were found in addition to already known pheromone components of this species. By using the solid-sample injection, the exact site of pheromone release could be determined in *M. brassicae*.

Key Words—Solid-sample injection, sex pheromone analysis, *Ostrinia nubilalis*, *Bombyx mori*, *Mamestra brassicae*, Lepidoptera, Pyralidae, Bombycidae, Noctuidae, site of pheromone release.

INTRODUCTION

In the investigations of substances present in extremely small quantities, a technique which permits direct application of the sample to gas chromatography offers a number of advantages over the more popular sample injection methods involving solutions. The most obvious advantage is that dilution of the sample

[1] Pheromones 52. Pheromones, 51: Bestmann, H.J., and Schmidt, M. 1985. *Tetrahedron Lett.* 26:6171–6174.

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is avoided. Furthermore, no impurities from the solvent are introduced into the samples, the lifetime of GC columns is increased, and the earlier-eluting GC peaks are not masked by the solvent peak. Conformational and structural changes in a substance, which in very occasional cases can be caused by contact with the solvent, can also be avoided.

Such a procedure, often referred by chromatographers as "solid sampling," is particularly useful to analyze volatile materials present in minute quantities in samples of animal tissue. When such a tissue is extracted with a solvent, only a small portion of the extract normally can be injected into the gas chromatograph. However, if the solvent is removed before chromatography, considerable quantities of the volatile substances are lost during the evaporation process and high-boiling impurities from the solvent are concentrated in the sample. But if the tissue can be directly introduced into the gas chromatograph such problems can be avoided.

A device for crushing small samples in the injection area of a gas chromatograph had been first described by Bowman and Karman as long ago as 1958. A similar injection technique described by Morgan and Wadhams (1972) has been extensively used in the study of glandular volatiles of ants (Attygalle et al., 1983, and references therein). This method, in which the sample is sealed in a glass tube and crushed in the heated injection area, although generally used with packed columns only, has been applied recently with capillary columns (Attygalle et al., 1987; Billen et al., 1986). A method has also been described in conjunction with capillary columns, where the glandular liquid present in a reservoir is withdrawn into a glass capillary and dropped into the heated injection port (Morgan et al., 1979). Bridges and Guinn (1980) described a solid-injection method for studying bark beetle pheromones.

A commercially available solid sampler (Perkin-Elmer, MS 41) has been used in conjunction with packed columns in the study of sex pheromones of Lepidoptera, first by Weatherston and MacLean (1974) and subsequently by Descoin's group in France (Descoins and Gallois, 1979, and references therein; Frerot et al., 1984). Although a modification of this system has been used with capillary columns (Buser and Widmer, 1979), no applications in the investigations of lepidopteran pheromones have been reported. Hitherto, the advantages of the solid-injection technique and the high-resolution power of fused silica capillary columns have not been utilized simultaneously in the analysis of lepidopteran pheromones. Here, we describe such a technique and its application in the investigation of sex pheromones of Lepidoptera. The method was successfully employed also in mass spectrometry linked with gas chromatography.

METHODS AND MATERIALS

Solid-Sample Injector. A solid-sample injector was built similar to that described by Morgan and Wadhams (1972) for packed columns. The sampler

consisted of a 11-cm-long stainless-steel tube (3 mm OD, 2 mm ID; 4 in Figure 1) closed at its lower end, with an elongated hole (12 mm; Figure 1, 12) on one side, a spike (Figure 1, 13) soldered to the bottom, and a stainless-steel plunger (Figure 1, 1) fitting inside the tubing. The plunger (1) and tubing (4) were tightened together by a silicone rubber septum and a screw nut (Figure 1, 2).

The splitless injector of the gas chromatograph was modified to take the solid sampler. The regular insert liner was replaced with a larger diameter glass

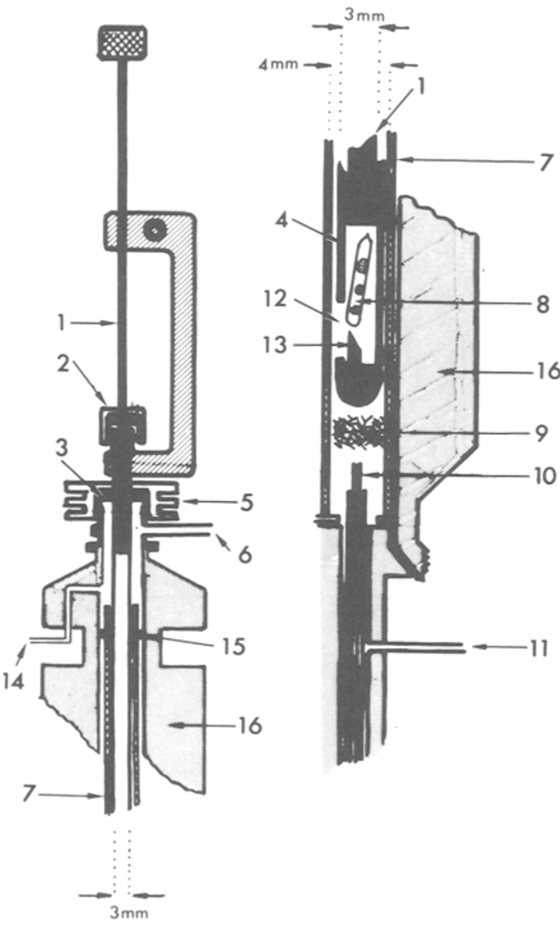

FIG. 1. Schematic presentation of the solid sample injector. 1 = plunger; 2 = screw nut; 3 = silicone rubber septum; 4 = metal tube; 5 = septum cap; 6 = septum flush line; 7 = glass insert liner; 8 = sample sealed in glass capsule; 9 = silanized glass wool plug; 10 = fused silica capillary column, 11 = split vent; 12 = elongated hole; 13 = spike; 14 = carrier gas line; 15 = rubber O ring; 16 = heated injector block.

tube (Figure 1, 7; 8 cm × 4 mm ID) that could accommodate the solid sampler. The sampler was tightened to the injection port by a silicon rubber septum (Figure 1, 3) and the septum cap (Figure 1, 5). A silanized glass wool plug (Figure 1, 9) in the glass tube (Figure 1, 7) prevented glass debris entering the GC column (Figure 1, 10).

Gas Chromatography with Solid-Sampling Technique. Gas chromatography (GC) was performed on a Packard-United Technologies-438A instrument equipped with a splitless injector, a flame ionization detector, and a Shimadzu Chromatopac C-R3A data system. A fused silica capillary column (12 m × 0.22 mm) with a chemically bonded CP-19 (≙OV-17) stationary phase of 0.2 μm film thickness was used for the analysis. The oven temperature was kept at 60°C for 2 min and increased to 270°C at 10°/min. Further analysis was carried out on a fused silica capillary column (25 m × 0.22 mm) coated with SP-2340 stationary phase, the oven temperature kept at 60°C for 2 min and increased to 195°C at 4°/min. Nitrogen was used as the carrier gas at a flow rate of 0.5 ml/min.

In use, the solid sampler was screwed tightly onto the hot injection port, the nut (2 in Figure 1) unscrewed, and the plunger (1 in Figure 1) withdrawn (under nitrogen flow). The glass capsule (Figure 1, 8; 2 cm × 2 mm) containing the sample to be analyzed was inserted, the plunger (Figure 1, 1) partly depressed, and the nut (Figure 1, 2) retightened. The capsule (Figure 1, 8) was kept in the injection port (220°C) for 2–3 min to ensure the volatilization of the substances from the tissue and allow the carrier gas flow to stabilize. The split vent was closed, and the plunger was lowered with a distinct push to crush the capsule against the spike (Figure 1, 13), thus releasing the volatile substances into the GC column. The split vent (Figure 1, 11) was opened after 0.5 min. After each injection, the sampler was removed and the glass debris was emptied from the glass insert liner.

The quantification of pheromone components was done by using a solution of (Z)-11-hexadecenyl acetate (Z11:HDA) as an external standard. All the chemicals used in this study were from our laboratory collection of pheromones.

Gas Chromatography–Mass Spectrometry. A Finnigan 9502 gas chromatograph, fitted with a split-splitless injector (Grob type), linked to a Finnigan 3200E quadrupole mass spectrometer with a Data System 6000 was used. A fused silica capillary column (CP-19, 38 m × 0.22 mm) was directly coupled to the mass spectrometer, and helium was used as the carrier gas at a flow rate of 1 ml/min. EI spectra (70 eV) were recorded at a rate of 2 sec/scan. The injection port of the GC was modified to take the solid sampler, and samples were injected as described above. The split vent was kept closed for 1 min. The oven was kept at room temperature for 3.5 min and then programmed from 120 to 260°C at a rate of 6°/min. Further analysis was performed on a fused silica capillary column (50 m × 0.22 mm) coated with SP-2340 stationary phase.

The oven temperature was kept at 60°C for 4 min and increased to 195°C at 6°/min.

Insect Rearing. The pupae of *Mamestra brassicae* and *Ostrinia nubilalis* were obtained from a laboratory colony maintained at Hoechst AG (Frankfurt/M.). The pupae of *Bombyx mori* originated from the Max Planck Institute, Seewiesen. The pupae were sexed, placed in plastic boxes containing saw dust, and maintained on a 16-hr light–8-hr dark cycle. After emergence, the insects were observed during the scotophase, under a red darkroom lamp, to study the calling behavior and period.

Encapsulation of Pheromone Glands. The samples were prepared from 2- to 4-day-old female moths. First, the abdominal tip (the last two abdominal segments) of a calling female was stripped off with a pair of scissors and the "pheromone gland" was excised carefully under a binocular microscope, using a pair of tweezers and a scalpel. For example, in *Mamestra brassicae* and *Ostrinia nubilalis*, the intersegmental glandular membrane between segments VIII and IX was excised and cleaned by removing the unfertilized eggs, gut contents, hemolymph, and other lipid material found underneath. In *Bombyx mori*, the pair of intersegmental scent sacs ("sacculi laterales") were removed and cleaned in the same way.

One to five glands prepared in this way were pushed into the bottom of a 2-cm × 2-mm, thin-walled soda glass capillary closed at one end, and the open end was sealed immediately in a micro-flame. (The thin-walled capillaries were specially made since ordinary melting point tubes are too strong for this purpose). The sealed samples were either used immediately or stored at −20°C until analyzed.

RESULTS AND DISCUSSION

Pheromone Gland Volatiles of Females of Ostrinia nubilalis *and* Bombyx mori. The two species, belonging to the families Pyralidae and Bombycidae, respectively, were selected to demonstrate the applicability of the solid sampling technique in GC analysis of sex pheromones of Lepidoptera. The glandular compositions of these two species are known.

In female European corn borers, *Ostrinia nubilalis* (Pyralidae), mixtures of geometric isomers of 11-tetradecenyl acetate have been found as the sex pheromone. The proportion of the isomers varies with the geographical origin of the moths (Klun et al., 1975). The main peak in the chromatogram of the sample investigated corresponded to (Z)-11-tetradecenyl acetate (Z11:TDA) (Figure 2), thus indicating our sample belonged to the "(Z)-strain." Furthermore, besides the main acetate, tetradecyl acetate (TDA), (*E*)-11-tetradecenyl acetate (*E*11:TDA), and (Z)-11-tetradecen-1-ol (Z11:TDOl), six hydrocar-

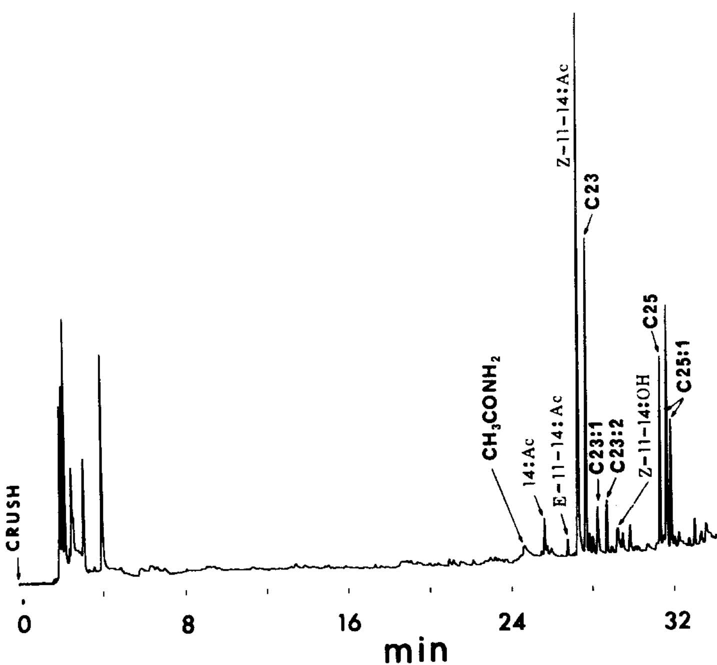

FIG. 2. GC-FID chromatogram obtained from one pheromone gland of female *Ostrinia nubilalis* by solid sampling on a 25-m × 0.22-mm fused silica capillary column SP-2340. Oven temperature was held at 60°C for 2 min and increased to 195°C at 4°/min. 14:Ac = tetradecyl acetate, *E*11–14:Ac = (*E*)-11-tetradecenyl acetate, Z11–14:Ac = (*Z*)-11-tetradecenyl acetate, Z11–14:OH = (Z)-11-tetradecen-1-ol, C23 = tricosane, C23:1 = tricosene, C23:2 = tricosadiene, C25 = pentacosane, C25:1 = pentacosene.

bons of C_{23} to C_{25} range, and the methyl esters of hexadecenoic and octadecanoic acid (not shown in the figure) were also found in the gland.

The stationary phase SP-2340, containing 100% cyanopropyl functionality, used in this study can resolve most of the positional and geometrical isomers of monounsaturated compounds found as lepidopteran pheromones (Heath and Tumlinson, 1984). The identification of the peaks was done by the comparison of retention times with those of authentic materials. Additional confirmation was provided by the mass spectrometric method described by Horiike and Hirano (1982). According to this method, the intensities of various peaks in an ion cluster of a spectrum of an unknown pheromone acetate, when compared to those obtained under the same spectroscopic conditions from a series of positional isomers of monounsaturated alkenyl acetates, can indicate the position of the double bond. The relative abundance of TDA, *E*11:TDA, *Z*11:TDA, and *Z*11:TDOl were 6, 3, 100, and 9, respectively.

In the sex gland of *Bombyx mori* (Bombycidae) (10*E*,12*Z*)-10,12-hexadecadien-1-ol (bombykol) (Butenandt et al., 1959), a trace of the isomeric (*E*,*E*)-alkadienol (Kasang et al., 1978a) and (10*E*,12*Z*)-10,12-hexadecadienal (bombykal) (Kasang et al., 1978b) have been found. However, when the composition of the gland volatiles of three *Bombyx* glands was investigated by this technique, bombykal could not be detected, by either GC or GC-MS. One major peak appeared in the chromatogram, identified as bombykol by its mass spectrum as well as retention time. A minor GC signal, with a shorter retention time (on CP-19) and mass spectrum identical to that of bombykol, was identified as the *E*,*E* isomer of bombykol by comparison with an authentic sample.

Pheromone Complex of Mamestra brassicae and Location of Pheromone. Very little is known about the exact site where the pheromones are located in female Lepidoptera. Thus, we wanted to reinvestigate the pheromone complex of *Mamestra brassicae* (Noctuidae) by the solid-sampling technique with a special emphasis on determining the exact location in the abdominal tip where pheromones are concentrated.

Mamestra brassicae has a distinct glandular region between segments VIII and IX (Otto et al., 1976) (Figure 3). During the calling period, the female insects raised their wings slightly and protruded the ovipositors, thus exposing the intersegmental glandular membrane. They do not show any calling activity during the first night after emergence (Subchev, 1983). Under our laboratory conditions most females exhibited the calling behaviour about 5 hr after the beginning of the dark period on the second night onwards. The calling period lasted about 2 hr, similar to that observed by Subchev (1980).

The present technique enabled the accurate determination of pheromone titers of individual glands. The females of *M. brassicae* use (*Z*)-11-hexadecenyl acetate (Z11 : HDA) as the main component of their specific sex pheromone (Bestmann et al., 1978; Descoins et al., 1978; Novak et al., 1979; Struble et al., 1980). The samples taken at 5 hr after the beginning of the dark period from 1-day-old females showed only traces of Z11 : HDA. Highest pheromone titers were observed in the samples taken under similar conditions from 2-, 3-, or 4-day-old females.

Figure 3 shows the amount of Z11 : HDA present in different parts of the abdominal tip of 3-day-old females sampled during the calling time. This clearly demonstrated that the pheromone components were present only in the intersegmental membrane. The traces of Z11 : HDA present in the other segments may be due to contamination during dissection or to diffusion from the membrane. It was interesting to discover the absence of any significant amounts of pheromone in the tissues beneath the glandular membrane. This proved the fact that the pheromones are concentrated in the glandular epithelium during the calling time. Hence the solid-sampling technique will be useful in biosynthetic studies in determining the fate of a precursor after a local application.

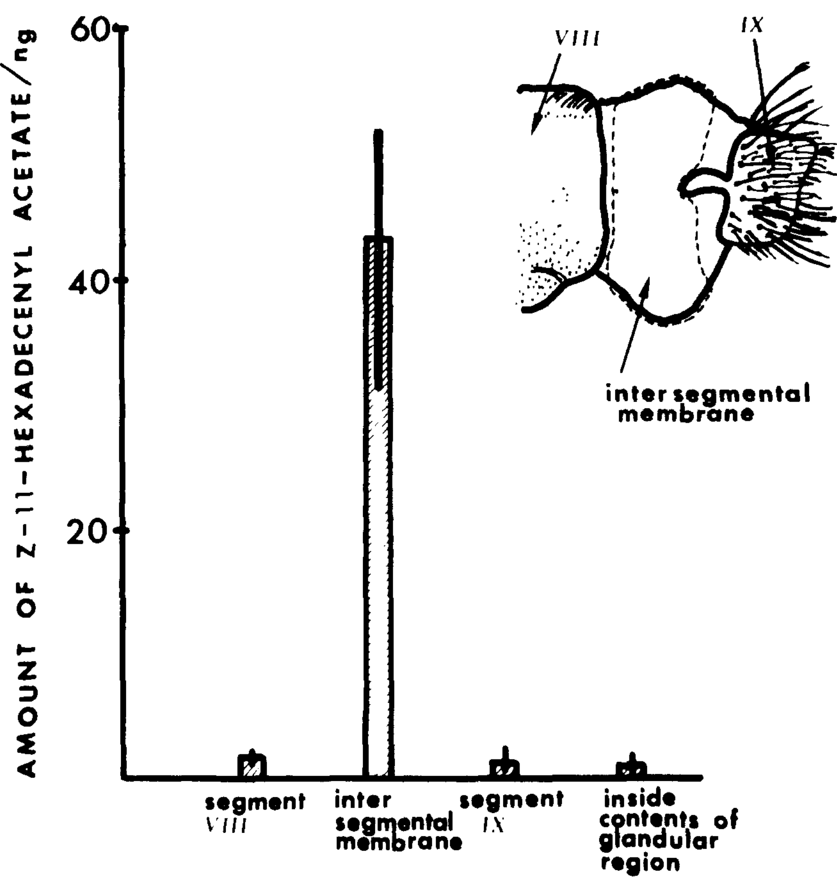

FIG. 3. The amounts of (*Z*)-11-hexadecenyl acetate present in various parts of the abdominal tip of female *Mamestra brassicae*. Each bar represents median from three determinations and the vertical line indicates the range of values. Samples were taken from "calling" insects, three days after emergence.

We also determined the pheromone titers of individual females ($N = 3$) and found a wide variation in the amount of Z11 : HDA present (31.3–51.9 ng per female on the third day). This variation may explain why certain females are more attractive to males than others. This result also shows that the term "female equivalent" (FE) often used by pheromone scientists must be treated with caution. Furthermore, the amount of pheromone found by this method was much more than that reported by Struble et al. (1980) by solvent extraction.

The solid-sampling method was compared with the solvent-extraction method. Only 20–25% of the pheromones were extracted when an excised glandular membrane was kept in 5 μl of hexane at room temperature for 20 min.

Extracting for a longer period of time introduced further impurities. This demonstrated the advantages of solid injection technique, where the "extraction" efficiency is considered very high.

This study renders obsolete the classic method of pheromone extraction, where large numbers of abdominal tips (last few segments) are disintegrated in a solvent followed by purification by liquid chromatography. As shown in Figure 3, the pheromones are located only in a small region of the abdominal tip; therefore when only this glandular region is taken, avoiding the other extraneous material, and extracted, such extracts are clean enough for direct GC examination. Figure 4 shows a chromatogram obtained by injecting one gland of *M. brassicae* by the solid-sampling technique.

The volatile substances identified from the gland of *M. brassicae* consisted of acetamide, tricosane, pentacosane, tetradecyl acetate, hexadecyl acetate, (Z)-9-hexadecenyl acetate, (*E*)-11-hexadecenyl acetate, (Z)-11-hexadecenyl acetate, and (Z)-11-hexadecen-1-ol (see Figure 4).

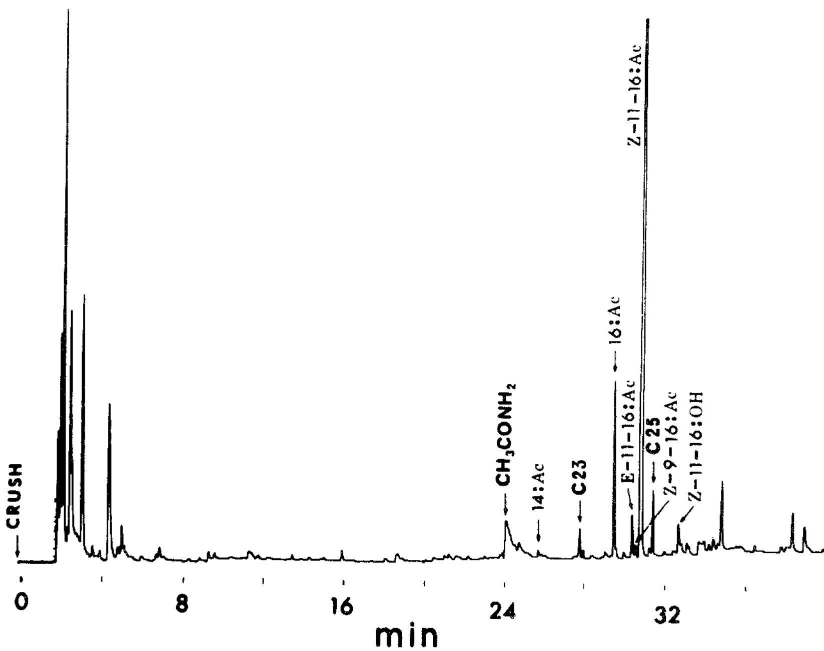

FIG. 4. GC-FID chromatogram obtained from one pheromone gland of female *Mamestra brassicae* by solid-sample injection. GC conditions given in Figure 2. 14:Ac = tetradecyl acetate, 16:Ac = hexadecyl acetate, *E*11-16:Ac = (*E*)-11-hexadecenyl acetate, Z11-16:Ac = (Z)-11-hexadecenyl acetate, Z9-16:Ac = (Z)-9-hexadecenyl acetate, Z11-16:OH = (Z)-11-hexadecen-1-ol, C23 and C25 see Figure 2.

Acetamide and the hydrocarbons are frequently found in lepidopteran glands (Attygalle, unpublished) and are therefore considered as nonspecific body contents, whereas the alkyl and alkenyl acetates and the alkenol were regarded as components of the pheromone complex or at least as biosynthetic precursors or degradation products. Of these substances, (Z)-11-hexadecenyl acetate, (*E*)-11-hexadecenyl acetate, tetradecyl and hexadecyl acetate have been identified already in *M. brassicae* (Bestmann et al., 1978; Descoins et al., 1978; Novak et al., 1979; Struble et al., 1980). The presence of (Z)-9-hexadecenyl acetate and (Z)-11-hexadecenol has not been indicated before. However, the formation of minute quantities of alcohols as artifacts from their respective acetates during sample preparation and chromatography was observed. In most of the comprehensive analytical investigations of lepidopteran sex pheromones (Tamaki, 1986), the presence of alcohols together with their corresponding acetates has been reported. Hence, it is difficult to regard unequivocally the (Z)-11-hexadecenol found as a primary gland constituent.

The solid-sampling technique can be used directly with GC-MS analysis. Figure 5 shows a reconstructed gas chromatogram (RGC) and four mass chromatograms obtained from three glands of female *M. brassicae*. The mass chromatogram of m/z = 222 ($C_{16}H_{30}^+$) has four maxima. Of them, the peaks 3, 4, and 5 have the corresponding maxima in the mass chromatogram for m/z = 61 ($CH_3COOH_2^+$), indicating these three peaks to be isomeric hexadecenyl acetates. Both saturated and unsaturated acetates yield an ion at m/z = 61 due to protonated acetic acid ($CH_3COOH_2^+$). Peak 6 has m/z = 31 and no m/z = 61; therefore it corresponds to a hexadecenol. Peak 2 has m/z = 224 and m/z = 61; therefore it corresponds to a hexadecyl acetate. Peaks 1–6 have the identical retention times to those of authentic tetradecyl acetate, hexadecyl acetate, (*E*)-11-hexadecenyl acetate, (Z)-9-hexadecenyl acetate, (Z)-11-hexadecenyl acetate, and (Z)-11-hexadecenol, respectively, on the SP-2340 column. The relative abundance of these compounds in the gland were 0.5, 8.9, 2.6, 0.8, 100, and 2.3, respectively.

By the applications presented, the advantages of a solid-sampling procedure in conjunction with fused silica capillary columns in the pheromone analysis of Lepidoptera could be demonstrated. There has been a lot of discussion about whether all Lepidoptera produce multicomponent pheromones. This technique is ideally suited for investigations of multicomponent mixtures consisting of compounds of different polarities because the method is not selective. Hence there is no extraction of a single compound or compounds of similar polarity which is an inherent disadvantage of the solvent-extraction technique. If a solvent extraction employing a nonpolar solvent was used, a very polar compound such as acetamide may not have appeared in the chromatogram presented in Fig. 4.

In order to identify biologically active components of the mixture, methods

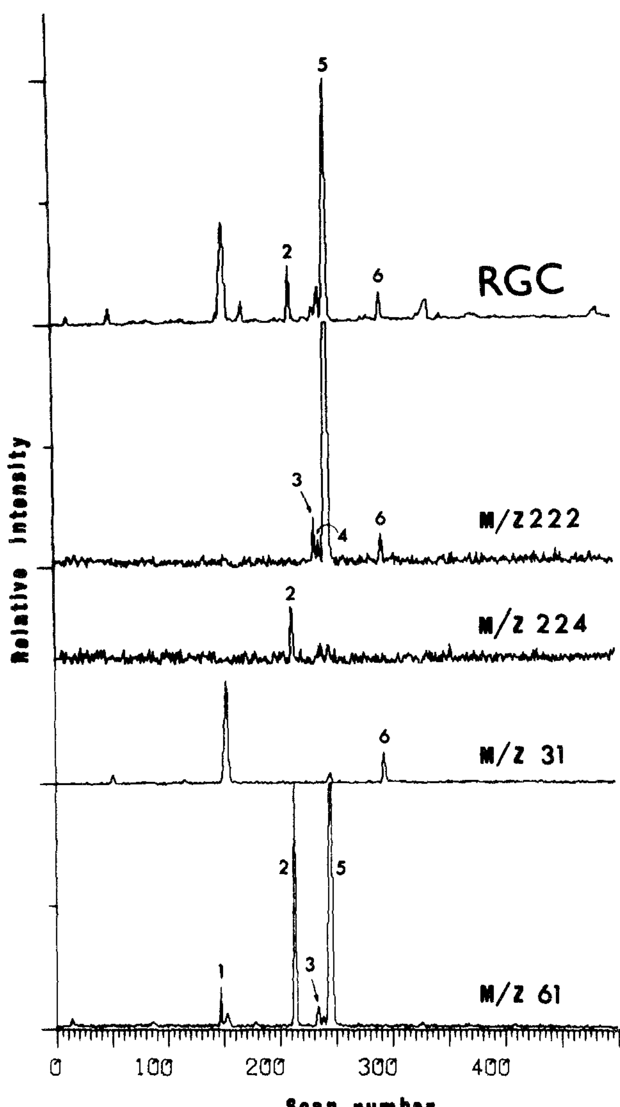

Fig. 5. A reconstructed gas chromatogram (RGC) and four mass chromatograms obtained from three glands of female *Mamestra brassicae* by solid sampling on a 50-m × 0.22-mm fused silica capillary column coated with SP-2340 linked to a mass spectrometer. The oven temperature was held at 60°C for four min and increased at 6°C/min to 195°C. 1 = tetradecyl acetate, 2 = hexadecyl acetate, 3 = (*E*)-11-hexadecenyl acetate, 4 = (Z)-9-hexadecenyl acetate, 5 = (Z)-11-hexadecenyl acetate, 6 = (Z)-11-hexadecen-1-ol.

such as electroantennography, field trials, and behavioral tests are required. This is supported by the fact that (*Z*)-11-hexadecen-1-ol is known to act as a strong attraction inhibitor for *M. brassicae* (Struble et al., 1980). Electrophysiological investigations with male antenna and the substances identified have been performed, the results of which will be published elsewhere.

Additionally, it is noteworthy that we could not detect the aldehyde component bombykal, (10*E*,12*Z*)-10,12-hexadecadienal, in *Bombyx mori* by this method. It was assumed that the aldehyde formation may take place either at a later stage of calling or perhaps is synthesized only just before pheromone release.

The application of solid-sampling technique in the studies of sex pheromones of a number of species with hitherto unknown pheromones is in progress.

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Short Communication

EFFECTS OF NONHOST-PLANT ODORS ON ANEMOTACTIC RESPONSE TO HOST-PLANT ODOR IN FEMALE CABBAGE ROOT FLY, *Delia radicum*, AND CARROT RUST FLY, *Psila rosae*

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Abstract—An inflatable polythene wind tunnel was used for behavioral assays of female *D. radicum* and *P. rosae* in diffuse host- and non-host-plant odor, alone and in combination. Host-plant odor caused an upwind anemotactic response in both species. Changes in fly distribution, relative to wind direction, occurred when onion odor was combined with host odor for *D. radicum* and *P. rosae*, and when sage odor was combined with carrot ordor for *P. rosae*. The assay has potential for screening volatiles for their behavior-modifying effects.

Key Words—Cabbage root fly, *Delia radicum*, carrot rust fly, *Psila rosae*, Diptera, Anthomyiidae, Psilidae, odor-induced upwind anemotaxis, host-plant odor, non-host-plant odor.

INTRODUCTION

The cabbage root fly, *Delia radicum* L. (Diptera; Anthomyiidae) and the carrot rust fly, *Psila rosae* Fab. (Diptera; Psilidae) have host plants within the Cruciferae and Umbelliferae, respectively. Each plant family has a distinctive plant chemistry (Kjaer, 1976; Harbourne, 1971) that is involved in the host-plant location and oviposition behavior of *D. radicum* (Finch, 1980; Städler, 1978) and *P. rosae* (Guerin et al., 1983; Städler and Buser, 1984). Gravid female *D. radicum* responded anemotactically to host-plant odor and allylisothiocyanate

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(Hawkes et al., 1978; Nottingham and Coaker, 1985). Propyl-benzenes from Umbelliferae attracted female *P. rosae* (Guerin et al., 1983), but anemotactic responses have not been demonstrated.

Both species were less exploitive of their host plants in diverse than simple plant plots in field experiments (Tukahirwa and Coaker, 1982; Uvah and Coaker, 1984), with onion odor being important in suppressing numbers of *P. rosae*.

The objectives of this study were (1) to establish whether *P. rosae* responded anemotactically to host-plant odor and (2) to adapt the behavioral assay (Jones et al., 1981; Nottingham and Coaker, 1985) to observe any behavior-modifying effects of non-host-plant odors.

METHODS AND MATERIALS

A series of behavioral assays presenting diffuse odor from host and non-host plants, both singly and combined, were done with female *D. radicum* and *P. rosae* in an inflatable polythene wind tunnel (Jones et al., 1981). The wind tunnel, 0.5 m diameter, with a 2-m-long flight chamber, was constructed from layflat plastic tubing supported by two metal hoops placed at each end of the flight chamber and covered with metal honeycomb (1 cm diameter), wire mesh (0.1 mm), and muslin. The tube was inflated by fans connected at each end and adjusted to give a wind speed of 0.3 m/sec. The tunnel was erected in a controlled environment room maintained at 22°C, 65% relative humidity, and illuminated by overhead fluorescent tubes providing 1500 lux within the tunnel. Ammonium chloride smoke mixed thoroughly with the air in the treatment chamber and passed uniformly through the first screen into the flight chamber.

The host plants used were cabbage (*Brassica oleracea* var. *capitata* cv. Primo) and carrot (*Daucus carota* cv. Danvers), for *D. radicum* and *P. rosae*, respectively. The non-host plants used were onions (*Allium cepa* var. Rhijneburger robusta) and sage (*Salvia officialis*). All the plants were grown under greenhouse conditions.

Ten grams of 1 to 2-month-old cabbage plants or 75 g of carrot plants of similar age were presented alone and together with the same weight of non-host plants of the same age. Odorless control treatments were included.

The plants were put into 100-ml glass beakers containing about 10 ml of water, with the foliage exposed above the rim of the beaker, and the beaker was placed on a tripod, within the treatment chamber, 20 cm from the first screen. In combined odor treatments, beakers containing host and non-host plants were arranged side-by-side on the tripod.

In each of the three replicates of the assay treatments, approximately 15 six- to nine-day-old mated and gravid female *D. radicum* or *P. rosae* were released from the center of the flight chamber after preconditioning for 2 hr in the release cage within the tunnel in the absence of odor. The release cage

consisted of a 15 × 5-cm glass tube enclosed at each end with muslin and suspended 20 cm from the tunnel floor. The flies were released 15 sec after the introduction of the treatment odor by gently removing the muslin from both ends of the tube.

The flies' response was scored by counting the number of flies in the upwind, middle, and downwind thirds of the flight chamber 5 min after their release. The data for the mean percentage of flies in each third of the flight chamber were analysed by chi-square contingency tables, done for paired treatments within each group of four treatments comprising odorless control, host odor and non-host odor alone, and host and non-host odor combined.

RESULTS AND DISCUSSION

Response to Host-Plant Odor. The odorless control elicited similar percentages of *D. radicum* in the upwind and downwind sections (18 and 21%), respectively; whereas the distribution of flies in the cabbage treatment was different from the odorless control ($P < 0.01$), with more than twice the percentage of flies in the upwind than the downwind section (44 vs. 19%) (Table 1).

This upwind anemotactic response supports previous findings that host odor stimulates upwind movement (e.g., Hawkes et al., 1978).

The odorless control elicited similar percentages of *P. rosae* in the upwind and downwind sections (18 vs. 22%); whereas the distribution of flies in the carrot treatment was different from the odorless control ($P < 0.01$), with more flies in the upwind than the downwind section (45 vs. 5.5%) (Table 1).

Host-plant volatiles attracted female *P. rosae* in the laboratory and the field (Guerin et al., 1983), and the present results suggest that they also initiated an upwind anemotactic response, which may be important in its host-finding behavior.

Effects of Non-Host-Plant Odor on Behavioural Response to Host-Plant Odor. The distribution of *D. radicum* in the onion and cabbage plus onion treatments was not different from the odorless control ($P > 0.01$). The distribution of flies in the cabbage treatment, however, was different from that in the cabbage plus onion odor treatment ($P < 0.01$), with the combined odor eliciting a lower percentage of flies in the upwind section (26 vs. 44%) and an increased percentage in the middle section (63 vs. 37%) compared to cabbage odor alone (Table 1).

The distribution of flies in the cabbage plus sage treatment, on the other hand, was not different from that in the cabbage treatment ($P > 0.01$) (Table 1).

The presence of clover odor was not thought to be an important factor causing the decreased exploitiveness of *D. radicum* when it was grown with host plants (Tukahirwa and Coaker, 1982); however, the present results suggest

TABLE 1. NUMBER OF FLIES AND MEAN PERCENTAGE OF FLIES IN UPWIND, MIDDLE, AND DOWNWIND SECTIONS OF FLIGHT CHAMBER IN DIFFUSE ODOR FROM HOST AND NON-HOST PLANTS, PRESENTED SINGLY AND COMBINED.

		Upwind third		Middle third		Downwind third	
	No. flies (±SE)	No. flies (±SE)	Mean %	No. flies (±SE)	Mean %	No. flies (±SE)	Mean %
Female *D. radicum*							
Odorless control (a)	12.7 ± 0.3	2.3 ± 0.7	18.1	7.7 ± 0.5	60.6	2.7 ± 0.3	21.3
Cabbage (b)	14.3 ± 0.5	6.3 ± 0.3	44.1	5.3 ± 0.3	37.0	2.7 ± 0.5	18.9
Onion (a)	14.7 ± 2.4	3.0 ± 0.5	20.4	10.7 ± 1.8	72.8	1.0 ± 0.5	6.8
Cabbage + onion (a)	15.4 ± 1.9	4.0 ± 0.5	26.0	9.7 ± 1.6	63.0	1.7 ± 0.3	11.0
Sage (a)	13.0 ± 1.4	3.3 ± 0.7	25.4	8.0 ± 0.8	61.5	1.7 ± 0.3	13.1
Cabbage + sage (ab)	17.0 ± 0.5	5.3 ± 0.5	31.2	10.0 ± 0.8	58.8	1.7 ± 0.3	10.0
Female *P. rosae*							
Odorless control (a)	11.0 ± 0.5	2.0 ± 0.5	18.2	6.7 ± 0.3	61.9	2.3 ± 0.3	21.9
Carrot (b)	12.7 ± 0.7	5.7 ± 0.9	44.8	6.3 ± 1.1	49.6	0.7 ± 0.3	5.5
Onion (a)	14.4 ± 0.7	3.7 ± 0.3	25.7	8.0 ± 1.2	55.5	2.7 ± 0.7	18.8
Carrot + onion (a)	17.7 ± 0.5	5.0 ± 0.5	28.2	9.0 ± 0.9	51.8	3.7 ± 0.7	20.0
Sage (a)	11.7 ± 0.3	2.7 ± 1.0	23.1	8.0 ± 0.5	68.4	1.0 ± 0.8	8.5
Carrot + sage (a)	16.3 ± 0.5	4.0 ± 0.5	24.5	10.0 ± 1.4	61.3	2.3 ± 0.5	14.1

[a]Treatments with different letters have significantly different distributions of flies ($P = 0.01$ chi-square). ($N = 3$ for all treatments).

that certain non-host odors (e.g., onion) may be capable of modifying aspects of *D. radicum's* host-location behavior.

The distribution of *P. rosae* in the onion, carrot plus onion, sage, and carrot plus sage treatments was not different from the odorless control ($P > 0.01$). However, the distributions of flies from both the carrot plus onion and the carrot plus sage treatments were different from the carrot treatment ($P < 0.01$), with lower percentages in the upwind section in the carrot plus onion (28%) and carrot plus sage (24.5%) than in the carrot treatment (45%) and higher percentages in the middle and downwind sections (Table 1).

Onions intercropped with carrots were successful in reducing carrot fly damage only when they were young and actively growing (Uvah and Coaker, 1984) which, together with the present data, suggests that onion odor modifies aspects of *P. rosae's* host-plant-finding behavior. Sage also affected movement with respect to the wind, suggesting that other non-host odors might be effective at modifying the behavior of *P. rosae* in a similar way.

Further Development of Assay. Further work should incorporate a wider selection of non-host-plant odors together with a range of host to non-host ratios. A larger number of replicates would also be desirable.

The results of these preliminary experiments with non-host odors suggest that a behavioral assay of this type might provide a basis for screening odors for their effectiveness in modifying the behavior of insects that use host-plant odor to locate their host plants. This could not only provide a rationale for selecting intercrops to suppress certain insect pests, but, probably more importantly for future insect pest control, also identify non-host volatiles which could be used in the field to modify insect behavior.

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USE OF A SEX ATTRACTANT AND AN INHIBITOR FOR MONITORING WINTER MOTH AND BRUCE SPANWORM POPULATIONS[1]

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Abstract—A single sex pheromone component was isolated from abdominal tip extracts of female Bruce spanworm. *Operophtera bruceata* (Hulst). This was identified as (Z,Z,Z)-1,3,6,9-nonadecatetraene by capillary gas chromatography (GC), electroantennography, and mass spectrometry. In addition, hexane extracts of female abdominal tips from Bruce spanworm and the winter moth. *O. brumata* L., were analyzed by GC coupled to an electroantennographic detector (GC-EAD). The extracts of *O. bruceata* and *O. brumata* females elicited only a single response, at the same retention time, from antennae of their conspecific and reciprocal males. In field tests conducted in Saskatchewan, traps baited with the synthetic tetraene captured Bruce spanworm males. In tests carried out on Vancouver Island, British Columbia, where the two species coexist, both Bruce spanworm and winter moth males were captured. The attractancy of lures containing the synthetic pheromone alone and in combination with several structurally related analogs was field tested at both locations. One of these, an isomer of the natural pheromone, (*E*,*Z*,*Z*)-1,3,6,9-nonadecatetraene, inhibited the capture of Bruce spanworm males but had no effect upon the number of winter moth males which were taken. Thus, populations of these two very similar species can be distinguished by employing traps baited with pheromone ± the inhibitor. Cone-orifice Hara traps were found useful for field trapping males of both species.

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Key Words—Lepidoptera, Geometridae, *Operophtera bruceata* (Hulst), Bruce spanworm, *Operophetra brumata* L., winter moth, sex pheromone, (*Z,Z,Z*)-1,3,6,9-nonadecatetraene, inhibitor, (*E,Z,Z*)-1,3,6,9-nonadecatetraene.

INTRODUCTION

The major, if not the only, sex pheromone component of the winter moth, *Operophetra brumata (L.), is* (*Z,Z,Z*)-1,3,6,9-nonadecatetraene (1,3Z,6Z,9Z-19:H) (Roelofs et al., 1982; Bestmann et al., 1982). Populations of this species in North America, where it has been accidentally introduced, are now known to occur in British Columbia and the Maritime provinces of Canada and in the northwestern regions of the United States (Embree, 1965; Gillespie et al., 1978). In field tests, Roelofs et al. (1982) found that the synthetic pheromone also attracted males of two closely related species, the Bruce spanworm, *O. bruceata* (Hulst) and *O. occidentalis* (Hulst). The Bruce spanworm occurs transcontinentally in Canada and in the northern United States (Brown 1962) and is very similar to the winter moth in life history, appearance, habits, and hosts. Since the winter moth is such a serious pest of fruit and shade trees and other hardwoods, and now also Sitka spruce (Stoakley, 1985), its spread in North America is of considerable concern. Thus, there is a need to develop a rapid and effective means of monitoring, separately, populations of each species. Light trapping is ineffective for such monitoring since females are wingless and males are only poorly attracted. Furthermore, adult males of these two species can only be distinguished by structural differences in their genitalia (Eidt et al., 1966). Thus, while it is possible to collect adult males of both species by field trapping with 1,3Z,6Z,9Z-19:H and to differentiate between them by dissection and examination of their genitalia, the utility of this method is severely limited.

The report of Roelofs et al. (1982) that Bruce spanworm males were attracted by the winter moth pheromone appeared while our studies to identify the Bruce spanworm and winter moth pheromones were in progress. The coattraction to traps baited with synthetic 1,3Z,6Z,9Z-19:H raises a number of questions: (1) Is 1,3Z,6Z,9Z-19:H also a component of the Bruce spanworm pheromone? (2) If 1,3Z,6Z,9Z-19:H is a component of the Bruce spanworm pheromone, is it the only component or does Bruce spanworm have a multicomponent pheromone which results in the reproductive isolation of the two species? (3) Is it possible to develop species-specific sex attractant lures for Bruce spanworm and winter moth males? In addressing these questions, we have examined and identified the sex pheromone of Bruce spanworm females, reexamined the winter moth pheromone, and have tested the effect of additions of several synthetic analogs to baits containing 1,3Z,6Z,9Z-19:H upon winter moth and Bruce spanworm male attraction in the field.

METHODS AND MATERIALS

Insects, Pheromone Isolation, and Identification. Late-instar Bruce spanworm larvae were collected from aspen near Stettler, Alberta, and allowed to pupate in cages containing aspen leaves. Pupae were sexed and stored separately in the laboratory at 22°C for three months and then at 0°C for two to three weeks to initiate adult emergence. Males were used for electroantennography and females as a source of sex pheromone. Pheromone was obtained by a 20-min pentane extraction of the terminal three to four abdominal segments from 2- to 4-day-old calling females. The extract was concentrated under a stream of N_2, fractionated by GC using an SP-2100 column (30 m × 0.25 mm ID), programmed from 40°C to 90°C at 30°C/min and then 230°C at 4°C/min. Timed fractions, 15 sec each, of the split column effluent were collected in Dry-Ice cooled capillaries and assayed by EAG (Wong et al., 1984a). The contents of EAG-active fractions were analyzed by combined gas chromatography–mass spectrometry (GC-MS) using a model 4000 (Finnigan) system equipped with an SP-2100 column (60 m × 0.32 mm ID). Both EI (70 eV) and chemical ionization (CI) (methane reagent gas) mass spectra were obtained. Spectra were acquired at 1 scan/sec over the range of 40–320 mass units. Retention times and mass spectra of components in the extract were compared with data obtained using synthetic standards.

In addition, antennal stimulatory components were detected as they eluted from a capillary column by employing a GC equipped with both a flame ionization detector (FID) and an electroantennographic detector (GC-EAD) (Arn et al., 1975). Detection of pheromone components by this technique permits the recognition of closely eluting stimulatory compounds which could have gone unrecognized using the timed collection method. Hexane extracts and surface washes of abdominal tips of Bruce spanworm and winter moth females (obtained by field collection) were prepared and analyzed using antennae from males of the same and the reciprocal species for component detection. A DB-210 column (30 × 0.32 mm ID), programmed as above, was employed in these analyses. Signals from the FID and EAD were displayed simultaneously on two Hewlett Packard model 3392A recording integrators. Two internal standards, heptadecane (17:H) and tetracosane (24:H), were added in each analysis to facilitate comparisons between analyses.

Synthetic Chemicals. Monoepoxides and other compounds used, except for the following, were synthesized as described previously (Wong et al., 1984b, 1985). The mixture of monoepoxytriene regioisomers [(CME)-1,3Z,6Z,9Z-19:H], prepared from 1,3Z,6Z,9Z-19:H, were not resolved but the three monoepoxydiene isomers, from 3Z,6Z,9Z-19:H, were separated by HPLC using a 50-cm Whatman Magnum 9 Partisil 10 silica column, eluted with 3% ether in pentane. (Z,Z,Z)-9,12,15-Octadecatrienal (9Z,12Z,15Z-18:ALD) was prepared

from linolenyl alcohol (9Z,12Z,15Z-18:OH, Nucheck Corp., Elysian, Minnesota) by treatment with pyridinium chlorochromate (Corey and Suggs, 1975). (Z,Z,Z)-1,3,6,9-Nonadecatetraene was initially prepared by a method similar to that previously described (Roelofs et al., 1982; Huang et al., 1983; Jain et al., 1983) and later, the series of $C_{18,19 \text{and} 20}$ (*Z*,*Z*,*Z*)-1,3,6,9-tetraenes and (*Z*,*Z*,*Z*)-1-bromo-3,6,9-nonadecatriene (1-bromo-3Z,6Z,9Z-19:H) were prepared by an alternate procedure (Millar and Underhill, 1986).

(*E*,*Z*,*Z*)-1,3,6,9-Nonadecatetraene (1,3*E*,6*Z*,9*Z*-19:H) was prepared as follows.

(A) Preparation of (E,Z,Z)*-2,5,8-Octadecatrienal.* (*Z*,*Z*,*Z*)-2,5,8-Octadecatrienol (Millar, unpublished) (132 mg. 0.5 mmol) in CH_2Cl_2 (0.5 ml) was added to a slurry of pyridinium chlorochromate (216 mg, 1 mmol) and sodium acetate (16 mg, 0.25 mmol) in CH_2Cl_2 (1.5 ml), and the mixture was stirred at 20°C for 3 hr under nitrogen. Ether (10 ml) was then added, and the mixture was stirred a further 10 min to allow the precipitate to coagulate. The liquid phase was decanted and passed through a column of Florisil (1 cm ID × 5 cm). The solid residues were washed with several portions of ether, and the washings were passed through the Florisil column. The combined column eluate was concentrated at reduced pressure, giving the crude aldehyde (80 mg, 61%) as an unstable yellow oil consisting of an 85:15 mixture of *trans* and *cis* isomers. The crude product was used without further purification. [^{1}H]NMR ($CDCl_3$) δ 9.51 (d, 1H, J = 7.9 Hz: H-1), 6.81 (dt, 1H, J = 15.6, 6.2 Hz; H-3) 6.13, (ddt, 1H, J = 15.6, 7.9, 1.6 Hz; H-2), 5.56 (dtt, 1H, J = 10.6, 7.2, 1.4 Hz; H-6), 5.44–5.34 (m, 2H; H-5, H-9), 5.39 (dtt, 1H, J = 10.6, 7.3, 1.4 Hz; H-8), 309 (m, 2H; H-4), 2.77 (m, 2H; H-7), 2.02 (m, 2H; H-10), 1.45–1.15 (m, 14H; H-11 to H-17), 0.86 (t,3H, J = 6.8 Hz; H-18). IR (neat): λ_{max} 3020 (m), 2960 (m, shoulder), 2935 (s), 2865 (s), 2825 (m, shoulder), 2740 (m), 1590 (s), 1460 (m), 1125 (m), 975 (m) cm^{-1}. MS (EI, 70 eV), *m/z* (relative intensity): 262 (M^+, 0.9), 218 (2.7), 208 (3.6), 149 (4.5), 135 (10,5), 121 (23.7), 109 (38.1), 96 (40.9), 95 (36.5), 93 (35.8), 92 (30.9), 91 (46.2), 81(94.5), 80 (52.2), 79 (89.3), 69 (36.1), 67 (100), 57 (42.9), 55 (90.9), 43 (90.2).

(B) Preparation of (E,Z,Z,)*-1,3,6,9-Nonadecatetraene.* To a solution of methyltriphenylphosphonium iodide (142 mg, 0.35 mmol) in THF (5 ml) at 0°C under nitrogen was added dropwise a hexane solution of *n*-butyllithium (1.4 M; 0.22 ml, 0.31 mmol). The mixture was stirred at 0°C for 30 min, followed by dropwise addition of a solution of crude aldehyde (80 mg, ≃ 0.31 mmol) in THF (2 ml). The mixture was stirred at 0°C for 20 min, then quenched by pouring into ice-water (10 ml). The mixture was extracted with hexane (1 × 20 ml, 2 × 10 ml), and the combined organic extracts were washed with brine (1 × 10 ml), dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography on silica gel (1 cm ID × 20 cm), eluting with pentane, followed by HPLC on a Whatman Magnum 9 SCX column (Ag^+

form), eluting with 0.1% dimethoxyethane in hexane, giving 1,3*E*,6*Z*,9*Z*-19:H (50.4 mg, 62%) free of 1,3*Z*,6*Z*,9*Z*-19:H. [^{1}H]NMR ($CDCl_3$): δ 6.30 (ddd, 1H, J = 17.2, 10.2, $\simeq$ 10 Hz; H-2), 6.06 (br dd, 1H, J = 15.3, $\simeq$ 10 Hz; H-3), 5.67 (dt, 1H, J = 15.3, 6.2 Hz; H-4), 5.25–5.45 (m, 4H; H-6, 7, 9, 10), 5.09 (d, 1H, J = 17.2 Hz; H-1, *cis*), 4.95 (d, 1H, J = 10.2 Hz; H-1 *trans*), 2.85 (t, 2H, J = 6.2 Hz; H-5), 2.77 (t, 2H, J = 6.1 Hz; H-8), 2.03 (m, 2H; H-11), 1.1–1.6 (m, 14H; H-12 to H-18), 0.86 (t, 3H, J = 6.7 Hz; H-19), IR (neat): λ_{max} 3020 (m), 2965 (m), 2935 (s), 2865 (s), 1000 (m) cm^{-1}. The mass spectrum corresponded with that of the 3*Z*-isomer (Bestmann et al., 1982; Huang et al., 1983).

Field Trapping. Field experiments were carried out in areas containing aspen near Saskatoon, Saskatchewan, where only Bruce spanworm occurs and these were repeated approximately six weeks later on Vancouver Island, British Columbia primarily within Victoria, where both species occur, and also 50 km north near Duncan. Two types of traps were used, namely, Pherocon 1CP® (Zoecon Corp., Palo Alto, California) and Hara traps (Hara Products Ltd., Swift Current, Saskatchewan) which are similar to the cone-orifice model 3 trap described by Steck and Bailey (1978). Candidate lures were impregnated into rubber septa via dilute solutions in hexane. Traps were placed 10–15 m apart on trees or wooden stakes at a height of 1.0–1.5 m and were set out in randomized block designs. Summed trap captures were transformed ($\sqrt{X + 1}$) and subjected to analysis of variance; significantly different means were separated by Duncan's (1955) multiple-range test. In all tables, values within a column followed by the same letter are not significantly different ($P > 0.05$).

Adult Bruce spanworm and winter moth males were distinguished by the method of Eidt et al. (1966).

RESULTS AND DISCUSSION

EAG assays of 15-sec fractions collected during GC fractionation of female spanworm extracts disclosed strong male antennal stimulatory activity present in a single fraction eluting from the SP-2100 column. The activity, on repeated analyses, was consistently associated with materials which eluted at a time just before nonadecane.

The amount of pheromone recovered from the abdominal tip extracts was approximately 1 ng/female. The active compound was identified from the following information. Bromination and hydrogenation destroyed the activity, indicating the presence of double or triple bonds. The compound was not affected by basic hydrolysis conditions, indicating the lack of ester or other base-sensitive groups. The EI mass spectrum, with base peak *m/z* 79 and ions corresponding to the series $(C_nH_{2n-5})^+$, gave a small molecular ion at *m/z* 260, confirmed by methane CI, for a possible molecular formula of $C_{19}H_{32}$, corre-

sponding to four sites of unsaturation and/or rings. The lack of any fragmentations typical of heteroatom-containing groups added further credence to the molecule being an unsaturated hydrocarbon. There were two significant even-numbered rearrangement ions in the higher mass range, at *m/z* 206 and 106. The former corresponds to loss of butadiene from the molecular ion, strongly suggesting the presence of a conjugated 1,3-diene unit at one end of the molecule. As there was no major fragment corresponding to hexatriene, or the loss of hexatriene, the diene system was not further conjugated, so that the other site(s) of unsaturation were beyond carbon 5. The other fragment, at *m/z* 106, corresponding to C_8H_{10}, was identified by comparison with the spectra of 3,6,9-alkatrienes (Underhill et al., 1983, and references cited), where there is a prominent fragment at *m/z* 108 due to structure-specific cleavage between carbons 8 and 9, accompanied by a hydrogen transfer to give the even-numbered rearrangement peak. By analogy, the ion at *m/z* 106 in the unknown compound could arise from the same type of rearrangement in a 1,3,6,9-tetraene. In 1982, it was clear that the EI spectrum matched that reported by Bestmann et al. (1982) for 1,3Z,6Z,9Z-19:H and, following this, its retention times on several different columns were found to coincide exactly with those of the synthetic compound.

GC-EAD analyses showed only a single response was elicited by Bruce spanworm males to female abdominal tip extracts (Figure 1, trace b), and this corresponded in elution time to that of 1,3Z,6Z,9Z-19:H (trace a). Using the antenna of the winter moth male for detection, a similar EAD response occurred, and no additional responses, which may have indicated the presence of inhibitory components to winter moth, were detected (trace c); the large amount of material extracted from the females used for this determination was likely due to an excessive amount of tissue removed. In agreement with data reported (Bestmann et al., 1982; Roelofs et al., 1982), tip extracts of female winter moths elicited in antennae of conspecific males a single antennal response corresponding to the elution of 1,3Z,6Z,9Z-19:H (data not shown). No additional responses were observed using antennae from Bruce spanworm males. Thus all of our analytical data indicated a single and identical pheromone component present in both species.

Field-trapping experiments were carried out to determine if Bruce spanworm or winter moth male attraction to 1,3Z,6Z,9Z-19:H could be modified by additions of structural analogs of the pheromone. Our major goal was to obtain a blend which would inhibit attraction of Bruce spanworm males but remain attractive to winter moth so that populations of the latter could be specifically monitored in the presence of Bruce spanworm. In our first experiment in 1984 in Saskatchewan (3X replicated), none of nine analog additions resulted in significantly different (5% level) trap captures of Bruce spanworm males. A total of 153 Bruce spanworm males were captured in traps baited with

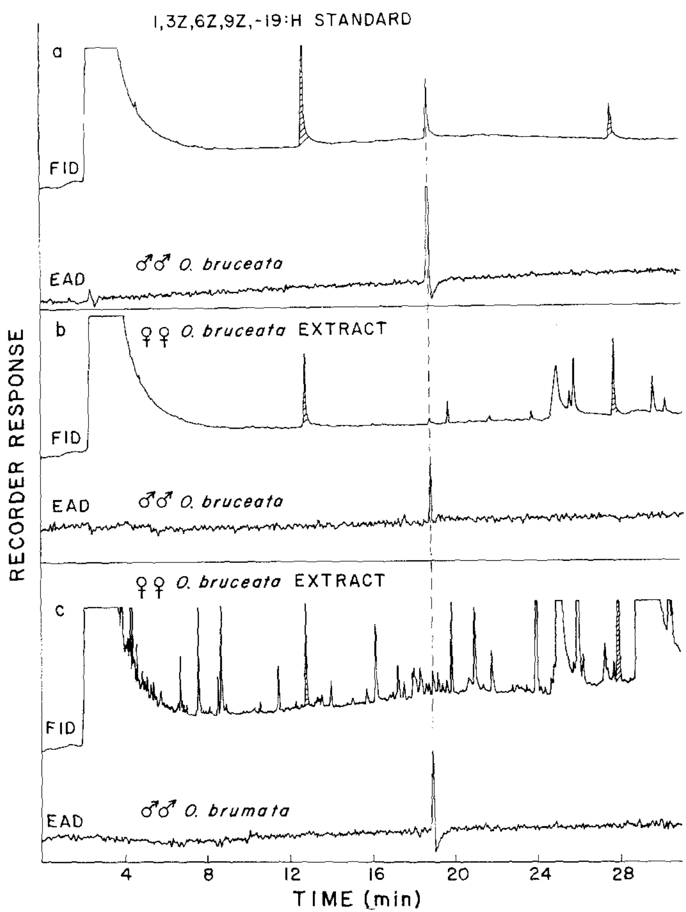

FIG. 1. Separately recorded flame ionization detector (FID) and electroantennographic detector (EAD) traces: upper pair (a) are in response in synthetic (*Z,Z,Z*)-1,3,6,9-nonadecatetraene and two internal standards (shaded); middle pair (b) to a female abdominal tip extract (1 FE) of Bruce spanworm; lower pair (c) to an abdominal tip extract of five Bruce spanworm females. EAD traces in a and b were obtained using Bruce spanworm male antennae; a winter moth male antenna was used for trace c.

1,3Z,6Z,9Z-19:H alone (100 μg) from October 6 to 9. The number of males taken in traps baited with the same amount of pheromone plus one of the nine structural analogs (20 μg each) ranged from 107 to 206. Analogs tested included nonadecane, (*Z,Z*)-6,9-nonadecadiene, (*Z,Z,Z*)-3,6,9-nonadecatriene, (*Z,Z,Z*)-6,9,12-nonadecatriene, (*Z,Z,Z,E*)-3,6,9,11-nonadecatetraene, (*Z,Z,Z,Z*)-3,6,9,11-nonadecatetraene, (*Z,Z*)-6,9-*cis*-3,4-epoxy-nonadecadiene, (*Z,Z*)-3,9-*cis*-6,7-epoxy-nonadecadiene, and (*Z,Z*)-3,6-*cis*-9,10-epoxy-nona-

decadiene. In tests at Victoria (2X replicated), where the winter moth/Bruce spanworm male population was ca. 19 : 1, there was no difference in the number of males captured by these treatments. Blank traps took no males at either location.

Additional structural analogs of 1,3Z,6Z,9Z-19:H were field tested in 1985. Data obtained from the Saskatchewan test (Table 1) showed additions of one analog, 1,3*E*,6Z,9Z-19:H, a geometric isomer of the natural pheromone, significantly inhibited Bruce spanworm captures. This was followed by a second field test where the effect of different amounts of added inhibitor on male attraction was examined. Additions from 10 μg to 300 μg significantly reduced the number of target males captured (Table 2). No males were taken in traps baited with the inhibitor alone or in blank traps. It should be noted that although the number of males taken in traps containing 100 and 300 μg of inhibitor plus 100 μg of pheromone were not significantly different from blank traps, low numbers of males were captured by at least one of the analog-containing replicates. In field tests at Victoria, none of the analog-containing lures inhibited the capture of winter moth males (Table 1): each of the lures containing increasing amounts of added 1,3*E*,6Z,9Z-19:H remained attractive to winter moth males

TABLE 1. EFFECT OF ADDITION OF SECOND COMPONENTS TO LURES CONTAINING (Z,Z,Z)-1,3,6,9-NONADECATETRAENE ON CAPTURE OF BRUCE SPANWORM AND WINTER MOTH MALES

Additive (μg)[a]	Males captured[b]	
	Saskatchewan site (Bruce spanworm)[c]	Victoria site (>93% Winter moth)[d]
None	173a	492a
1,3*E*,6Z,9Z-19:H (20)	59b	504a
1,3Z,6Z,9Z-18:H (20)	243a	527a
1,3Z,6Z,9Z-20:H (20)	194a	600a
1-Bromo-3Z,6Z,9Z-19:H (20)	175a	429a
(CME)-1,3Z,6Z,9Z-19:H (20)	142a	413a
(CME)-1,3Z,6Z,9Z-19:H (100)	231a	585a
9Z,12Z,15Z-18:ALD (20)	259a	519a
9Z,12Z,15Z-18:OH (20)	245a	449a
Blank trap	Oc	Ob

[a] All lures contained 100 μg of 1,3Z,6Z,9Z-19:H.
[b] Values followed by the same letter are not significantly different ($P > 0.05$).
[c] Captures of Bruce spanworm males in Pherocon 1CP traps (3 × replicated), October 1-4, 1985.
[d] Males captured in Hara traps (3 × replicated), December 6-11, 1985, were >93% winter moths, the others were Bruce spanworm.

TABLE 2. EFFECT OF ADDITION OF 1,3*E*,6*Z*,9*Z*-NONADECATETRAENE TO LURES CONTAINING 1,3*Z*,6*Z*,9*Z*-NONADECATETRAENE ON CAPTURE OF BRUCE SPANWORM AND WINTER MOTH MALES

Lure composition (μg)		Males captured[a]	
1,3*Z*,6*Z*,9*Z*-19:H	1,3*E*,6*Z*,9*Z*-19:H	Saskatchewan site (Bruce spanworm)[b]	Victoria site (>93% Winter moth)[c]
100	0	185a	273bcd
100	3	168a	331abc
100	10	34b	442a
100	30	28b	378ab
100	100	13bc	369ab
100	300	16bc	223cd
100	600	—	230d
0	100	0c	0e
Blank trap		0c	0e

[a] Values followed by the same letter are not significantly different ($P > 0.05$).
[b] Total number of Bruce spanworm males captured in Pherocon 1CP traps (3 × replicated) October 4–15, 1985.
[c] Males caught in Hara traps (3 × replicated) December 5–8, 1985, were >93% winter moths, the others were Bruce spanworm.

(Table 2). In tests similar to these, Knauf et al. (1984) also were unable to find synthetic inhibitors of winter moth male attraction to its pheromone.

Clearly, both species are attracted to the major pheromone component, but only one, the winter moth, is attracted to this component plus inhibitor. The discovery that attraction of Bruce spanworm males to 1,3*Z*,6*Z*,9*Z*-19:H is inhibited by the presence of 1,3*E*,6*Z*,9*Z*-19:H, raises the question: Is this a naturally occurring component in winter moth females which may be used for reproductive isolation? Tests were made to determine if these two isomers could be separated by GC, and four columns of differing polarity (DB-5, DB-1701, DB-Wax, and DB-210, each 30 m × 0.32 mm ID) were examined. Both isomers coeluted on the first three columns but partial separation was effected using the DB-210 column (19.14 min for 1,3*Z*,6*Z*,9*Z*-19:H and 19.21 min for 1,3*E*,6*Z*,9*Z*-19:H), the column which had been used for the GC-EAD analyses. With such a small difference in retention times it would not have been possible to detect the presence of small amounts of the *E* isomer, if indeed it did occur, in the winter moth female pheromone extract. Field evidence has confirmed that traps baited with live Bruce spanworm females from the Okanagan Valley of British Columbia attracted winter moth males in Victoria (Ring, unpublished), but the reciprocal cross-attractancy experiments using winter moth fe-

males in a known habitat of Bruce spanworm have been inconclusive to date. Furthermore, in caged experiments it was shown that winter moth females could breed successfully with Bruce spanworm males, but the reciprocal mating did not occur. The resulting progeny were viable to at least the F_2 generation (the end of the breeding experiment), and pupal cremaster characteristics were intermediate between the two species (Smith and Ring, unpublished). With the close morphological, behavioral, ecological, and phenological similarities between the two species, one could speculate that these two closely related species have been isolated by mainly geographical barriers until recent times.

The 1985 data indicated that populations of winter moth and Bruce spanworm may be distinguished by deploying two different trap baits, one with pheromone alone, the other with pheromone plus inhibitor. This was clearly demonstrated in an experiment set out in Victoria and in Duncan (Table 3). In Victoria, there was no significant difference between the numbers of male moths taken in traps baited with the two different bait compositions, indicating the presence of a predominantly winter moth population, which was confirmed by examination of captured males' genitalia. At Duncan, significantly fewer males were captured in traps with pheromone plus inhibitor than in traps with pheromone alone, indicating that Bruce spanworm dominated the population. Again, this was confirmed by examination of captured males' genitalia. It should be noted, until such time as captures of Bruce spanworm can be reduced to zero in traps containing pheromone plus inhibitor, detection of low populations of winter moth in regions of high Bruce spanworm populations will require examination of the captured males' genitalia (Eidt et al., 1966) to distinguish

TABLE 3. CAPTURE OF WINTER MOTH AND BRUCE SPANWORM MALES AT TWO LOCATIONS ON VANCOUVER ISLAND IN 1985

	Victoria site[a]			Duncan site[b]		
Lure composition (μg)	Total caught	Number dissected[c]	Winter moth (%)	Total caught	Number dissected[c]	Bruce spanworm (%)
1,3*Z*,6*Z*,9*Z*-19:H (100)	252a	39	92	223a	56	100
1,3*Z*,6*Z*,9*Z*-19:H (100) + 1,3*E*,6*Z*,9*Z*-19:H (100)	258a	48	100	61b	58	100
1,3*E*,6*Z*,9*Z*-19:H (100)	Ob			Oc		

[a] Number and species of males caught in Hara traps, 4 × replicated, December 8–13, 1985. Values followed by the same letter are not significantly different ($P > 0.05$).
[b] Number and species of males caught in Hara traps, 4 × replicated, December 13-23, 1985. Values followed by the same letter are not significantly different ($P > 0.05$).
[c] Number of males dissected and genitalia examined for species identification.

TABLE 4. CAPTURES OF BRUCE SPANWORM AND WINTER MOTH MALES WITH PHEROCON 1CP AND HARA TRAPS

Lure composition (μg)	Trap type	Number of males caught[a]	
		Bruce spanworm[b]	Winter moth[c]
1,3Z,6Z,9Z-19:H (100)	Pherocon	110a	278b
1,3Z,6Z,9Z-19:H (100)	Hara	143a	271b
1,3Z,6Z,9Z-19:H (100) + Vapona®	Hara	NT[d]	714a
Blank + Vapona®	Hara	NT	0c

[a] Values followed by the same letter are not significantly different ($P > 0.05$).
[b] Captures at Saskatchewan site in 3 × replicated traps, October 15–25, 1985.
[c] Captures at Victoria, British Columbia, site in 3 × replicated traps, November 19–December 5, 1985. Males caught were >93% winter moth, the remainder were Bruce spanworm.
[d] Not tested.

between these species. However, fewer males will be required for examination since only those captured in traps with inhibitor need be dissected.

It had been noted during these investigations that captures of Bruce spanworm and winter moth males in Pherocon 1CP traps often approached or appeared to exceed saturation levels during an evening even in areas where no appreciable defoliation had been noticed during the preceding June, an indication of a moderaltely low infestation. Cone-orifice Hara traps, which have a high trap capacity, were tested for field use as they should require less frequent inspections. Tests carried out in Saskatchewan and at Victoria showed no difference in the number of Bruce spanworm or winter moth males retained by these two trap types (Table 4). In the Victoria test, Hara traps which also contained Vapona® strips, were tested, and significantly more winter moth males were recorded in these traps than in Hara traps without the insecticidal strips or in Pherocon 1CP traps. Clearly, large numbers of males can be expected to escape from Hara traps unless an insecticide is included.

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ANALYSIS OF LONG-RANGE REPRODUCTIVE BEHAVIOR OF MALE *Diabrotica virgifera virgifera* LECONTE AND *D. Barberi* SMITH AND LAWRENCE TO STEREOISOMERS OF 8-METHYL-2-DECYL PROPANOATE UNDER LABORATORY CONDITIONS

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Abstract—Behavioral analysis of male attraction to stereoisomers of 8-methyl-2-decyl propanoate was conducted with *Diabrotica virgifera virgifera* and *D. barberi* using a flight tunnel. *D. barberi* males were attracted to the 2*R*,8R isomer only, and the response was inhibited by the addition of 2*S*,8*R*. *D. v. virgifera* males were also attracted to the 2*R*,8*R* isomer and were neither attracted nor inhibited by any of the other isomers. Males attracted to the lures displayed a series of behaviors characterized by a slow hovering flight upwind towards the odor source. While significant numbers of males of both species landed on the pheromone lure, none attempted copulation, indicating that other cues are necessary to induce this behavior.

Key Words—*Diabrotica virgifera virgifera, Diabrotica barberi,* Coleoptera, Chrysomelidae, sex pheromone, 8-methyl-2-decyl propanoate, flight-tunnel bioassays.

INTRODUCTION

The sex pheromone of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (WCR), was identified by Guss et al. (1982) as 8-methyl-2-decyl propanoate. Indirect evidence indicates that this is the compound used in pher-

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omone communication by the northern corn rootworm, *D. barberi* Smith and Lawrence (NCR) as well (Guss et al., 1985).

The chirality of this molecule plays an important role in determining its biological activity, a phenomenon that has been documented for pheromones of other Coleoptera (Tumlinson et al., 1977, Lanier et al., 1980). Guss et al. (1982) showed that both NCR and WCR males were attracted to as little as 10 ng of racemic pheromone per lure, although NCR response was inhibited by concentrations over 0.1 μg. Trapping in the field with individual isomers revealed that the WCR was attracted to the 2*R*,8*R* isomer and, to a lesser extent, the 2*S*,8*R* isomer, while NCR was attracted to the 2*R*,8*R* isomer only. The addition of 0.1 μg of 2*S*,8*R* or 0.5 μg of 2*S*,8*S* to 1 μg of 2*R*,8*R* caused complete inhibition of the NCR response; however, the 2*S*,8*R* and 2*S*,8*S* isomers were not inhibitory to WCR males (Guss et al., 1984, 1985).

Although the isomeric specificity of 8-methyl-2-decyl propanoate was documented in the field (Guss et al., 1984, 1985), no behavioral studies have been performed under controlled laboratory conditions to verify these findings. In addition, volatility of the pheromone (i.e., release rates from rubber septa) was not examined, making it difficult to compare these results with those obtained using different systems under different climatic conditions. The following reports the results of quantitative behavioral analyses of male NCR and WCR responses to known release rates of the four isomers of 2-methyl-8-decyl propanoate in a flight tunnel.

METHODS AND MATERIALS

The four isomers of 8-methyl-2-decyl propanoate (designated 2*R*,8*R*, 2*S*,8*R*, 2*R*,8*S*, and 2*S*,8*S*) were obtained from J.H. Tumlinson (USDA, Gainesville, Florida) and were the same preparations used by Guss et al. (1984, 1985). The isomeric purity of individual isomers was as follows: 2*R*,8*R* (97.8%); 2*S*,8*S* (98.8%); and 2*S*,8*R* (97.4%). Impurities consisted of mixtures of the other isomers and are listed in Guss et al. (1984). To formulate lures, rubber septa (catalog No. 8753-D22, A.H. Thomas Co., Philadelphia, Pennsylvania) were extracted for 24 hr in a Soxhlet extraction apparatus with methylene chloride and air dried for an additional 24 hr. Individual isomers were diluted to the appropriate concentrations with hexane (Fisher Scientific Ltd., HPLC grade) and added either singly or in mixtures to the large wells or rubber septa in 200-μl volumes. After allowing 24 hr for solvent evaporation, the septa were ready to use.

Bioassays for analysis of male behavior were conducted in the laboratory at 24°C using a 1.2 × 0.5 × 0.5 m Plexiglas flight tunnel through which air was pulled at a constant flow rate. Pheromone was dispensed from a rubber septum mounted in a copper funnel 3 cm in diameter at the large end. The

narrow end of the funnel was connected to a regulated air supply via rubber tubing, and air was passed over the lures at a constant volume of 500 ml/min. Titanium tetrachloride fumes emitted from a rubber septum in the funnel were used to visualize the shape of the pheromone plume, and adjustments were made by altering the airflow entering the chamber through the funnel as well as the speed of the fan removing air (0.3 m/sec) at the other end. This enabled us to release known quantities of pheromone by regulating airflow over the lures. The plume used in all studies had a diameter of 15 cm at the downwind end. Assays were carried out between 0800 and 1130 hr using feral males collected from corn and goldenrod plants immediately prior to use.

Individual males were placed in small wire cages and allowed to acclimate for approximately 5 min prior to being tested. A septum containing an isomer or mixture of isomers to be tested was mounted in the funnel at the upwind end of the tunnel. The assay began by placing a wire cage containing a male in the downwind end of the flight tunnel on a steel platform 15 cm high with the open end of the cage facing upwind and into the pheromone plume. Subsequent behaviors exhibited by males were then tabulated in first-order, preceding–following, behavioral transition matrices and comprehensive ethograms were formulated. The assay was discontinued after 3 min or when the male landed on the lure. Statistical significance of behavioral sequences with frequencies greater than zero was assessed by calculating chi-square values as outlined by Stevenson and Poole (1976).

The probability of a particular behavioral transition occurring was significantly greater than chance if (observed − expected)/(expected)$^{0.5}$ values were greater than $(\chi^2_{0.05}, 36\ df)^{0.5}/36^{2df}$. Standard normal deviates were then calculated from significant chi-squared values, and the most probable behavioral sequences in the ethograms ($P \leq 0.05$) were determined using the binomial test (Siegel, 1956).

The rate of volatilization of 8-methyl-2-decyl propanoate from rubber septa was determined by quantitive gas chromatographic analysis of volatiles released over a period of time. Rubber septa were loaded with pheromone concentrations of 0.1, 0.25, 0.5, 1.0, 2.0 μg/lure and volatiles were collected using the technique of Tumlinson et al. (1982). Individual septa were each placed in a silanized glass aeration chamber (Teal et al., 1986) that was connected to a compressed nitrogen gas source. The other end of the aeration chamber was fitted with a small charcoal trap made by enclosing 3–5 mg of charcoal between two 325-mesh stainless-steel frits in a 2-cm section of glass tubing (6.0 mm OD) (Tumlinson et al., 1982). Volatiles evaporating from the septa were collected on the charcoal traps for a period of 2–8 hr at 24°C with nitrogen flow rates between 300 and 700 ml/min.

Following volatile collection, the charcoal traps were extracted with four 50-μl aliquots of methylene chloride and, after the addition of 15 μl of isooctane, the solution was concentrated to about 8–10 μl under a stream of N_2 gas.

An external standard was prepared by applying a known amount of 8-methyl-2-decyl propanoate onto the charcoal trap and extracting by the procedure outlined above.

Quantitative analysis of samples was done using a Hewlett Packard 5890 gas chromatograph interfaced to an Apple IIe computer via an IMI Chromatochart data analysis stream (Interactive Microwave Inc., State College, Pennsylvania). The chromatograph was run in splitless mode with a flame ionization detector. A fused silica 30-m × 0.25-mm (ID) SPB-1 (Supelco) capillary column was used with hydrogen as the carrier gas at a linear flow velocity of 38 cm/sec. Operating conditions were as follows: initial temperature = 80°C; injector purge time = 0.5 min; temperature program (at 1 min) = 25°C/min; final temperature = 140°C. Data were analyzed with the Chromatochart software package using the charcoal trap extract of a known amount of pheromone as an external standard. Results were expressed as nanograms of pheromone released per hour with a nitrogen flow rate of 500 ml/min and were plotted against the concentration of pheromone on the rubber septa.

RESULTS AND DISCUSSION

In the behavioral ethograms for NCR and WCR males (Figure 1), parentheses were used to distinguish those transitions which occurred with a frequency

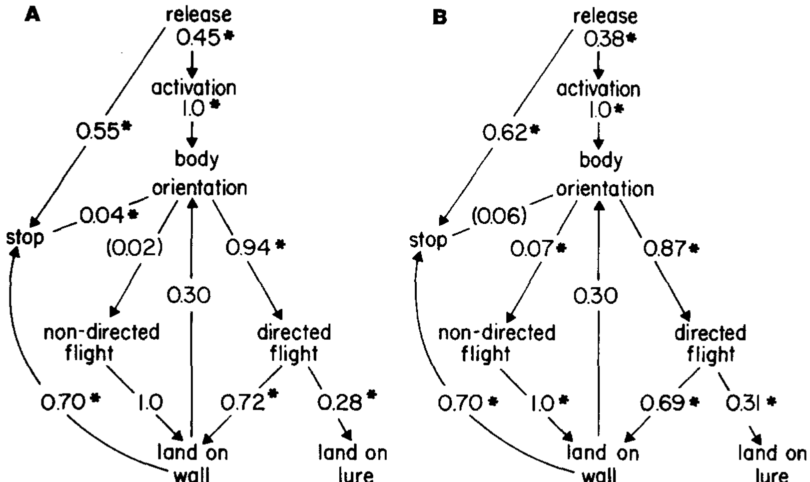

FIG. 1. Behavioral ethograms of male NCR and WCR to 1 μg of 8*R*-methyl-2*R*-decyl propanoate in the flight tunnel. Parentheses indicate those transitions not occurring with a frequency greater than chance ($P \leq 0.50$). Asterisks (*) denote the most probable transitions determined from the standard normal deviates ($P \leq 0.05$). (A) Response of NCR males ($N = 93$); (B) Response of WCR males ($N = 94$).

that was not significantly greater than chance ($P \geq 0.05$), as determined by chi-squared analysis. Among the significant transitions, those which had the highest probability of occurring, as determined by the binomial test of the standard normal deviates ($P \leq 0.05$), were indicated with an asterisk (*). Similar ethograms and statistical comparisons were made for all data given in Table I.

The ethograms produced for NCR indicated that males responded to the 2*R*,8*R* isomer of 8-methyl-2-decyl propanoate (Figure 1) but not to any of the other three isomers (Table I). When 1 μg of 2*R*,8*R* was combined in a 1 : 1 ratio with 2*S*,8*R*, response was suppressed (Table 1). However, males responded

TABLE 1. RESPONSES OF ADULT MALE CORN ROOTWORMS TO ENANTIOMERS AND BLENDS OF ENANTIOMERS OF 8-METHYL-2-DECYL PROPANOATE, BASED ON BINOMIAL TEST OF STANDARD NORMAL DEVIATES[a]

		Males Responding (%)				
Lure	*N*	Activation	Body orientation	Directed flight	Land on upwind wall	Land on lure
Northern corn rootworm						
1 μg 2*R*,8*R*	93	45a	45a	42a	30a	12a
1 μg 2*S*,8*S*	40	0b				
1 μg 2*R*,8*S*	38	0b				
1 μg 2*S*,8*R*	35	0b				
1 μg 2*R*,8*R* + 1 μg 2*S*,8*S*	66	45a	45a	45a	37a	18a
1 μg 2*R*,8*R* + 1 μg 2*R*,8*S*	69	43a	43a	40a	32a	8a
1 μg 2*R*,8*R* + 1 μg 2*S*,8*R*	46	0				
Western corn rootworm						
1 μg 2*R*,8*R*	94	38a	38a	33a	23a	10a
1 μg 2*S*,8*S*	36	0b				
1 μg 2*R*,8*S*	42	0b				
1 μg 2*S*,8*R*	35	0b				
1 μg 2*R*,8*R* + 1 μg 2*S*,8*S*	62	45a	45a	31a	20a	11a
1 μg 2*R*,8*R* + 1 μg 2*R*,8*S*	70	51a	51a	42a	29a	13a
1 μg 2*R*,8*R* + 1 μg 2*S*,8*R*	70	44a	44a	40a	27a	13a

[a]Numbers in the same column followed by the same letter are not significantly different in a chi-squared test of independence of adjusted values.

TABLE 2. RESPONSE OF MALE NCR AND WCR TO SELECTED ISOMERS AND ISOMERIC MIXTURES OF 8-METHYL-2-DECYL PROPANOATE IN FLIGHT TUNNEL[a]

Species	Isomer	Amount (μg)	Number tested	Directed flight response (% of males)
NCR	2*R*,8*R* + 2*S*,8*R*	1.0 0.1	55	51
NCR	2*R*,8*R* + 2*S*,8*R*	10.0 1.0	25	0
NCR	2*R*,8*R*	0.25	25	48
WCR	2*R*,8*R*	0.25	27	41
NCR	2*R*,8*R*	0.1	32	3
WCR	2*R*,8*R*	0.1	27	0

[a] Airflow rate = 500 ml/min; temperature = 24°C.

readily to a 10:1 ratio of 1 μg 2*R*,8*R* with 0.1 μg 2*S*,8*R* (Table 2). A 10:1 ratio consisting of 10 μg of 2*R*,8*R* with 1 μg of 2*S*,8*R* again suppressed male resonse (Table 2), demonstrating that the inhibition was due to the actual amount of 2*S*,8*R* present (i.e., a threshold effect) rather than its ratio with 2*R*,8*R*. The 2*S*,8*S* and 2*R*,8*S* isomers had no inhibitory effect when each was combined with 2*R*,8*R* in a 1:1 ratio (Table 1). Although Guss et al. (1985) showed that 2*R*,8*R* was the active isomer and 2*S*,8*R* was inhibitory, their results indicated that inhibition was caused by 2*S*,8*S* as well.

The ethograms depicting the response of WCR males showed that 2*R*,8*R* was again the active isomer (Figure 1B), whereas the other three isomers exerted no effect, either alone or in combination with 2*R*,8*R* (Table 1). Guss et al. (1984) found 2*S*,8*S* and 2*R*,8*S* to be neither attractive or inhibitory in the field, but both 2*R*,8*R* and, to a lesser degree, 2*S*,8*R* attracted males in significant numbers. The attractiveness of 2*S*,8*R* in the field is difficult to explain. While a related species, *D. porracea* Harold, responds exclusively to 2*S*,8*R*, this isomer does not appear to be a component of WCR pheromone (Guss et al., 1984). The fact that no response to 2*S*,8*R* was found in the flight tunnel may have been due to the relatively low number of beetles tested (N = 35) compared to the potentially large population tested in the field. However, the possibility of attraction caused by impurities (i.e., the 2*R*,8*R* isomer) in the lures which were used in the field cannot be discounted.

In reviewing the role of chirality in the pheromone communication of Co-

leoptera, Silverstein (1979) proposed nine possible ways in which stereoisomers could mediate male response. The results presented here, as well as by Guss et al. (1985), showed that NCR pheromone was best described by category 5 (Silverstein, 1979) in which the insect produces only one enantiomer (2*R*,8*R*) and is inhibited in the presence of the other enantiomer (2*S*,8*R*). Futhermore, the data from the flight tunnel and the results of Guss et al. (1984) both suggest that WCR pheromone consists of a single enantiomer (2*R*,8*R*) and that any response to other enantiomers is an artifact (category 2, Silverstein, 1979).

Males responding to the active isomer or mixtures showed stereotyped behaviors. Although there was some variation in frequencies of behavioral transitions, the actual sequence of behaviors for both species was very similar in all cases where a positive response was observed, Upon being released, males left the cage and walked out to the upwind edge of the platform while waving their antennae in a rapid, agitated manner (activation). This was followed by ambulation back and forth along the platform edge until the body pointed directly into the odor plume. The prothoracic legs were then extended, elevating the head and prothorax (body orientation), and after taking a few steps, flight was initiated. A positive response was characterized by slow hovering flight with the body in a vertical position. Flight of this type was either upwind towards the lure (directed) or lateral with little forward motion (nondirected). The probability of the latter occurring was usually not significant.

A negative response (stop) consisted of either no flight or very fast, darting flight with no hovering, usually towards the roof of the chamber. Responses of this type could occur immediately upon release, after body orientation, or after landing on the wall.

Beetles engaged in directed flight landed on the air outlet containing the lure or, more commonly, on a nearby wall. Those individuals landing on the wall occasionally reorientated and flew to the lure, but walking out of the odor plume and "losing interest" was the most probable course of action. Males landing on the air outlet invariably located the lure, but no attempts at copulation were observed. Although attempted copulation with artificial lures is a well-documented phenomenon among other taxa (e.g., Greenblatt et al., 1976, Teal et al., 1986), apparently some other visual or chemical cues are necessary to elicit this response in *Diabrotica*.

While the behavioral responses of males to pheromone source have been well documented for several species of Lepidoptera (Bartell, 1977; Baker and Linn, 1984), few such studies have been performed with Coleoptera. An investigation by Greenblatt et al. (1976) of the mating behavior of *Trogoderma glabrum* (Herbst) revealed that male activation involved prothoracic leg extension and elevation of the head and prothorax, a feature displayed by both NCR and WCR males. In a field study of *Diabrotica balteata* LeConte, Cuthbert and Reid (1964) observed that activated males approached lures upwind in a char-

acteristic hovering flight. Attempts to elicit this response from males under laboratory conditions were unsuccessful (Cuthbert and Reid, 1964).

The success of the flight-tunnel assay for NCR and WCR males was largely dependent on the time of day when assays are done. NCR males were responsive only during morning hours on days preceded by cool nights. This period corresponded to the main response peak for males under these conditions in the field (Dobson and Teal, 1986). The response was very poor on mornings preceded by warm nights. Similarly, males did not respond in the laboratory during the nightly response period documented in the field (Dobson and Teal, 1986), despite efforts to maintain the flight chamber in complete darkness.

WCR males only responded during morning hours as well, although the response was high regardless of temperatures during the preceding night. Since WCR activity in the field was high throughout the day (Dobson and Teal, 1986), the reason for the loss of response under laboratory conditions is unknown. However, the increased agitation and "flightiness" of beetles in the field which was observed as daytime temperatures rose was no doubt an important factor. Furthermore, success of this assay was limited to feral males collected from the field immediately prior to testing. An explanation for the failure of laboratory-reared males to respond awaits further investigation.

The flight tunnel used in this investigation was a promising tool for the assay of chemicals involved in long-range attraction in other Coleoptera. Prior to this study, chemicals involved in attraction of Coleoptera have been assayed by various means including field trapping (Guss et al., 1984, Tumlinson et al., 1977), devices which permitted ambulation towards an odor source (Payne et al., 1976, Sharpas and Burkholder, 1978), and static systems which produced only close-range activation responses (Ball and Chaudhury, 1973, Guss et al., 1982). Baker and Linn (1984), however, concluded that bioassays done in a flight tunnel provide the greatest discrimination when investigating the response of insects which normally orient by flight. While there are no previous reports of the use of flight tunnel for pheromone studies with Coleoptera, there are definite advantages to using this system. In particular, use of a flight tunnel enables one to monitor, in a controlled environment, individual males performing orientation behaviors similar to those observed in the field. Previous authors assessed the attractiveness of various substances to WCR males by placing an individual in a Petri dish with a filter paper chip impregnated with the test substance. A positive response was recorded for individuals that exhibited antennal waving and orientation towards the filter paper (Ball and Chaudbury, 1973; Guss et al., 1982). The behaviors indicative of a positive response in the flight tunnel clearly provide a more objective means of assessing attraction and/or inhibition than does this latter approach.

The response threshold for both NCR and WCR males in the flight tunnel at 24°C occurred when the concentration of the *2R,8R* isomer was between 0.1 and 0.25 μg per lure (Table 2). This was considerably higher than the threshold

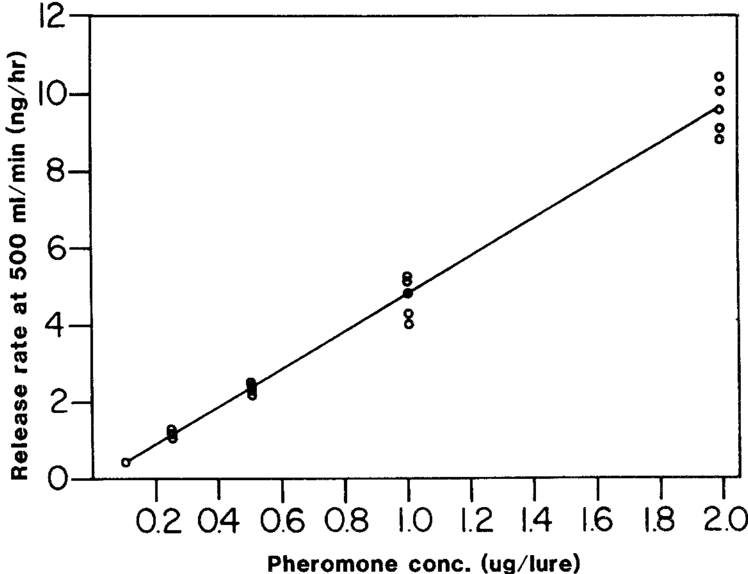

FIG. 2. Release rates of 8-methyl-2-decyl propanoate from rubber septa at 24°C in the laboratory.

value of 10 ng reported by Guss et al. (1982) in the field. This discrepancy may have been caused by different rates of pheromone release in the field and the laboratory.

A standard curve was constructed to estimate release rates for septa loaded with various concentrations of pheromone (Figure 2). The relationship between pheromone concentration and release rate was linear ($r^2 = 0.98$, $N = 25$). The relationship between release rate and airflow was also linear for flow rates within the range of 300–700 ml/min ($r^2 = 0.95$), permitting corrections for differences in airflow between tests. Consequently, a typical 1-μg lure used in the flight tunnel with an airflow of 500 ml/min at 24°C released about 4.7 ng/hr, and the lowest release rate which could elicit a positive response, as indicated by directed flight, was 0.44 ng/hr for NCR males and between 0.44 and 1.18 ng/hr for WCR males (Table 2).

In summary, this study demonstrated that both NCR and WCR males were attracted to the 2*R*,8*R* isomer of 8-methyl-2-decyl propanoate when it was released from rubber septa at levels as low as 1.18 ng/hr. Furthermore, with the exception of the inhibitory effects of the 2*S*,8*R* isomer on NCR males, none of the other isomers elicited any behavioral response in either species. Consequently, reproductive isolation of these two species is probably not achieved through differences in long-range communication using pheromones. Since the attraction of WCR males to 2*R*,8*R* was not inhibited by other isomers, racemic

mixtures of the pheromone (containing all four isomers) could be used in monitoring programs for this species. Inhibition of NCR males should be considered, however, when using pheromone preparations containing the 2*S*,8*R* isomer.

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EFFECTS OF MONOTERPENE ODORS ON FOOD SELECTION BY RED DEER CALVES (*Cervus elaphus*)[1]

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Abstract—The response of red deer calves (*Cervus elaphus*) to the odors of Sitka spruce (*Picea sitchensis*) and lodgepole pine (*Pinus contorta*) needles and five monoterpene constituents of their essential oils was measured by simple two-choice feeding trials. All odors were significantly rejected ($P < 0.05$) except Sitka spruce and lodgepole pine by females and Sitka spruce and alpha-terpineol by males. Females had a stronger dislike of all odors except lodgepole pine than males. Rejection of monoterpene odors weakened slightly with time. A strong logarithmic relationship between the strength of the rejection response and vapor pressure of the compounds tested suggested that the odors were rejected on the basis of concentration rather than quality.

Key Words—*Cervus elaphus*, red deer, terpenoids, monoterpenes, olfaction, *Picea sitchensis*, *Pinus contorta*.

INTRODUCTION

Monoterpenes are a group of secondary metabolites found widely throughout the plant kingdom. They are common constituents of volatile or essential oils, and individual monoterpenes are characterized by highly distinctive odors. Aromatic substances in plants, such as essential oils, may influence food selection by herbivores by acting on the sense of smell. Ruminants such as deer may have good reason to avoid plants rich in essential oils, because some are known

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to inhibit the activity of symbiotic rumen microorganisms, upon which ruminants rely for the digestion of plant material. Essential oils from *Artemisia* spp. (Nagy et al., 1964; Welch et al., 1982), *Pseudotsuga menziesii* (Oh et al., 1967, 1970; Radwan, 1972; Conolly et al., 1980) and *Juniperus* spp. (Painter, 1971; Schwartz et al., 1980a) have all been shown to inhibit rumen microorganisms, Longhurst et al. (1968) reported that black-tailed deer fawns (*Odocoileus hemionus columbianus)* rejected the odor of essential oils from *Ubellularia california*, and Schwartz et al. (1980b) suggested that mule deer (*Odocoileus hemionus hemionus*) used smell to discriminate between feeds contaminated with varying levels of juniper oils. Narjisse (1981) reported that sheep also rejected the odor of essential oils of *Artemisia tridentata*. However, essential oils consist of many monoterpene constituents, and the objective of the experiment reported here was to measure the response of red deer to both whole essential oils from conifers and also to pure monoterpenes.

METHODS AND MATERIALS

Odor trials were carried out on 20 red deer calves (*Cervus elaphus*) using methods adapted from Arnold et al. (1980), in which food is contaminated by the odors of test materials absorbed in pads of cotton wool. The calves were all 3–4 months old and had been reared at Reedie Hill Deer Farm, Fife, Scotland. They had no previous experience of conifers or monoterpenes in their diet. A group of 10 males and another of 10 females were housed in separate rooms. In each room, eight buckets mounted in wall brackets 1 m above the floor were arranged in four pairs. The members of each pair were placed 2.5 m apart. Throughout the experiment, a pelleted ration (Calfrearer Cakelets, 16% protein, 8% fiber) was offered ad libitum in these buckets, which were topped up with pellets twice daily. Water and hay were available ad libitum throughout the experiment. The deer were allowed eight days to become accustomed to the system, while the effect of bucket positioning on the mass of pellets consumed from each bucket was assessed (Table 1). Seven treatment periods, each lasting

TABLE 1. MEANS AND CONFIDENCE LIMITS FOR PERCENTAGE OF TOTAL FOOD CONSUMPTION TAKEN FROM TREATMENT BUCKETS DURING ACCLIMATION PERIOD

	Food Consumption (%)			
	Initial position	Reverse position	SE	99% Cl
Females	45.7	54.3	3.3	50 ± 11.7
Males	50.8	49.2	0.8	50 ± 2.8

72 hr, then followed consecutively. The treatments were three monoterpene hydrocarbons: limonene, α-pinene and terpinolene; two monoterpene alcohols: α-terpineol and borneol (monoterpenes supplied by R.C. Treatt and Co., Bury St. Edmunds); and the crushed foliage from two conifer species: lodgepole pine (*Pinus contorta*) and Sitka spruce (*Picea sitchensis*).

The food in one bucket of each pair was contaminated by a monoterpene odor by sprinkling 1 ml of the pure compound onto three pads of cotton wool, two of which were taped to the rim of the bucket and one was loose in the food. The control bucket of each pair received three similar pads of cotton wool but with no monoterpenes applied. Borneol (a solid) and the conifer foliage were placed in bags of nylon gauze which replaced the cotton wool pads. The mass of pellets consumed from all buckets was recorded 3, 6, 12, 24, 48, and 72 hr after starting the treatment. The cotton wool pads were recharged with 1 ml of the test compounds every 24 hr. In order to eliminate the effects of bucket positioning on food consumption from each bucket, the positions of the treatment and control buckets were interchanged at 12, 24, and 48 hr.

The mass of pellets consumed from the treatment buckets was expressed as a percentage of total food consumption for each group of animals. If the deer had shown no discrimination between treatment and control buckets, this figure would have been 50%. Consumption of less than or greater than 50% from the treatment buckets would indicate rejection or preference of the test odor, respectively. During the acclimation period, 99% confidence limits were calculated for the mean mass of pellets consumed from the treatment buckets with no monoterpenes present (Table 1). During the treatment periods, significant rejection was said to have occurred when the percentage of pellets consumed from the treatment buckets was below the appropriate 99% confidence limit for the acclimation period.

RESULTS AND DISCUSSION

Over the experimental period as a whole, total consumption of pellets rose gradually as the calves grew accustomed to an ad libitum diet. Females consistently consumed significantly less than males ($P < 0.001$, 1.19 kg/day per head $\pm$ SE 0.42 for females compared with 1.67 kg/day per head $\pm$ SE 0.38 for males).

The overall responses of the calves to the odors during the 72-hr treatment periods are presented in Table 2. The odors of all pure monoterpenes were significantly rejected ($P < 0.01$) by both males and females (except α-terpineol by males). The only significant response to crushed conifer foliage was the rejection of lodgepole pine needles by males. The weakness of the rejection response to conifer foliage was probably due to weakness of the odor they emitted compared with the pure monoterpenes. Females rejected all treatments more strongly than males (except lodgepole pine).

TABLE 2. EFFECTS OF MONOTERPENE ODORS ON FOOD SELECTION BY RED DEER CALVES—CONSUMPTION FROM ODOR-TREATED BUCKETS OVER 72 HR

	Consumption (kg/head)			
	Females		Males	
Treatment	Total	From treatment (%)	Total	From treatment (%)
Sitka spruce	3.79	1.70 (44.9)	4.67	2.26 (48.4)
Lodgepole pine	3.78	1.65 (43.7)	5.01	2.25*(44.9)
Alpha-terpineol	3.96	1.18*(29.8)	5.59	2.76(49.4)
Borneol	3.86	1.46*(37.9)	5.25	2.35(44.9)
Limonene	4.33	1.20*(27.7)	5.79	2.23(38.6)
Terpinolene	3.83	0.94*(24.6)	5.42	2.30(42.4)
Alpha-pinene	3.80	0.76*(20.0)	5.33	1.96(36.8)
Nondiscrimination zones (99% CL)		(38.3–61.7)		(47.2–52.8)

*Significant rejection of treatment odor ($P < 0.01$).

Evidence of partial adaptation by the deer to some of the odors is presented in Figure 1. Between 24 and 72 hr, both males and females reduced their initially strong rejection of α-pinene, limonene, and terpinolene, and females also reduced their rejection of α-terpineol and borneol. The monoterpene hydrocarbons limonene, terpinolene, and α-pinene were more strongly rejected by both males and females than the monoterpene alcohols. α-terpineol and borneol. There was a strong logarithmic relationship between the vapor pressure of the compounds tested (vapor pressure data from Jordan, 1954) and initial response ($r = 0.95$ for males and 0.98 for females) (Figure 2).

These results suggest that deer may reject monoterpenes on the basis of odor concentration rather than odor quality. It is likely that the rejection of monoterpene odors by deer is an innate response. Since the deer in this experiment had never encountered monoterpenes or conifers in their diet before, their response could not have been based on learning from previous experience. An innate rejection of foods containing monoterpenes may have selective advantages for deer since several authors (Schwartz et al., 1980; Conolly et al., 1980; Welch and Pederson (1981) have demonstrated that monoterpenes inhibit digestion in ruminants, such as deer, by inhibiting the activity of rumen microorganisms.

Olfactory adaptation is the process whereby odors initially perceived as strong, gradually seem to weaken when exposure to them is prolonged. Originally, olfactory adaptation was thought to be due to the inability of the olfactory receptors to respond to prolonged stimulation, but now it is believed that olfactory stimuli may be suppressed at a central level (Ottoson, 1963). In domestic

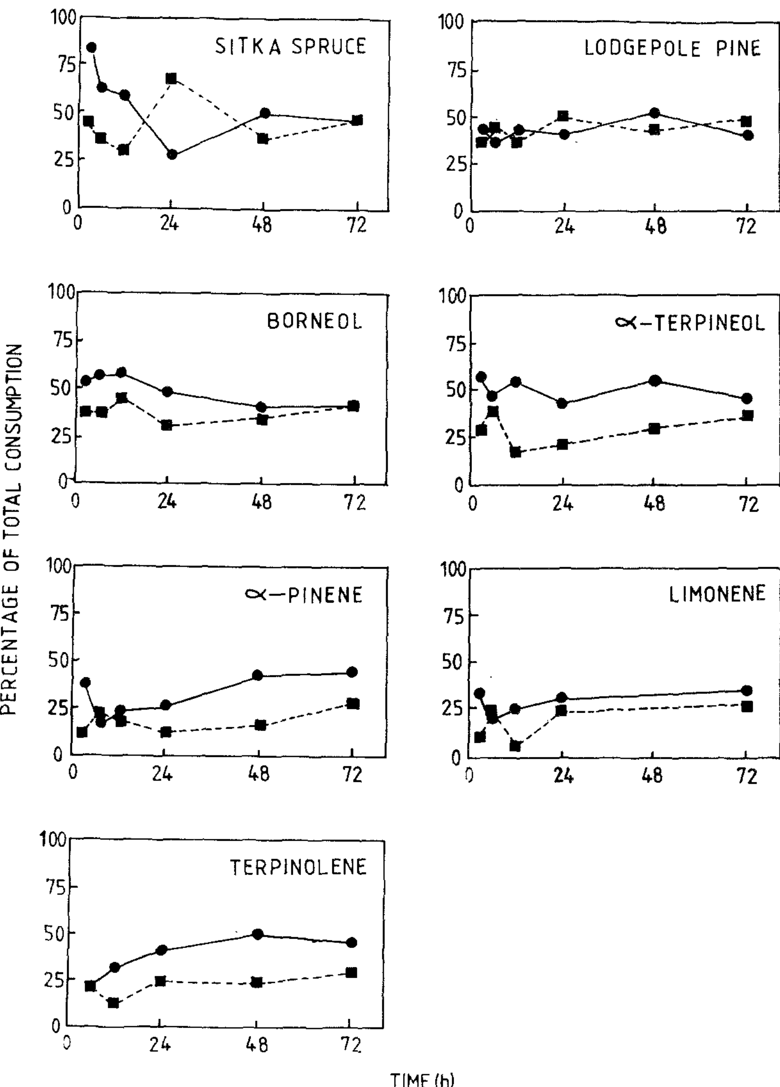

FIG. 1. Variations in response of red deer calves to odors over time. —●—, males; --■--, females.

ruminants, olfactory adaptation has been demonstrated to occur rapidly. Arnold and Hill (1972) and Arnold et al. (1980) showed that olfactory adaptation in sheep could occur within 6–24 hr, and Tribe (1949) found that sheep could adapt in less than an hour to several odors, including two which contained monoterpenes (eucalyptus and cedar wood oils). Figure 1 shows that for red deer calves, olfactory adaptation either did not occur or occurred only partially

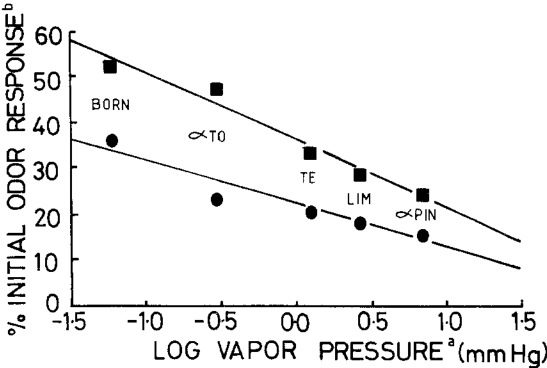

FIG. 2. Relationship between strength of rejection response and vapor pressure for five monoterpene odors: borneol (BORN), alpha-terpineol (αTO), terpinolene (TE), limonene (LIM), and alpha-pinene (αPIN). ■, males, $r = 0.95$; ●, females, $r = 0.98$. [a]Based on data of Jordan (1954) at 30°C. [b]Percentage of total consumption taken from odor-treated buckets 0–24 hr.

over 72 hr. The slowness with which olfactory adaptation occurred in deer compared with published data for sheep suggests that wild ruminants may rely more heavily on their sense of smell when feeding then domestic ruminants. The effect of domestication on olfaction has been demonstrated by Kruska and Stephan (1973), who found that olfactory structures in the forebrain of domestic pigs were about 30% smaller than in wild pigs. Stoddart (1980) suggested that "reductions in the olfactory part of the brain are related to the obvious behavioral changes wrought by domestication." It may be that domestication, in selecting for high productivity, has selected against those animals which reduce consumption in response to adverse olfactory stimuli.

The higher degree of rejection of food contaminated by monoterpene odors by females compared with males may have been due to bullying behavior which was common between males and may have reduced the opportunity for smaller males to select food from the preferred odorless buckets. Bullying between females was rarely observed. Alternatively, this result may reflect a genuine difference between males and females in their ability to detect and respond to olfactory stimuli. Sex differences in olfactory sensitivity have also been demonstrated in several mammals including humans (Doty et al., 1984) and black-tailed deer (*Odocoileus hemionus*) (Rice and Church, 1974). Male and female black-tailed deer differ markedly in their responses to butyric acid and putrefied fish extract.

This clear demonstration of the effects of monoterpene odors on the feeding behavior of deer may be of ecological significance in determining food selection in the wild. It is therefore important to extend these observations to feeding trials under natural conditions.

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IDENTIFICATION OF UNIQUE PHEROMONE COMPONENTS FOR SOYBEAN LOOPER MOTH *Pseudoplusia includens*

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Abstract—Analysis of sex pheromone glands from individual female soybean looper moths showed that in addition to the previously identified main component (Z)-7-dodecenyl acetate, the compounds dodecyl acetate, 11-dodecenyl acetate, (Z)-7-dodecenyl propionate, and (Z)-7-dodecenyl butanoate were also produced. Two of the components, 12 : OAc and 11–12 : OAc, were not detected in a single analysis of female effluvium. Flight-tunnel tests showed that the five-component and three-component blends were equal to each other and to a female extract (> 80% source contacts) and that the mixtures were superior to Z7–12 : OAc alone. Field tests indicated that the five-component blend was significantly more attractive than Z7–12 : OAc alone at a dosage of 1 mg, but that the blend was only slightly better at 3 mg.

Key Words—Sex pheromone, Lepidoptera, Noctuidae, *Pseudoplusia includens*, sustained-flight tunnel, (Z)-7-dodecenyl acetate, dodecyl acetate, 11-dodecenyl acetate, (Z)-7-dodecenyl propionate, (Z)-7-dodecenyl butanoate.

INTRODUCTION

Males of a large number of noctuid species are attracted to (Z)-7-dodecenyl acetate (Z7–12 : OAc) alone or in combination with other compounds (Steck et al., 1982). This includes many species in the subfamily Plusiinae, such as the

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cabbage looper moth, *Trichoplusia ni* (Berger, 1966) and the soybean looper moth, *Pseudoplusia includens* (Tumlinson et al., 1972). Although both *T. ni* and *P. includens* males are attracted to relatively high release rates of Z7–12:OAc in pheromone traps, the males of both species readily distinguish conspecific females in pheromone traps (Tumlinson et al., 1972; Kaae et al., 1973; Leppla, 1983), and the presence of female *T. ni* reduces the response of male *P. includens* (Mitchell, 1972; Leppla, 1983). These results were explained in part with the identification of five additional pheromone components for *T. ni*, including 12:OAc, Z5–12:OAc, 11–12:OAc, Z7–14:OAc, and Z9–14:OAc (Bjostad et al., 1980, 1984; Linn et al., 1984). We now have investigated *P. includens* and report on its unique sex pheromone blend.

METHODS AND MATERIALS

Extracts. The insects were reared on a semisynthetic diet (Shorey and Hale, 1965), at 26°C, 70% relative humidity, 16:8 light–dark photoperiod. The sexes were separated as pupae, and males were held at conditions similar to those used in rearing. Pheromone glands were dissected from 3- to 4-day-old females at the midpoint of the 8-hr scotophase, placed in 10 μl of glass-distilled dichloromethane, and soaked for 1 hr prior to GLC analysis.

Volatile extracts were prepared as in Bjostad et al. (1984), by trapping volatiles from the gland (20 min) onto glass wool under a nitrogen stream. Collections were made during the fifth hour of the 8-hr scotophase. The volatile extract was then analyzed by capillary GLC.

Chemical Identification. Capillary GLC was conducted with a polar 45-m Carbowax 20 M column, and a nonpolar 49-m OV-101 column, used with splitless injection and programmed from 80 to 180°C at 10°/min after an initial delay of 1 min. Quantification of relative abundances of compounds was performed by electronic integration of peak areas (flame ionization detection). The packed GLC column was a 3% OV-101 (methyl silicone) on 100- to 120-mesh Gas-Chrom Q in a 2-m glass column (inside diameter, 4 mm) at 180°C. Fractions were collected with 30-cm glass capillary tubes.

Hydrolysis of the pheromone acetates was performed by adding 100 μl of a 0.5 M NaOH in aqueous ethanol solution to crude gland extract in a 4-ml vial. Hexane (0.2 ml) was added after 30 min at room temperature, and the mixture was shaken. The hexane layer was removed and reduced in volume by evaporation with a nitrogen stream, and the retention time of the hydrolysis product was determined by capillary GLC.

Acetylation was carried out on the hydrolysis products by adding a drop of acetyl chloride to a 4-ml vial in which a portion of the hexane layer from hydrolysis had been evaporated to dryness. After 1 hr, the acetyl chloride was evaporated to dryness, and a drop of hexane was added to recover the acetyla-

tion product. After concentration with a stream of nitrogen, the solution was analyzed by capillary GLC.

Mass spectra were obtained with a HP-5985 GC-MS equipped with a 25-m Carbowax 20 M glass capillary column. Chemical ionization was performed with isobutane as a reagent gas.

Synthetic compounds used for standards and for the behavioral studies were as follows: (*Z*)-7-dodecenyl acetate (Farchan Co., Willoughby, Ohio); 11-dodecenyl acetate (Nu Chek Prep, Inc., Elysian, Minnesota); (*Z*)-7-dodecenyl propionate and butanoate were prepared by reaction of (*Z*)-7-dodecen-1-ol with propionyl chloride or butyryl chloride. The compounds were found to have greater than 99% isomeric purity by capillary GLC analysis (Carbowax 20 M column).

Flight-Tunnel Tests. Individual 3- to 4-day-old males were tested in the sustained-flight tunnel described by Miller and Roelofs (1978), during the fifth and sixth hours of the 8-hr scotophase period. Males were placed in the room housing the tunnel at the beginning of the scotophase to acclimate to light and temperature conditions in the tunnel: 0.3 lux and 21–23°C. Other conditions in the flight tunnel were 70% relative humidity and 50–55 cm/sec wind velocity. Males were handled during testing as described in Linn et al. (1984). Each male was allowed 1 min to respond and was scored for the following behaviors: taking flight, oriented flight in the odor plume, initiation of upwind flight, and source contact. Males were tested only once and then discarded.

Sources for synthetic compounds in all tests were polyethylene caps, and these were prepared by adding Skelly B solutions of the synthetics to the inside of the caps with Pasteur pipets. After 1 hr, the caps were closed and left in the laboratory hood for 36 hr. Caps were held in glass vials at −10°C when not in use. In addition, pheromone gland extracts were tested by soaking three female glands in 30 μl of dichloromethane, placing 10 μl of this solution onto a 0.75-cm^2 piece of Whatman No. 1 (qualitative, lot No. 308306) filter paper, and positioning this on the end of an insect pin placed in a cork stand.

Field Tests. Field trials were conducted during the growing seasons of 1983 and 1984, at the Louisiana State Penitentiary farm, Angola, Louisiana. Pherocon 1C (Zoecon Co., Palo Alto, California) traps were baited with the desired pheromone blends or dosage of Z7–12:OAc and placed 30 m apart on aluminum poles ca. 1.5 m high. Trap height was no greater than 20 cm above the crop canopy. Tests were conducted in a variety of crops, including soybean, cabbage, and broccoli. Each treatment was replicated three times. Traps were monitored daily or on alternate days over a two-week period, and treatments were randomized each collection day. Captured specimens were returned to the laboratory and identified to species, using genitalia characteristics described by Eichlin (1975). Data for each replicate were combined over all days, and the mean trap catch for the three replicates was calculated. Treatment means were compared by Duncan's multiple-range test.

RESULTS

Chemical Identification. Analysis of single female pheromone glands by capillary GLC showed the presence of four compounds in addition to the main component Z7–12:OAc (Figure 1). The ratios of these components in eight different gland analyses is given in Table 1. The earliest eluting compound was determined to be dodecyl acetate (12:OAc) by comparison of its retention times to a standard on the Carbowax and OV-101 capillary GLC columns, and by hydrolysis to dodecen-1-ol and reacetylation to 12:OAc as determined by capillary GLC analysis.

The compound eluting just after Z7–12:OAc was found to be 11-dode-

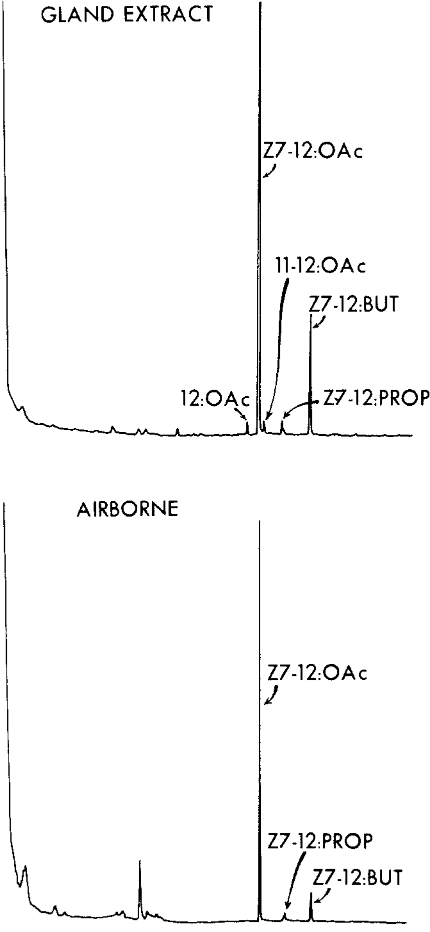

FIG. 1. Capillary GLC traces of gland extract and volatile extract of *P. includens*.

TABLE 1. PROPORTIONS OF COMPOUNDS FROM GLAND EXTRACTS AND VOLATILE EXTRACT OF INDIVIDUAL SEX PHEROMONE GLANDS OF *P. includens*

	Gland Extract								Mean	SD	Volatile
Compound	1	2	3	4	5	6	7	8			
12:OAc	0.1	6.8	0.3	0.4	0.5	0.6	0.5	0.4	1.2	2.3	—
Z7–12:OAc	95.5	93.2	95.0	89.9	93.0	91.9	93.7	93.0	93.2	1.7	88.8
11–12:OAc	0.4	0.7	0.6	1.1	0.9	0.6	0.6	0.6	0.7	0.2	—
Z7–12:Prop	0.4	1.0	0.6	2.6	0.4	0.5	1.0	0.4	0.9	0.7	3.4
Z7–12:But	3.6	5.2	3.6	6.8	5.6	7.0	4.7	5.8	0.2	1.2	7.8

cenyl acetate (11–12:OAc) by comparison of its retention times to a standard on the Carbowax and OV-101 capillary GLC columns. Although the retention times of Z9–12:OAc and 11–12:OAc are the same on Carbowax, they were separated (25.02 and 24.86 min, respectively) on the OV-101 column using a program rate of 4°C/min.

The two remaining compounds were determined to be (Z)-7-dodecenyl propionate (Z7–12:Prop) and (Z)-7-dodecenyl butanoate (Z7–12:But). Analysis of retention times for these compounds from crude extracts on the capillary GLC columns suggested that they were not standard 12- or 14-carbon acetates, alcohols, or aldehydes. Hydrolysis of the components from female extracts collected from the packed methyl silicone column, however, showed that the alcohol products had identical retention times on the capillary Carbowax column to that of the alcohol from the main component Z7–12:OAc. The alcohols were acetylated to give a product with the same GLC retention time as that of Z7–12:OAc. The propionate and butanoate moities were characterized with CI and EI mass spectral data. In addition to the fragmentation pattern expected for the long-chain alcohol portion of the compounds, the propionate compound produced a (M + 1) ion at *m/z* 241 with CI, and characteristic propionate fragmentation ions at *m/z* 75 and 57 with both CI and EI. The butanoate compound exhibited a (M + 1) ion at *m/z* 255 with CI, and characteristic butanoate fragmentation ions at *m/z* 89 and 71 with both CI and EI. All spectra were identical to those of synthetic standards.

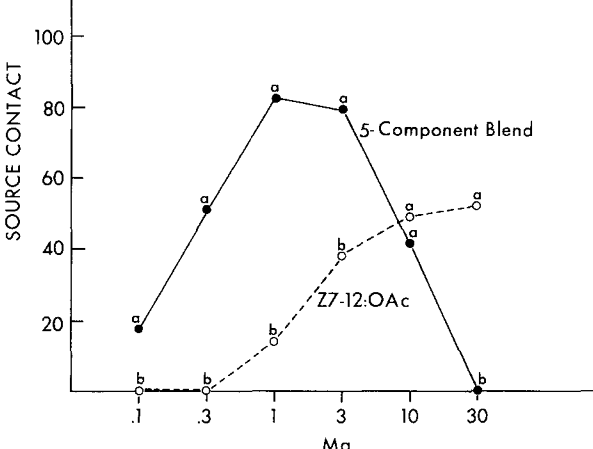

FIG. 2. Response, as proportion of males reaching source, of *P. includens* to a dosage series of the five-component blend (as defined in Figure 1 from gland extracts) versus Z7–12:OAc alone. $N = 40$ for each data point. Values for each dosage with different letters are significantly different ($P < 0.05$) according to the method of adjusted significance levels for proportions (Ryan, 1960).

Whereas analysis of gland extracts showed the presence of four additional compounds, only the Z7–12 : OAc, propionate, and butanoate components were detected in an analysis of the female effluvium (Figure 1, Table 1). Several additional airborne collections were atttempted, but the quantity of compounds trapped was too low for analysis.

Flight-Tunnel Tests. Male *P. includens* were tested in the flight tunnel to a dosage series of Z7–12:OAc, and the five-component blend identified from female glands (Figure 2). Males responded in peak percentages (80%) to the 1- and 3-mg dosages of the blend, whereas response to Z7–12:OAc at these dosages was 16 and 38% respectively.

Male *P. includens* then were tested to several treatments at the 3-mg dosage to determine the effect of removing various compounds on male flight behavior (Figure 3). Male response was >80% to the female-produced blend and the blend minus 12:OAc and 11–12:OAc, and this was not significantly different from the response level to the extract (1 FE). Removal of either the propionate or butanoate from the five-component blend, however, resulted in

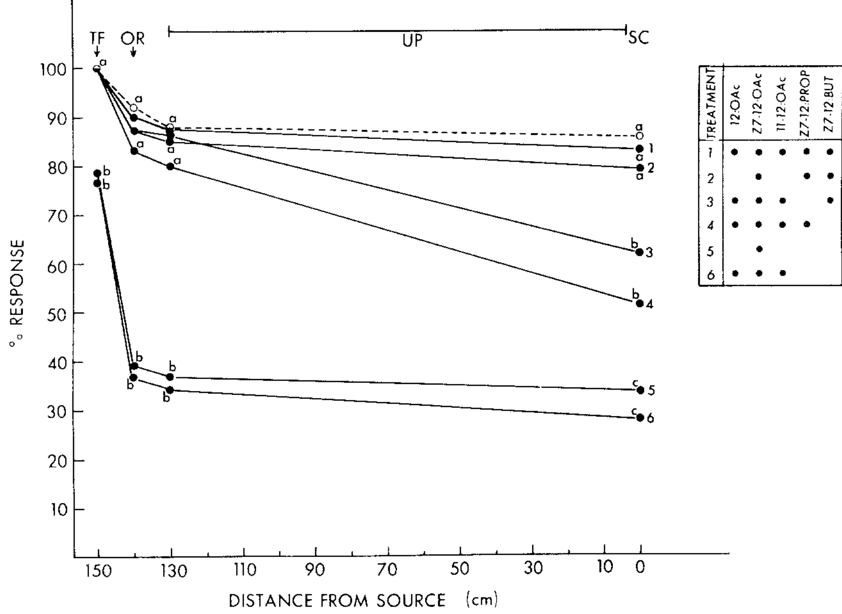

FIG. 3. Response of male *P. includens* in the flight tunnel to a 3-mg dosage of selected blends, Z7–12:OAc alone, and a 1-FE gland extract. Behaviors are: taking flight (TF), stationary orientation flight (OR), upwind flight (UP), and source contacts (SC). Dashed line represents response to 1 FE gland extract. $N = 70$ for each treatment. Values for any behavior with different letters are significantly different ($P < 0.05$) according to the method of adjusted significance levels for proportions (Ryan, 1960).

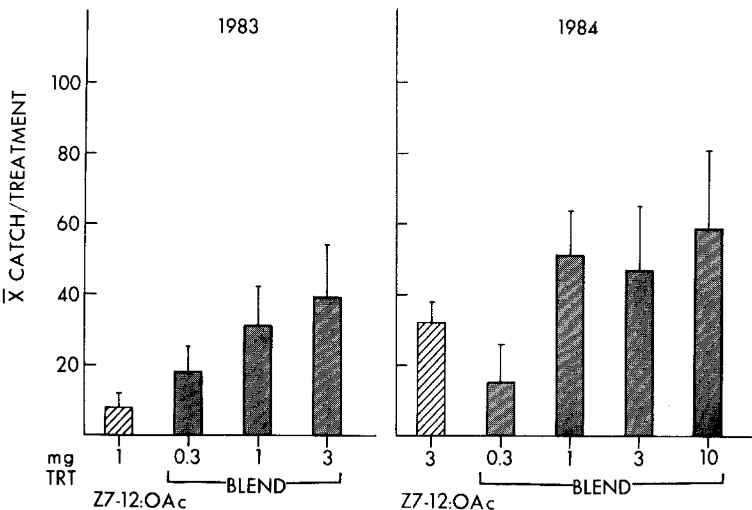

FIG. 4. Trap catch of male *P. includens* to a dosage series of the five-component blend (as defined in Figure 1 from gland extracts) versus a 1- (1983) or 3- (1984) mg dosage of Z7–12 : OAc. Values are mean + SD, N = 3 replicates for each treatment, with each replicate representing total catch over a two week period.

significantly fewer males reaching the source, as a result of arrestment of upwind flight. Male response was significantly lower at all steps in the sequence to Z7–12 : OAc alone compared to other treatments, primarily due to fewer males taking flight and orienting to the pheromone plume. The results in Figure 3 also show that addition of 12 : OAc and 11–12 : OAc to Z7–12 : OAc did not significantly affect male behavior over that observed to Z7–12 : OAc alone.

Field Tests. Field trials in 1983 showed that male *P. includens* were caught in significantly greater numbers to the 1- and 3-mg dosages of the five-component blend compared to the 1-mg dosage of Z7–12 : OAc alone (Figure 4). Tests in 1984, however, showed that trap catch with a 3-mg dosage of Z7–12 : OAc was lower, but not significantly so, compared to the 1-, 3-, or 10-mg dosages of the blend.

DISCUSSION

In addition to Z7–12 : OAc, four compounds were observed in pheromone gland extracts of female *P. includens*. These compounds were identified as 12 : OAc, 11–12 : OAc, Z7–12 : Prop, and Z7–12 : But. The mixture of five components elicited response levels in the flight tunnel that were equal to those with female extracts, and both were superior to Z7–12 : OAc alone.

Two of the minor components, 12:OAc and 11–12:OAc, found in *P. includens* gland extracts also are part of the *T. ni* sex pheromone blend. They play a questionable role with *P. includens*, however, since they were not detected in the female effluvium assay, and full activity was obtained in flight tunnel tests when they were omitted from the blend. The remaining three components, Z7–12:OAc, Z7–12:Prop, and Z7–12:But, for *P. includens* represent a unique pheromone system. Although all can be produced from the same biosynthetic route of Z11–16:acid to Z9–14:acid to Z7–12:acid to Z7–12:OH as found in *T. ni* (Bjostad and Roelofs, 1983), we are not aware of any other lepidopteran species that utilizes this interesting ester series of acetate, propionate, and butanoate.

We have shown that *T. ni* and *P. includens* both use Z7–12:OAc as a major pheromone component and that both produce two minor components in common. However, *T. ni* has three additional specific components and *P. includens* has two additional unique components. The importance of these blends in effecting specific mate recognition signals for these two species is presented in a companion paper (Linn et al. 1987).

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ISOLATION AND STRUCTURE DETERMINATION OF NEW NEMATICIDAL TRIGLYCERIDE FROM *Argemone mexicana*

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Abstract—The petroleum ether extract of *Argemone mexicana* seeds was found to possess nematicidal activity against the plant parasitic nematode *Meloidogyne incognita*. The active nematicidal compound has been isolated from the crude extract by column chromatographic techniques and purified by TLC. Chemical structure has been determined by chemical and spectroscopic methods to be that of a triglyceride, sn-glycerol-1-eicosa-9, 12-dienoate-2-palmitoleate-3-linoleate.

Key Words—Nematoda, *Meloidogyne incognita*, *Argemone mexicana*, Papaveraceae, triglyceride, nematicide.

INTRODUCTION

Recent ecological studies have shown that some plant species escape attack by plant-parasitic nematodes (Huang, 1985). The number of plant-parasitic nematodes capable of attacking a given plant species is controlled by the presence of plant defense mechanisms. The mechanisms involved in plant resistance to nematodes have been reviewed (Kaplan and Keen, 1980; Veech, 1981, 1982). Chemical investigations of plants that escape nematode attack have led to the isolation and identification of several natural nematicides (Figure 1).

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FIG. 1. Selected natural nematicides isolated from plants: (**1**) cucurbitacin, isolated from cucumber (Huang, 1985); (**2**) pyrocatechol, isolated from roots of *Eragrostis curvala* (Scheffer et al., 1962); (**3**) glyceollin, isolated from soybeans (Veech, 1981); (**4**) α-terthienyl, isolated from roots of *Tagestes erecta* (Veech, 1981); (**5**) polyacetylene, isolated from safflower, *Carthamus tinctorius* (Kogiso et al., 1976); and (**6**) asparagusic acid, isolated from asparagus (Takasugi et al., 1975).

As a part of our ongoing studies on isolation and characterization of natural products which are bioactive against agricultural pests (Saleh, 1984, 1985, 1986; Saleh et al., 1984, 1986b, c; Abdel Rahman et al., 1986), we report here the isolation of a nematicidal triglyceride from *Argemone mexicana* L. (Papaveraceae). The most active nematicidal component in the crude petroleum ether extract of *A. mexicana* seeds was isolated by chromatographic techniques, with the isolation sequence being guided by a laboratory bioassay against the root-knot nematode *Meloidogyne incognita*. The active compound has been identified by chemical and spectroscopic methods.

METHODS AND MATERIALS

Isolation of Nematicidal Components. Seeds of *Argemone mexicana* were collected from the Nile Bank at Aswan, Egypt, during the spring of 1984. The seeds (400 g) were ground to a fine powder and extracted successively with petroleum ether (40–60°C), then with acetone, and finally with methanol in a Soxhlet apparatus. The extracts were dried over anhydrous sodium sulfate, filtered, and the solvents removed at reduced pressure to give 95 g (24% yield) of a light yellow viscous oil from the petroleum ether fraction, 24 g (6% yield) of a yellow oil from the acetone fraction, and 18 g (4.5% yield) of an orange oil from the methanol fraction.

The crude extract obtained using petroleum ether was chromatographed on a column containing silica gel, (AR-100-mesh, Mallinkrodt, Inc., St. Louis, Missouri) preactivated by heating for 24 hr at 110°C and packed with hexane. Major column fractions were eluted with hexane, followed by mixtures of 5%, 10%, 20%, and 50% ether in hexane, then ether and acetone, and finally methanol.

Column fractions were monitored for nematicidal activity and examined by thin-layer chromatography (TLC). The most active column fraction (10% ether in hexane) was further fractionated and purified by preparative TLC on precoated silica gel plates of 1 mm thickness (EM Laboratories, Inc., Elmsford, New York) developed three times with 10% ether in hexane. Analytical TLC was carried out on 0.25-mm silica gel plates with the solvent system hexane–ether–acetic acid (90 : 10 : 1); spots were detected in iodine vapor or after spraying with anisaldehyde reagent and heating at 100°C (Stahl, 1973).

Nematicidal Bioassay. Aqueous solutions of the crude seed extracts, column fractions, and the purified compound were prepared by solubilizing them in a solvent–surfactant–water medium that is nontoxic to nematodes (Feldmesser et al., 1983). The nematicidal activities of the crude seed extract, column fractions, and the purified compound were investigated using the wet-screen method (McBeth and Bergeson, 1953) and by final viability determination (Feldmesser et al., 1983).

Wet-screen bioassay was carried out in a multicavity dish; each cavity was filled with 5 ml of the aqueous solution containing the test compound and one drop of water–nematode suspension containing ca. 100 nematodes, *Meloidogyne incognita*. Observations were made daily for one week by microscopic examination. Normal unstressed *M. incognita* are in continuous rapid motion, and the esophageal areas are hyaline. Exposure to nematicides results in reduced motility, immotility, and death; when the nematodes are moribund or dead, the esophageal structures disintegrate and darken. When reduction or cessation of motility appeared to be the sole or major effect of exposure, nematodes were held for 24 hr or longer after the end of the exposure period to determine re-

covery of motility. Untreated control samples were run along with experimental extracts; the control mortality was $< 10\%$.

Visual examination is not completely reliable for determining mortality; therefore final viability determinations on tomato plants were carried out according to Feldmesser et al. (1983). The inoculated tomato seedlings were examined after three weeks to determine the viability of the nematode inocula expressed as root infection. Five replicates of each treatment were carried out, and the results are reported as an average of the five replicates. Infections were evaluated on an arbitrary basis (the root-knot index) by assigning values of 0 = no infection, 1 = 1–25% of the roots galled, 2.0 = 26–50% galled, 3.0 = 51–75% galled, and 4.0 = 76–100% root infection.

Instruments and Conditions. Gas chromatography–mass spectrometry (GC-MS) was performed on a Finnigan-Mat 5300 GC-MS data system equipped with a 30-m (0.25 mm ID) DB-1 fused silica capillary column. Helium was used as the carrier gas and methane as the makeup gas for chemical ionization (CI) runs. GC separations of fatty acid methyl esters were carried out at a column temperature which was started at 100°C and raised to 200°C at a rate of 5°C/min. Spectra in the EI mode were recorded at 70 eV. Mass spectra (MS) of the triglyceride were determined using direct exposure probe (DEP) techniques. High-resolution MS were recorded on a VG Zab-4F analytical instrument (DEP technique). Infrared (IR) spectra were recorded on an IBM IR/32 FT spectrophotometer. High-resolution proton magnetic resonance spectra ($[^{1}H]$NMR) were recorded in $CDCl_3$ with a Bruker 500-MHz NMR spectrometer. $[^{13}C]$NMR and two-dimensional chemical shift correlations spectra were recorded on a 300 MHz Bruker instrument.

RESULTS AND DISCUSSION

Seeds of *Argemone mexicana* were collected from the Nile Bank of Aswan, Egypt, in May 1984. The seeds were extracted successively with petroleum ether, then with acetone, and finally with methanol. All extracts were bioassayed for their nematicidal activity against the root-knot nematode *Meloidogyne incognita* as described in Methods and Materials. The nematicidal activity was found to reside in the petroleum ether extract. This extract was fractionated by column chromatography on silica gel; the fractionation and isolation sequences were guided by bioassay. The column fraction eluted with 10% ether in hexane was the most active, being four times more active than the crude extract. Final purification of this column fraction by TLC yielded the active compound which was found to be five times more active than the crude extract. Solutions of 200 ppm of the purified compound showed a strong killing effect (LC_{50} 90 ppm, LC_{90} 150 ppm) in the wet-screening bioassay. In the final viability test, solutions of 100 ppm of the purified compound prevented formation of nematode

galls or root-knots in the roots of tomato plants, corresponding to a root knot index of zero. The potency increased fivefold during isolation, but the component isolated is only 2% of the seed oil, suggesting either that considerable loss of the nematicidal principal during isolation or that a synergist component(s) is removed during the isolation process. It is interesting in this context that *A. mexicana* is known to contain several biologically active components. Sanguinarine, a liver-toxic alkaloid, also a potent inhibitor to cytochrome P-450, and benzphetamine *N*-demethylase were isolated from the seeds of *A. mexicana* (Dalvi, 1985). Norsanguinarine, herberine, protopine, and β-sitosterol were isolated from the roots and 3-methoxyquercein and vanillic acid from the flowers (Pathok et al., 1985); aqueous extract of the leaves was antiinflammatory (Sukumar et al., 1984).

The active fraction was found to be pure by TLC; its exact mass from the high-resolution mass spectrum was found to be 880.7475, suggesting a possible component(s) of $C_{57}H_{100}O_6$. The NMR, IR, and MS data are consistent with a triglyceride structure of high purity of the type sn-glycerol-1-eicosa-9,12-dienoate-2-palmitoleate-3-linoleate (Figure 3). Infrared spectrum showed absorption bands characteristic of aliphatic ester groups (1750.6 cm^{-1}) and nonconjugated *cis* C=C double bonds (3010.3, 1653.2, 750.5 cm^{-1}). The molecular formula of the active compound, $C_{57}H_{100}O_6$, indicates eight sites of unsatura-

TABLE 1. NMR DATA OF NEMATICIDAL COMPOUND **1**[a]

Position	[^{1}H]NMR	[^{13}C]NMR (ppm)
1		173.35
1′		172.93
2	2.30 t (7.51)	34.22
2′	2.31 t (7.50)	34.39
3	1.59 br m (7.50, 6.93)	25.06
4	2.04 q (6.69, 6.93)	27.39
5	5.35 m	128.06–130.38
6	2.76 t (6.54)	25.82
7	1.34 m	22.27–32.11
7′	1.27 m	22.27–32.11
8	0.87 t (5.06)	14.29
9	4.13 dd (5.93, 11.87)	62.28
9′	4.28 dd (4.30, 11.87)	62.28
10	5.10 m	69.09

[a] Spectra were run in $CDCl_3$, and Me_4Si was used as the internal standard. Values are recorded in parts per million (ppm) downfield from Me_4Si; coupling constants (hertz) are in parentheses. Multiplicity is designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet whose center is given; br, broad. Spectra were determined on a 500 MHz instrument for protons and on a 300 MHz instrument for ^{13}C.

$CH_2OCOC_{19}H_{35}$
|
$CH\ OCOC_{15}H_{29}$
|
$CH_2OCOC_{17}H_{31}$

m/e 880 ($M^{\oplus}$), (6)

m/e 573 ($M - C_{19}H_{35}C\overset{\oplus}{O}O$), (11) m/e 625 ($M - C_{15}H_{29}CO\overset{\oplus}{O}$), (6) m/e 601 ($M - C_{17}H_{31}CO\overset{\oplus}{O}$), (15)

559 ($M - C_{19}H_{35}COOC\overset{\oplus}{H_2}$), (3) 587 ($M - C_{17}H_{31}COOC\overset{\oplus}{H_2}$), (3)

313, ($C_{15}H_{29}COO$–[⊕ ring]–OH), (6) 354, ($C_{15}H_{29}COO$–[⊕ ring with two O]=CH_2), (2)

368, ($C_{15}H_{29}COO$–[⊕ ring with two O]=$CHCH_3$), (3)

109, ($C_8H_{13}^{\oplus}$), (27) 123, ($C_9H_{15}^{\oplus}$), (14) 137, ($C_{10}H_{17}^{\oplus}$), (8) 149, ($C_{11}H_{17}^{\oplus}$), (10)

55, ($C_4H_7^{\oplus}$), (100) 67, ($C_5H_9^{\oplus}$), (82) 81, ($C_6H_9^{\oplus}$), (68) 95, ($C_7H_{11}^{\oplus}$), (47)

FIG. 2. Mass spectral data of the active compound.

9 → $CH_2OCOCH_2\,CH_2\,(CH_2)_4\,CH_2\,CH{=}CHCH_2\,CH{=}CH\,CH_2\,(CH_2)_5\,CH_3$

1 2 3 7 4 5 6 5 4 7′ 8

10 → $CH\,O\,COCH_2\,CH_2(CH_2)_4CH_2\,CH{=}CH\,CH_2\,(CH_2)_4CH_3$

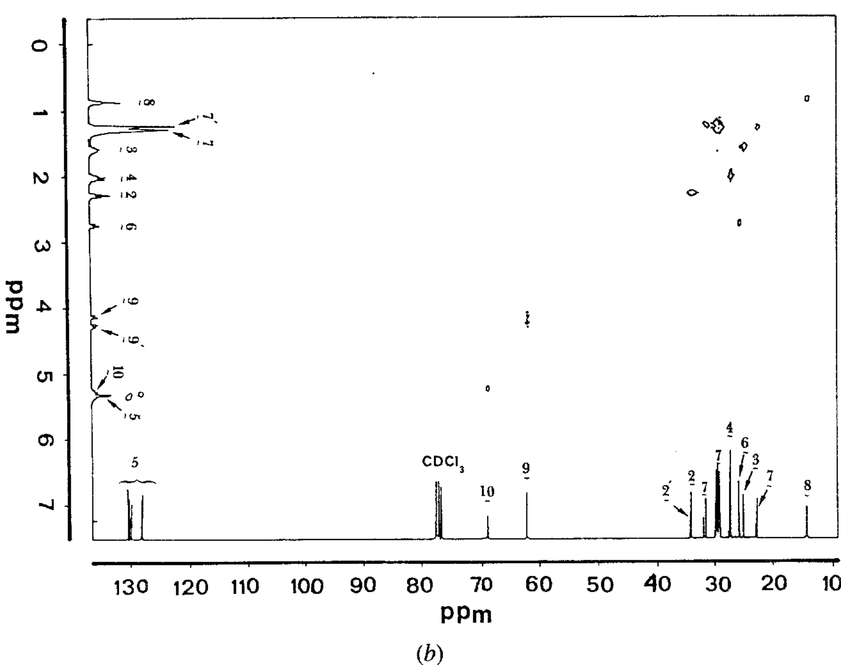
1′ 2′ 3 7 4 5 4 7′ 8

9 → $CH_2O\,COCH_2\,CH_2(CH_2)_4CH_2\,CH{=}CH\,CH_2CH{=}CH\,CH_2\,(CH_2)_3CH_3$

1 2 3 7 4 5 6 5 4 7′ 8

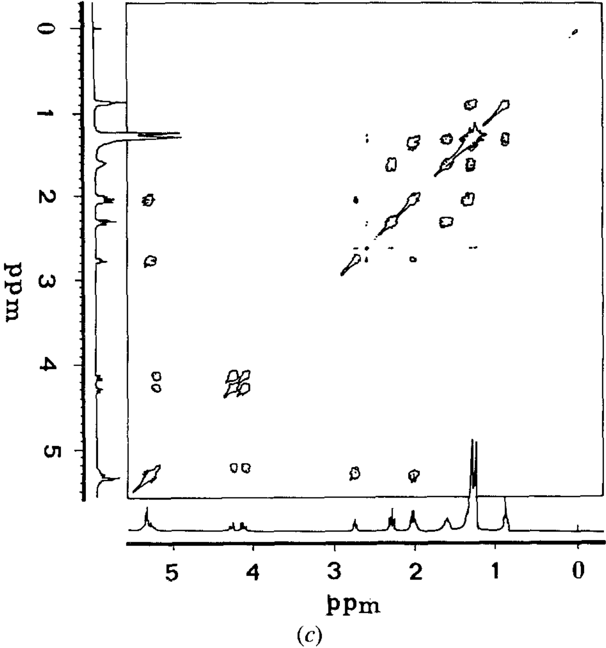
9′ H
9 H—C—O—
H—C—O—
9 H—C—O—
9′ H

(*a*)



(*b*)

FIG. 3. Two-dimensional ^1H–^{13}C and ^1H–^1H NMR data.



(*c*)

FIG. 3. Continued.

tion. Three carbonyl carbons in ester form (173.35 and 172.93 ppm) together with 10 olefinic carbons (128.06–130.36 ppm) can be accounted for from [^{13}C]NMR.

The NMR data of (**1**) are shown in Table 1. The assignments of hydrogen atoms and carbon atoms in the proposed structure were confirmed by the $^{13}C-^{1}H$ and $^{1}H-^{1}H$ two-dimensional spectra shown in Figure 2. Mass spectral data (Figure 3) are also consistent with the proposed structure. The absence of the ion $[M-R_2COOCH_2]^+$ relative to the $[M-R_1COOCH_2]^+$ and $[M-R_3COOCH_2]^+$ ions suggested that the fatty acid $C_{16:1}$ is esterified at the 2-position of the glyceride. The identities of the fatty acids in the triglyceride were confirmed by GC-CIMS analyses of the methyl esters of the fatty acids obtained by saponification of the triglyceride.

The fatty acid composition of *A. mexicana* seed oil has been found to be mostly linoleic, oleic, and palmetic (Mani and Lakshminarayana, 1970; Haussein et al., 1983). Minor amounts of myristic acid, 9- and 11-keto C_{28} and C_{30} acids, and hydroxy and epoxy fatty acids were also identified (Gunstone et al., 1977; Mani and Lakshiminarayana, 1972). The present results represent the first report of a triglyceride with nematicidal effect. This may provide a lead for an investigation of a new class of nematicidal compounds. Several saturated

and unsaturated C_9–C_{16} fatty acids isolated from roots of sweet daphne, *Daphne odora* (Thymelaeceae), were shown to have nematicidal activity to rice white-tip nematode *Aphelenchoides besseyi* (Munakata, 1983); it is also interesting to note that several compounds containing oleic acid, i.e., ethylene glycol mon-oleate, 1-monoolein, oleic acid, and oleyl alcohol, were found to be attractive to pine wood nematodes (Tominaga et al., 1982).

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SEX PHEROMONE COMPONENTS OF THREE SPECIES OF *Semiothisa* (GEOMETRIDAE), (*Z,Z,Z*)-3,6,9-HEPTADECATRIENE AND TWO MONOEPOXYDIENE ANALOGS[1]

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Abstract—Adult males of *Semiothisa signaria dispuncta* (Walker) were attracted to field traps baited with (*Z,Z,Z*)-3,6,9-octadecatriene and (*Z,Z*)-6,9-*cis*-3,4-epoxy-octadecadiene. However, analyses of sex pheromone gland extracts of females of this species by GC-MS and by GC in combination with an electroantennograph detector (GC-EAD) showed the pheromone to be comprised of a mixture of the next lower homologs: (*Z,Z,Z*)-3,6,9-heptadecatriene and (*Z,Z*)-6,9-*cis*-3,4-epoxy-heptadecadiene. Blends of these two C_{17} compounds were subsequently found to be more attractive to males in the field than the corresponding C_{18} mixtures. Sex pheromones of two other *Semiothisa* species were also found to contain C_{17} components. (*Z,Z,Z*)-3,6,9-Heptadecatriene, detected by GC-EAD analysis of a female abdominal tip extract of *S. bicolorata* (Fabricius), attracted conspecific males, and this attraction was significantly reduced by additions of (*Z,Z*)-6,9-*cis*-3,4-epoxy-heptadecadiene, the major pheromone component of *S. signaria dispuncta*, to the lure. (*Z,Z*)-3,9-*cis*-6,7-Epoxy-heptadecadiene was detected by GC-EAD analysis as the primary male antennal stimulatory component present in abdominal tip extracts of *S. ulsterata* (Pearsall), and males of this species were attracted to traps baited with this epoxide. Each of these three C_{17} compounds constitute previously unknown lepidopteran sex pheromone components. Blends of (*Z,Z,Z*)-3,6,9-heptadecatriene and (*Z,Z*)-3,9-*cis*-6,7-epoxy-heptadecadiene attracted males of a fourth species, *S. delectata* Hulst, but no females of this species were obtained to permit analysis of its sex pheromone. The occurrence of (*Z,Z,Z*)-3,6,9-heptadecatriene in *S. neptaria* (Guenee) females was indicated by GC-MS analysis of an abdominal tip extract; how-

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ever, no males were attracted to any of the fielded mixtures containing this hydrocarbon.

Key Words—Lepidoptera, Geometridae, *Semiothisa signaria dispuncta*, *Semiothisa bicolorata*, *Semiothisa ulsterata*, *Semiothisa delectata*, *Semiothisa neptaria*, (Z,Z,Z)-3,6,9-heptadecatriene, (Z,Z)-6,9-*cis*-3,4-epoxy-heptadecadiene, (Z,Z)-3,9-*cis*-6,7-epoxy-heptadecadiene, sex attractant, sex pheromone.

INTRODUCTION

In a previous publication (Wong et al., 1985) adult males of several noctuid and geometrid species were shown to be attracted to C_{18}–C_{21} (Z,Z,Z)-3,6,9-triene hydrocarbons (I in Figure 1), to mixtures of their corresponding *cis*-3, 4-, *cis*-6,7-, and *cis*-9,10-monoepoxydiene analogs (II, III and IV, respectively), and to blends of these trienes and their monoepoxydiene mixtures. Wong et al. (1985) also showed additional noctuid and geometrid species were captured when enantiomers of (Z,Z)-3,6-*cis*-9,10-monoepoxydienes (IV) were tested as single lure components or as blends with triene hydrocarbons. During these field tests, low numbers of *Semiothisa signaria dispuncta* (Walker) males were found to be attracted to lures containing (Z,Z,Z)-3,6,9-octadecatriene (3Z,6Z,9Z–18:H) and to mixtures of its *cis*-3,4-, *cis*-6,7-, and *cis*-9,10-monoepoxydienes. Although the larval form of this species has not been associated with serious damage (Ferguson, 1974), we were interested in determining which of the positional epoxide isomers was responsible for the attraction of these males.

We report here that the sex pheromone of *S. signaria dispuncta* consists of C_{17} compounds and that C_{18} compounds tested previously were functioning as pheromone mimics. We also report the synthesis of the C_{17} series of compounds (I–IV) and data derived utilizing them in field screening tests.

METHODS AND MATERIALS

Synthetic Chemicals

Compounds were synthesized and purified by methods previously reported (Underhill et al., 1983; Wong et al., 1985), except for (Z,Z,Z)-3,6,9-heptadecatriene (3Z,6Z,9Z–17:H) and *cis*-monoepoxydienes described below.

Preparation of (Z,Z,Z)-3,6,9-Heptadecatriene. 1-Nonyne (1.49 g, 12 mmol) was added dropwise to a freshly prepared solution of EtMgBr (13 mmol) in THF (20 ml), and the resulting solution was heated at 40–45°C for 1.5 hr. The solution was then cooled to 0°C, and CuI (95 mg, 0.5 mmol) was added. The mixture was stirred 15 min, then cooled to −15°C, and 1-tosyloxy-2,5-

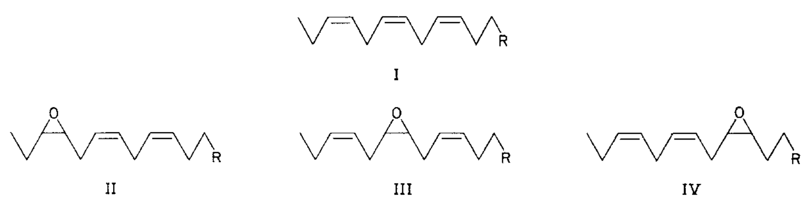

FIG. 1 Structures of some triunsaturated hydrocarbon and monoepoxydiene male sex attractants of arctiid, geometrid, and noctuid males. They include 3*Z*,6*Z*,9*Z*-trienes (I), 6*Z*,9*Z*-*cis*-3,4-epoxydienes (II), 3*Z*,9*Z*-*cis*-6,7-epoxydienes (III) and 3*Z*,6*Z*-*cis*-9,10-epoxydienes (IV) with chain lengths of 18–21 carbons. $R = C_6H_{13}$ to C_9H_{19}.

octadiyne (2.21 g, 8.0 mmol) in THF (10 ml) was added dropwise over 30 min. The mixture was warmed to 20°C over 3 hr, and stirred overnight. The mixture was then poured into aqueous 2 M NH_4Cl (50 ml), and extracted with ether (3 × 50 ml). The combined ether extracts were dried (Na_2SO_4), filtered through a short column of silica gel (2.5 × 5 cm), rinsing the silica well with ether, and the filtrate was concentrated, giving the crude triyne as a yellow oil. This was used directly in the next step.

Dicyclohexylborane (40 mmol) was prepared by the published procedure (Brown et al., 1977) from borane–dimethyl sulfide complex (10 M; 4.0 ml, 40 mmol) and cyclohexene (8.1 ml, 80 mmol) in THF (50 ml). The suspension was cooled to 0°C and the crude triyne in THF (10 ml) was added dropwise. The mixture was warmed to 20°C over 1 hr, and stirred an additional 3 hr. Acetic acid (20 ml) was then added dropwise, and the mixture was stirred overnight. The mixture was then made basic by dropwise addition of aq. NaOH (4 M, 100 ml), with efficient cooling to maintain the temperature below 10°C. Aqueous H_2O_2 (30%, 30 ml) was then added dropwise (very exothermic!), maintaining the temperature below 20°C. The mixture was then extracted with hexane (3 × 100 ml), the combined extracts were washed with brine, dried (Na_2SO_4), and concentrated. Volatile by-products were removed by warming to 50°C at 0.2 Torr. Purification was achieved by flash chromatography on $AgNO_3$ impregnated silica gel (10%; 3.5 cm ID × 20 cm), eluting with 15% ether in hexane, followed by Kugelrohr distillation (bp ≃ 140°C/0.1 Torr), giving the triene (1.08 g, 58%) as a colorless oil.

MS, *m*/*z* (relative intensity) was 234 (M^+, 1.8), 178 (14.2), 108 (42.3), 95 (32.7), 93 (35.2), 80 (52.9), 79 (100), 67 (68.6), 55 (46.2), 43 (43.2). [^{1}H]NMR (360 MHz, $CDCl_3$) δ 5.35 (m, 6H; H-3, H-4, H-6, H-7, H-9, H-10), 2.79 (br t, 4H, J = 5.9 Hz; H-5, H-8), 2.05 (m, 4H; H-2, H-11), 1.45–1.15 (m, 10H; H-12 to H-16), 0.96 (t, 3H, J = 7.5 Hz; H-1), 0.87 (t, 3H, J = 6.8 Hz; H-17). IR (neat) λ_{max}: 3020 (m), 2965 (s), 2935 (s), 2860 (m) cm^{-1}. Final confirmation of the all-*cis* structure was obtained by comparison of the

olefin region of the [^{1}H]NMR spectrum with that of a reference sample of 3*Z*,6*Z*,9*Z*–18:H.

Preparation of C_{17} cis-Monoepoxydienes. The monoepoxides were prepared from 3*Z*,6*Z*,9*Z*–17:H as previously described for the homologous C_{18}–C_{22} compounds (Wong et al., 1985). Thus, metachloroperbenzoic acid (tech. grade, 85% pure; 199 mg, 0.98 mmol) was added to a solution of 3*Z*,6*Z*,9*Z*–17:H (230 mg, 0.98 mmol) in dry CH_2Cl_2 (15 ml) at −10°C. The mixture was warmed to 20°C over 3 hr, then washed with 5% aq. $NaHCO_3$ (2 × 10 ml), dried (Na_2SO_4), and concentrated. The crude product was flash chromatographed on silica gel (2 cm ID × 20 cm), eluting with 3% ether in pentane, giving a combined monoepoxides fraction (157 mg, 63%). A portion of this was then separated into the individual monoepoxides by HPLC (Partisil M9 10/50 ODS-3, 0.9 × 50 cm), eluting with 3% ether in hexane. The elution order was 6,7-epoxide < 9,10-epoxide < 3,4-epoxide. Purified compounds were >99% pure by capillary GC (DB-5, 30 m × .25 mm ID). Baseline separation of the three isomers was obtained with this GC column. The GC elution order was 6,7-epoxide < 3,4-epoxide < 9,10 epoxide.

The EI mass spectra and the NMR spectra (360 MHz) of the isolated positional isomers were as follows: (*Z*,*Z*)-6,9-*cis*-3,4-epoxy-heptadecadiene (6*Z*,9*Z*-*cis*-3,4-epoxy-17:H) *m/z* (relative intensity): 250 (M^+, 0.2), 232 (trace), 221 (0.5), 203 (1.1), 192 (2.9), 178 (11.8), 163 (0.8), 147 (1.8), 135 (2.9), 124 (4.1), 121 (5.6), 109 (6.5), 107 (11.9), 105 (6.2), 95 (16.4), 94 (19.4), 93 (39.0), 91 (26.0), 80 (85), 79 (100), 69 (18.9), 67 (47.6), 59 (22.9), 57 (26.4), 55 (41.8), 43 (14.1). [^{1}H]NMR ($CDCl_3$): δ 5.49 (dtt, 1H, J = 10.7, 7.0, 1.4 Hz; H-7), 5.46-5.35 (m, 2H; H-6, 10), 5.30 (dtt, 1H, J = 10.7, 7.0, 1.4 Hz; H-9), 2.94 (dt, 1H, J = 6, 4, 4.2 Hz; H-4), 2.87 (dt, 1H, J = 6.2, 4.2 Hz; H-3), 2.78 (br. t, 2H, J = 6.7 Hz; H-8), 2.39 (m, 1H; H-5), 2.20 (m, 1H; H-5′), 2.03 (dt, 2H, J = 6.8, 6.4 Hz; H-11), 1.65–1.45 (m, 2H; H-2), 1.4–1.2 (m, 10H; H-12 to H-16), 1.04 (t, 3H, J = 7.5 Hz; H-1), 0.86 (t, 3H, J = 6.9 Hz; H-17).

(3*Z*,9*Z*-*cis*-6,7-epoxy-17:H) *m/z* (relative intensity): 250 (M^+, 0.2), 235 (0.3), 232 (0.2), 221 (1.3), 207 (0.8), 193 (0.6), 181 (2.4), 167 (2.5), 165 (1.9), 163 (2.3), 151 (2.9), 138 (4.1), 137 (3.9), 123 (5.2), 121 (5.5), 111 (18.9), 109 (14.6), 97 (19.4), 95 (29.6), 93 (18.9), 83 (52.5), 82 (29.5), 81 (51.9), 79 (32.4), 69 (46.8), 68 (37.1), 67 (99.7), 57 (29.1), 55 (100), 54 (42.0), 53 (22.8), 43 (64.6). [^{1}H]NMR ($CDCl_3$): δ 5.52 (dtt, 2H, J = 10.8, 7.2, 1.5 Hz; H-3, H-10), 5.40 (m, 2H; H-4, H-9), 2.92 (m, 2H; H-6, H-7), 2.38 (m, 2H; H-5, H-8), 2.18 (m, 2H; H-5, H-8), 2.03 (m, 4H; H-2, H-11) 1.4–1.1 (m, 12H; H-12 to H-16), 0.95 (t, 3H, J = 7.5 Hz; H-1), 0.84 (t, 3H, J = 7.5 Hz; H-17).

(3*Z*,6*Z*)-*cis*-9,10-epoxy-17:H, *m/z* (relative intensity): 250 (M^+, trace), 232 (trace), 221 (0.2), 207 (0.3), 203 (0.2), 195 (0.3), 181 (0.5), 167 (1.5), 151 (0.8), 133 (2.1), 123 (2.8), 122 (7.0), 108 (37.6), 95 (9.8), 94 (8.4), 93

(45.0), 91 (15.2), 81 (17.5), 80 (28.7), 79 (100), 69 (22.2), 67 (36.5), 57 (20.8), 55 (47.3), 43 (31.9). [^{1}H]NMR ($CDCl_3$): δ 5.54–5.24 (m, 4H; H-3, 4, 6, 7), 2.92 (m, 2H; H-9, 10), 2.78 (br t, 2H, $J \simeq 7$ Hz; H-5), 2.38 (m, 1H; H-8), 2.20 (m, 1H; H-8), 2.04 (br dq, 2H, $J \simeq 7, 7$ Hz; H-2), 1.50 (m, 2H; H-11), 1.45–1.1 (m, 10H; H-12 to H-16), 0.96 (t, 3H, $J = 7.5$ Hz; H-1), 0.86 (t, 3H, $J = 6.7$ Hz; H-17).

Insects, Isolation, and Identification of Pheromone Components

Antennae used for electrophysiological work (EAD and EAG) were obtained from adult males captured in field traps baited with sex attractants. Female moths, used as a source of sex pheromone, were recovered from two portable UV light traps, one located 100 km northeast of Saskatoon, Saskatchewan, the other 10 km south. Abdominal tips (terminal 3–4 segments) of field-collected females were excised and extracted with hexane for 30 min. Two internal standards, heptadecane (17 : H) and tetracosane (24 : H), were added to the extracts which were concentrated under a stream of N_2 and fractionated by GC. DB-1701 or DB-5 capillary columns were used (30 m × 0.32 mm ID). Following splitless injection at 40°C for 30 sec, the oven temperature was raised to 90°C at 40°C/min and then programmed at 4°C/min to 225°C. A Hewlett Packard 5710 GC equipped with both a flame ionization detector and an electroantennographic detector (GC-EAD) (Arn et al., 1975) was employed for the simultaneous detection of pheromone compounds. The effluent from either a DB-1701 or a DB-5 capillary column (30 m × 0.32 mm ID) was split 7 : 3 in favor of the FID detector and signals from both detectors were fed to two Hewlett Packard model 3392A recording integrators. Male antennal responses to mixtures of synthetic reference compounds and internal standards were measured and recorded in a similar manner. The reference mixture used for most species contained 17 : H and 24 : H, plus C_{17}–C_{22} 3*Z*,6*Z*,9*Z*-trienes and their corresponding *cis*-3,4-, *cis*-6,7-, and *cis*-9,10-monoepoxydienes; 2–5 ng amounts of each compound were injected. Male antennal stimulation by synthetic compounds was also measured by EAG (Chisholm et al., 1975) using 1 μg of test sample applied to filter paper discs.

Chemical ionization (CI) mass spectra were obtained using a Finnigan 3300 gas chromatograph–mass spectrometer equipped with a DB-5 column (60 m × 0.32 mm ID). Hydrogen was used as the carrier gas and methane as reagent gas. For the detection of pheromone components, the instrument was operated in the multiple-ion detection (MID) mode where a few selected ions were repeatedly scanned; the lower limit of compound detection using this method on our instrument is ca. 50–100 pg. Routinely, a "blank" sample containing only internal standards (17 : H and 19 : H) was run first, followed by the insect extract containing the internal standards, and finally a reference standard which contained 1 ng each of the C_{17} triene and three monoepoxydienes plus 17 : H and

19:H was run for retention-time purposes. Ions used for MID included $(M-1)^+$ corresponding to each of the internal standards, and $(M + 1)^+$, $(M - 1)^+$, $[(M + 1) - 18]^+$ and $[(M - 1) - 18]^+$, where applicable, for the C_{17} triene and monoepoxydienes.

Field Trapping

Field trapping was carried out approximately 100 km northeast of Saskatoon, Saskatchewan in an area containing spruce, pine, birch, aspen, and a variety of herbaceous shrubs. Pherocon 1CP traps, baited with synthetic compounds impregnated in rubber septa, were hung from branches approximately 30 m apart. Traps were set out in a randomized block design and trap captures were recorded at least twice weekly. Summed trap captures were transformed $(X + 1)$, analyzed by an analysis of variance test, and significantly different means were separated by Duncan's (1955) multiple-range test. In all tables, values within a column followed by the same letter are not significantly different ($P > 0.05$).

RESULTS AND DISCUSSION

Following earlier observations (Wong et al., 1985) of captures of *S. signaria dispuncta* in traps baited with 3*Z*,6*Z*,9*Z*–18:H and mixtures of its *cis*-monoepoxydiene analogs, traps (3× replicated) were set out containing the C_{18} triene and each of its three positional *cis*-epoxide isomers (II–IV), separately and as binary mixtures. Only traps which contained 6*Z*,9*Z*-*cis*-3,4-epoxy-18:H as a component attracted significant numbers of males of this species, and those traps which contained both the triene hydrocarbon and *cis*-3,4-epoxide captured significantly more males than any other treatment. Mixtures of the triene and 3,4-epoxide were further tested (Table 1) and optimum attraction was observed in traps baited at 9:1 and 4:1 ratios (epoxide–triene). Attraction of moths by lures at these two ratios was very species specific; all but one of 293 specimens taken were *S. signaria dispuncta* males. The results of EAG and combined GC-EAD analyses using C_{18-22} 3,6,9-triene hydrocarbons, and their derived monoepoxides were consistent with the field data in that the greatest antennal stimulation was elicited in response to 6*Z*,9*Z*-*cis*-3,4-epoxy-18:H (4.9 mV, EAG, $N = 4$). The response to 3*Z*,6*Z*,9*Z*–18:H, while only 25% as intense as that from the *cis*-3,4-epoxide, was the highest response given by the series of triene hydrocarbons.

Combined GC-EAD analysis of a hexane abdominal tip extract obtained from a field-collected *S. signaria dispuncta* female (Figure 2) disclosed no male antennal stimulation at retention times corresponding to any of these C_{18} compounds but strong activity was clearly associated with an earlier-eluting component. By extrapolation of the retention times of reference standards towards

TABLE 1. EFFECT OF DIFFERENT RATIOS OF 3Z,6Z,9Z-18:H AND 6Z,9Z-*cis*-3,4-EPOXY-18:H ON LURE ATTRACTANCY FOR *S. signaria dispuncta* MALES

Attractant formulation (μg)	Total males captured[a]
6Z,9Z-*cis*-3,4-epoxy-18:H (500)	40cd
6Z,9Z-*cis*-3,4-epoxy-18:H (500) + 3Z,6Z,9Z-18:H (5)	69cd
6Z,9Z-*cis*-3,4-epoxy-18:H (450) + 3Z,6Z,9Z-18:H (50)	154a
6Z,9Z-*cis*-3,4-epoxy-18:H (400) + 3Z,6Z,9Z-18:H (100)	138ab
6Z,9Z-*cis*-3,4-epoxy-18:H (250) + 3Z,6Z,9Z-18:H (250)	85bc
6Z,9Z-*cis*-3,4-epoxy-18:H (100) + 3Z,6Z,9Z-18:H (400)	16ef
3Z,6Z,9Z-18:H (500)	0f

[a] Each treatment 3× replicated June 4–21, 1985. Values followed by the same letter are not significantly different ($P > 0.05$).

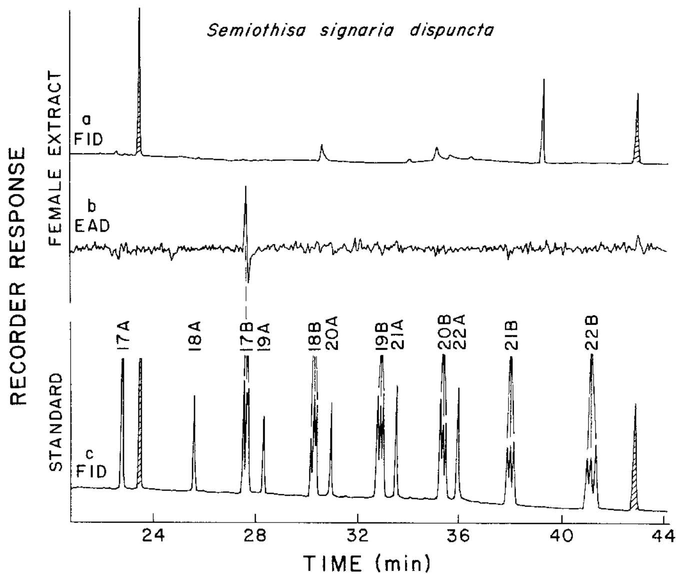

FIG. 2. Separately recorded flame ionization detector (FID) and electroantennographic detector (EAD) traces using antennae of male *S. signaria dispuncta*. Upper pair (a and b) are in response to an abdominal tip extract of a conspecific female containing internal standards, 17:H and 24:H (shaded). Lower trace (c) is the GC chromatogram of a mixture of C_{17-22} 3Z,6Z,9Z-triene hydrocarbons (designated by the letter A and preceded by the carbon chain length) and their monoepoxydiene analogs (designated by the letter B): the latter eluted from the DB-5 column in the order 6,7-, 3,4-, and 9,10-monoepoxydiene.

shorter times, the retention time of the antennal stimulatory component appeared to coincide with the postulated retention time of a 17-carbon monoepoxide. Following the synthesis of 3*Z*,6*Z*,9*Z*–17:H and the corresponding C_{17} *cis*-monoepoxydienes, it was found that the elution time of the EAD-active component corresponded exactly with that of 6*Z*,9*Z*-*cis*-3,4-epoxy-17:H. GC-MS analysis of an abdominal tip extract derived from five field-collected females provided data which supported the presence of 3*Z*,6*Z*,9*Z*–17:H; ions corresponding to $(M - 1)^+$ and $(M + 1)^+$ of this triene were simultaneously detected in the column eluate at the elution time of synthetic 3*Z*,6*Z*,9*Z*–17:H. By comparison of the intensity of the ions derived from a solution of known concentration of synthetic triene hydrocarbon with those from the female component, the amount present in the female extract was estimated to be ca. 1 ng/female. The amount of 6*Z*,9*Z*-*cis*-3,4-epoxy-17:H present in the extract was below the limits of instrument detection; we were not able to detect ions in the correct ratios and at the appropriate retention time for this component.

The attractancy of lures containing 3*Z*,6*Z*,9*Z*–17:H and 6*Z*,9*Z*-*cis*-3,4-epoxy-17:H (Table 2) were compared with the optimum C_{18} attractant blend which had been found previously. Here also, 9:1 epoxide–triene blends resulted in the greatest number of target males captured, and these blends were significantly more attractive than the corresponding C_{18} mixture. However, the data clearly illustrate the ability of the C_{18} lures to mimic the activity of the C_{17} pheromone components. All lures (Table 2), except those containing 3*Z*,6*Z*,9*Z*–17:H alone, attracted ony *S. signaria dispuncta* males; traps baited with 3*Z*,6*Z*,9*Z*–17:H alone caught males of *S. bicolorata*. Traps (3× replicated) baited with 100-, 300-, and 1000-μg amounts of C_{17} epoxide–triene blends (9:1)

TABLE 2. ATTRACTION OF *S. signaria dispuncta* MALES TO BLENDS CONTAINING C_{17} AND C_{18} TRIENES AND *cis*-3,4-EPOXYDIENES

Attractant formulation (μg)	Total males captured[a]
6*Z*,9*Z*-*cis*-3,4-epoxy-18:H (450) + 3*Z*,6*Z*,9*Z*–17:H (50)	76b
6*Z*,9*Z*-*cis*-3,4-epoxy-17:H (500)	58b
6*Z*,9*Z*-*cis*-3,4-epoxy-17:H (500) + 3*Z*,6*Z*,9*Z*–17:H (5)	122ab
6*Z*,9*Z*-*cis*-3,4-epoxy-17:H (450) + 3*Z*,6*Z*,9*Z*–17:H (50)	235a
6*Z*,9*Z*-*cis*-3,4-epoxy-17:H (400) + 3*Z*,6*Z*,9*Z*–17:H (100)	143ab
6*Z*,9*Z*-*cis*-3,4-epoxy-17:H (250) + 3*Z*,6*Z*,9*Z*–17:H (250)	122ab
6*Z*,9*Z*-*cis*-3,4-epoxy-17:H (100) + 3*Z*,6*Z*,9*Z*–17:H (400)	69b
3*Z*,6*Z*,9*Z*–17:H (500)	1c

[a]Each treatment 3× replicated from June 24 to July 10, 1985. Values followed by common letters are not different ($P > 0.05$).

captured 34, 74, and 150 *S. signaria dispuncta* males, respectively, from July 12 to 26, 1985; captures at the highest dose, although significantly different from the lowest dose, were not different from those baited with 300 μg ($P > 0.05$).

Traps (2× replicated) baited with 3*Z*,6*Z*,9*Z*–17:H and its three structurally related *cis*-monoepoxydiene analogs (II–IV), as single lure components and in mixtures (Table 3), captured males of three species of *Semiothisa* in addition to *S. signaria dispuncta*. *S. bicolorata* males were attracted by several lure formulations and, although the greatest captures occurred in traps baited with 10:1 mixtures of the triene and 6,7-monoepoxydiene, the number caught was not different ($P > 0.05$) from captures by the triene alone. In a 3×-replicated test which followed (June 28–July 15 1985), the number of *S. bicolorata* males taken in traps baited with 500 μg 3*Z*,6*Z*,9*Z*–17:H alone (85 males caught) and with binary lures containing 500 μg of triene plus 10% additions of the *cis*-6,7-monoepoxydiene (94 males) and *cis*-9,10-monoepoxydiene (39 males) were not significantly different ($P > 0.05$). However, 10% additions of the *cis*-3,4-epoxide resulted in significantly reduced trap captures (three males). Both the EAG and combined GC-EAD (Figure 3) analyses using C_{17-22} synthetic 3*Z*,6*Z*,9*Z*-trienes and corresponding monoepoxydienes supported the field data; the greatest antennal stimulation was elicited by 3*Z*,6*Z*,9*Z*–17:H with lower responses given, in decreasing order, by its *cis*-6,7-, *cis*-9,10-, and *cis*-3,4-monoepoxides. GC-EAD analyses of tip extracts from two field-collected *S. bicolorata* females provided the data which supported the triene hydrocarbon as a pheromone component. Analyzed separately, a single male antennal stimulatory component was found in both extracts which eluted from the capillary column at the same retention time as 3*Z*,6*Z*,9*Z*–17:H (Figure 3). Thus, the combined analytical and trapping data provide fairly conclusive proof that 3*Z*,6*Z*,9*Z*–17:H is a pheromone component for *S. bicolorata*, and there is no evidence of any other components.

Males of a third species, *S. ulsterata*, were captured (Table 3) in traps containing 3*Z*,9*Z*-*cis*-6,7-epoxy-17:H alone or as the major lure component. In a separate field test (3× replicated), traps baited with 500 μg of 3*Z*,9*Z*-*cis*-6,7-epoxy-17:H captured 56 *S. ulsterata* males between June 28 and July 12 1985; there was no significant difference ($P > 0.05$) in the number of males taken in these traps and in those which also contained 5 μg (35 males taken) and 50 μg of 3*Z*,6*Z*,9*Z*–17:H (51 males). Species specificity was again high; of the 143 males captured in the nine-trap experiment, 142 were *S. ulsterata*. When assayed by EAG and GC-EAD using synthetic compounds, *S. ulsterata* males gave the largest response to synthetic 3*Z*,9*Z*-*cis*-6,7-epoxy-17:H and lower, but well above background, responses to the C_{18} and C_{19} 6,7-epoxydiene homologs and to 3*Z*,6*Z*,9*Z*–17:H (Figure 4). Additional data in support of 3*Z*,9*Z*-*cis*-6,7-epoxy-17:H as a pheromone component was obtained by GC-

TABLE 3. CAPTURE OF FOUR SPECIES OF *Semiothisa* IN TRAPS BAITED WITH 3Z,6Z,9Z-17:H AND ITS MONOEPOXYDIENE ANALOGS[a]

Lure composition (μg)	Total number of males caught			
	S. signaria dispuncta	*S. bicolorata*	*S. ulsterata*	*S. delectata*
3Z,6Z,9Z-17:H (500)	2	25	0	6
3Z,6Z,9Z-17:H (500) + 6Z,9Z-*cis*-3,4-epoxy-17:H (50)	0	1	0	0
3Z,6Z,9Z-17:H (50) + 6Z,9Z-*cis*-3,4-epoxy-17:H (500)	114	5	0	0
6Z,9Z-*cis*-3,4-epoxy-17:H (500)	17	3	0	0
3Z,6Z,9Z-17:H (500) + 3Z,9Z-*cis*-6,7-epoxy-17:H (50)	3	83	0	2
3Z,6Z,9Z-17:H (50) + 3Z,9Z-*cis*-6,7-epoxy-17:H (500)	4	2	64	10
3Z,9Z-*cis*-6,7-epoxy-17:H (500)	0	1	70	3
3Z,6Z,9Z-17:H (500) + 3Z,6Z-*cis*-9,10-epoxy-17:H (50)	4	14	0	0
3Z,6Z,9Z-17:H (50) + 3Z,6Z-*cis*-9,10-epoxy-17:H (500)	0	0	0	0
3Z,6Z-*cis*-9,10-epoxy-17:H (500)	0	0	0	0

[a]Each treatment 2× replicated from June 24 to August 26, 1985.

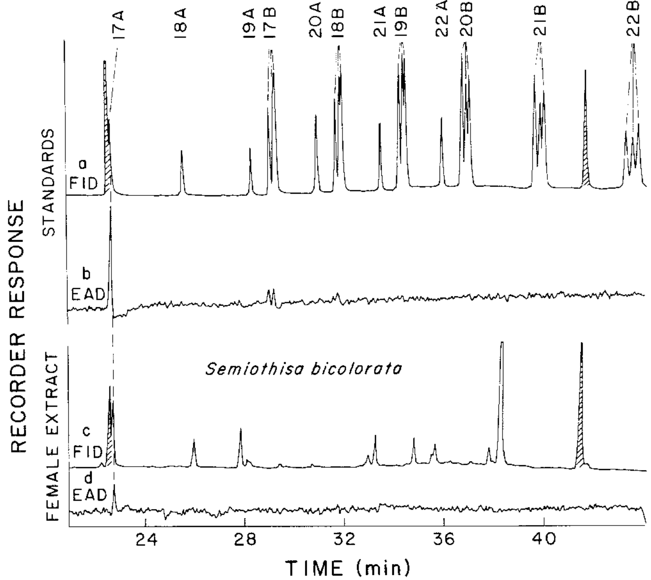

FIG. 3. FID and EAD (antennae of male *S. bicolorata*) traces: upper pair (a and b) are in response to C_{17-22} triene hydrocarbons (A), monoepoxydiene analogs (B) and two internal standards (shaded); lower pair (c and d) are in response to an abdominal tip extract (1 FE) from a conspecific female. A DB-1701 column was used; epoxides eluted in the order of 6,7-, 3,4-, and 9,10-monoepoxydiene.

EAD analyses of abdominal tip extracts from two field-collected females. These extracts were chromatographed separately on DB-1701 and DB-5 columns. In each case two antennal responses were observed, the major response occurring at the retention time of 3*Z*,9*Z*-*cis*-6,7-epoxy-17 : H and a minor response at a retention time corresponding to 3*Z*,9*Z*-*cis*-6,7-epoxy-18 : H. On the basis of the GC-EAD data, the sex pheromone of *S. ulsterata* appears to be comprised of at least two 6,7-epoxydiene components. The flight of this species had ended before mixtures of the C_{17} and C_{18} 6,7-epoxides could be properly tested.

Only a few males of *S. delectata* were captured in the survey traps (Table 3), but they also were taken in traps containing 3*Z*,6*Z*,9*Z*–17 : H, 3*Z*,9*Z*-*cis*-6,7-epoxy-17 : H, or mixtures of the two. Males of this species had also been captured earlier in the season in traps which had been set out to test C_{18-22} 6*S*,7*R*- and 6*R*,7*S*-enantiomers of 3*Z*,9*Z*-*cis*-6,7-epoxydiene hydrocarbons as lepidopteran sex attractants (Underhill et al., 1985). Of 20 *S. delectata* taken, 3*Z*,9*Z*,6*S*,7*R*-epoxy-18 : H was a lure component common to the capture of 19 and 3*Z*,6*Z*,9*Z*–18 : H was common to the capture of 15 of these. There was no indication that the 6*R*,7*S*-enantiomer was biologically active either as an at-

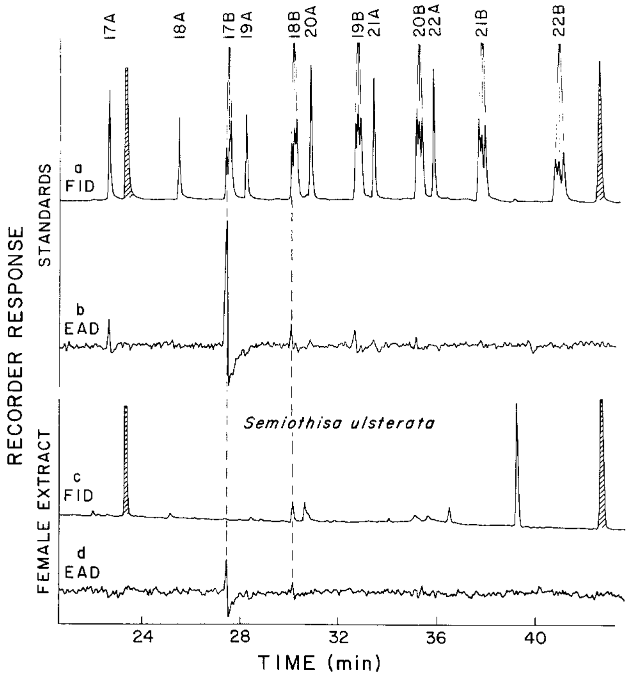

FIG. 4. FID and EAD (antennae of male *S. ulsterata*) traces; upper pair (a and b) are in response to C_{17-22} triene hydrocarbons (A), monoepoxydiene hydrocarbon analogs (B), and two internal standards (shaded); lower pair (c and d) are in response to an abdominal tip extract (1 FE) from a conspecific female. A DB-5 column was used; epoxides eluted in the order of 6,7-, 3,4-, and 9,10-monoepoxydiene.

tractant or inhibitor. At present, we have not been successful in obtaining *S. delectata* females to investigate their pheromone chemistry.

Two *S. neptaria* females were field collected and an abdominal tip extract was prepared. Lacking a conspecific male for GC-EAD analysis, the extract was submitted for CI-MS analysis by the MID technique. The presence of 3*Z*,6*Z*,9*Z*-17 : H in this extract was indicated by the appearance of ions at *m/z* 233 and 235, corresponding to $(M - 1)^+$, and $(M + 1)^+$. Both ions were present in the same ratio and occurred simultaneously at the same retention time as was found using a reference standard of this triene hydrocarbon. No evidence was obtained for the presence of the corresponding monoepoxydienes.

The foregoing field-trapping and analytical data, showing 3*Z*,6*Z*,9*Z*-17 : H and two of its structurally related monoepoxydiene hydrocarbons as sex attractants of four species of *Semiothisa*, has extended the range of geometrid sex attractants as outlined in Figure 1 to include compounds with chain lengths of 17–21 carbons. The occurrence of 3*Z*,6*Z*,9*Z*-17 : H in nature appears to be rare

and, to our knowledge, it has been only tentatively identified as a component of tomato (Garnero and Joulain, 1981). Neither 6*Z*,9*Z*-*cis*-3,4-epoxy-17 : H nor 3*Z*,9*Z*-*cis*-6,7-epoxy-17 : H have been previously reported. At the present time enantiomers of each of the three positional epoxide isomers are being synthesized in this laboratory for future field screening tests.

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PHEROMONAL BASIS FOR AGGREGATION BEHAVIOR OF PARASITOIDS OF THE GYPSY MOTH: *Brachymeria intermedia* (Nees) and *Brachymeria lasus* (Walker) (Hymenoptera: Chalcididae)

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(Received August 11, 1986; accepted August 14, 1986)

Abstract—The parasitoids *B. intermedia* and *B. lasus* aggregate at sites previously frequented. Extracts from filter paper conditioned by aggregated parasitoids were chromatographed (adsorption column and gas-liquid chromatography) and bioassayed. An active component that elicited aggregation response for *B. intermedia* was isolated and identified as 3-hexanone. This molecule was found in trace quantities (5–10 ng/female or male) on filter paper conditioned by either male, female or a combination of the sexes.

Key Words—Aggregation, *Brachymeria*, Hymenoptera, Chalcididae, 3-hexanone, parasitoid, pheromone.

INTRODUCTION

Intraspecific aggregations in animals are well-documented phenomena, with many known examples in Insecta. Massive aggregations of individuals have been observed in butterflies and moths (Brower et al., 1977; Capinera, 1979), aphids (Hayamizu, 1984), beetles (Crins, 1980; Faustini et al., 1982; Harris and Todd, 1980; Lee, 1980), flies (Atkinson and Shorrocks, 1984), bees (Hefetz et al., 1979), ants (Cavill et al., 1979), locusts (Gillet, 1973, 1975), and wasps (Evans and Gillaspy, 1984). The effects of such behavior include mating, host attack, defense, and thermoregulation. To orchestrate such aggregations, a system of communication, usually involving a chemical mode, has evolved to synchronize it. In one of the most studied groups, the scolytid beetles, this is achieved by pheromones. For instance, 4,8-dimethyldecanal (Suzuki and Mori, 1983), 4-methyl-3-heptanol (Gore et al., 1977; Blight et al., 1979), the tricyclic molecule, lineatin (Borden et al., 1979), and 6-methyl-5-hepten-2-ol

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(Byrne et al., 1974) are all aggregation pheromones. Beyond this group, little is known of either the function or basis of aggregation behavior.

Overwintering aggregations of the parasitoid *Brachymeria intermedia* under bark (Waldvogel and Brown, 1978) and subsequent laboratory studies (Simser and Coppel, 1980) on *Brachymeria lasus* provide the first possibility of an aggregation pheromone in parasitoids. In this paper, evidence is provided for the existence of aggregation pheromones in both species. This is accomplished by elucidation of techniques for sequestering the pheromone, the sex(es) producing and responding to it, and some aspects of the chemistry.

METHODS AND MATERIALS

The parasitoids were reared from previously parasitized cocooned pupae of the greater wax moth, *Galleria mellonella* (L.). Parasitized pupae were reared singly in 4-ml shell vials sealed with perforated caps. Freshly eclosed parasitoids were sexed, and the species confirmed. Each sex, or a combination of the two, were transferred to large screened cages (38 × 22 × 22 cm) one side of which was made of a Plexiglas sliding door. Filter paper (Whatman No. 40) -lined vials (5.5 × 2.3 cm) were placed in each cage. Parasitoids aggregated in the vials during scotophase. This provided filter paper conditioned by either, or a combination of, the sexes. Colony sizes of 200–300 parasitoids per cage each with 10 filter paper-lined vials were maintained all year. The parasitoids were fed water and honey daily and incubated at 28.5°C, 55–65% relative humidity, and a light–dark photoperiod of 16:8.

The filter papers were replaced every two months for a three-year period, utilizing approximately 4000 parasitoids of each sex and sex combination for each species. Filter paper replacement was based on the longevity of parasitoids under our laboratory conditions and the fact that these parasitoids took at least four to five weeks to aggregate and condition it. Crude extracts were obtained by placing the conditioned filter papers in 250-ml Erlenmeyer flasks and shaking them sequentially with hexane, hexane–ethyl acetate (50:50 mix), and methanol, each for 8 hr. Pooled extracts were filtered over sodium sulfate and concentrated to a few milliliters with a Buchi evaporator.

Bioassays were conducted with conditioned filter papers under the regime mentioned above. Clean vials were lined with conditioned filter paper and placed in wooden boxes (15 × 15 × 10 cm). Conditioned filter paper from *B. intermedia* colonies of virgin females, males, or males plus females, were each tested against 25 of each sex, or a combination thereof, of the same species. Interspecific assays were also conducted with *B. lasus* adults and *B. intermedia*-conditioned filter papers. Each bioassay was replicated six times. Similar intraspecific and interspecific tests were conducted with *B. lasus*-conditioned filter paper and *B. intermedia* adults.

Attempts to isolate an active aggregation component(s) were made through bioassays of crude, absorption column chromatographic, and gas-liquid chromatographic fractions (GLC). Because of the large number of replicates involved, a different bioassay technique was developed for testing for the aggregation pheromone. It consisted of pipetting the test material or solvent (control) into a 4-ml glass shell vial lined with filter paper and with a disc (1.27 cm) at the base. Two vials thus treated, experimental and control, were placed in a glass Petri plate (9 cm diam.) and held in place with modeling clay. Ten parasitoids (virgin females or a combination of the sexes) aged 3-7 days were added to the plate. The plate was covered and incubated about 1 hr prior to the onset of aggregation, which usually took place 8-9 hr after photophase started. The vials were checked 4-5 hr later for the numbers of parasitoids aggregating. Incubator conditions were the same as those previously mentioned. Each source of pheromone (crude, absorption column, and gas chromatographic) was bioassayed for $N = 12$ replicates.

Percentage aggregation was calculated from a ratio of numbers in the experimental vial (or control) over the sum of parasitoids aggregating in both vials. Data were analyzed for normal distribution by normal probability plots. Significant differences in means were culled using Student's t test.

Crude samples were chromatographed on silica gel (1.1 g), in a glass column (11.5 × 1 cm ID), using an elution series of: hexane, 0.1%, 1%, 10%, 20%, 40% ether-hexane, with fractions taken at 3-ml intervals. The active fraction was further fractionated on a 1.8 m × 2 mm ID, 10% OV-101 on 80/100 Gas Chrom Q, with helium flow rate of 30 ml/min. The temperature program used was: 40°C (10 min), 3°C/min to 65°C (10 min), and then 20°C/min to 245°C, with injection port at 40°C. Further analyses of samples were accomplished with a 15 m × 0.32 mm ID, 1-μm film thickness, DB-5 capillary column (J&W). Conditions of use of this column were: helium at 1.5 ml/min, and a temperature program of 45°C (10 min), 15°C/min to 285°C (hold), with an injection port temperature of 95°C.

GLC was conducted with a Spectra Physics 7100 equipped with flame ionization detectors. The gas chromatograph was fitted with a splitter modified from Brownlee and Silverstein (1968). Components from the packed column were shunted via the splitter to a glass capillary tubing. The tubing was encased in finely crushed Dry Ice held in place by a glass cylinder tapered at one end. The capillary tubing and tapered glass cylinder were held in place with Teflon tape. Condensed effluents within the tubing were desorbed with several washes of 500 ml ether in a 4-ml vial using a 10-ml Cornwall glass syringe. Samples were concentrated under argon and then bioassayed or further analyzed. The latter included monitoring for purification by capillary chromatography and mass spectrometry of active isolates. Samples were subject to electron impact (70 eV) and chemical ionization with isobutane using a Finnigan 4510 GC-MS.

RESULTS

Intraspecific bioassays showed significantly different responses for any combination of conditioned filter paper and sex of either species versus controls (Tables 1 and 2). A somewhat similar result was obtained in the response of *B. lasus* females or males to *B. intermedia*-conditioned filter paper, with the one exception being to the female and male source in interspecific assays. *B. intermedia* males or females did not respond to conditioned filter papers of *B. lasus*.

Crude extracts of conditioned filter papers of either species did not elicit aggregation response from conspecifics. However, column chromatography with silica gel did allow the isolation of an active fraction (Table 3) at 40% ether–hexane for *B. intermedia*. No other fraction elicited this response. Several areas and components of the active fraction were further fractionated by preparative GLC on a 10% OV-101 column. Only the volatile region elicited any aggregation activity and, subsequently, a component at 4.4 min. Analyses by capillary chromatography from our three sources of extracts showed the presence of this component at 2.6 min in trace quantities (5–10 ng/female and/or male, approximately).

Electron impact GC-MS of of the active area and single-component isolates gave mass spectra of a compound of molecular weight of 100, which was confirmed by chemical ionization (Figure 1). The structure that fits this spectrum is 3-hexanone, which is consistent with the reported principal fragments of this molecule (Stenhagen et al., 1974). Additional rationale comes from the ion at m/z 72 $(CH_2{=}COH\ C_2H_5)^+$ and loss of $CH_2{=}CH_2$ by the McLafferty rearrangement. This is compatible only with a linear structure, and, by the same token, excludes isomers resulting from α-substitution (e.g., 4-methyl-3-pentanone). The intense peak at m/e 71 rules out another linear isomer, 2-hexanone. Our unknown spectrum, compared to that of another isomer, 2,3-epoxy-4-methyl-pentane, was incompatible. A combination of bioassays (Table 3), congruence of an authentic sample with the corresponding active peak by capillary chromatography, and the mass spectrum of 3-hexanone which was identical to our unknown, established this molecule as the aggregation pheromone of *B. intermedia*.

DISCUSSION

The basis for the aggregation behavior of the parasitoid *B. intermedia* is pheromonal. Conditioned filter paper and a chromatographic fraction elicited aggregation response consistently different from either controls or other fractions. The facts that 3-hexanone was isolated and identified from extracts of conditioned filter paper and that a positive aggregation response to it was obtained allow us to conclude that this is the major component in the aggregation

TABLE I. INTRASPECIFIC AND INTERSPECIFIC BIOASSAYS OF *B. intermedia*-CONDITIONED FILTER PAPER FOR AGGREGATION RESPONSE

Source of conditioned filter paper: *B. intermedia*	Percent aggregated ($X \pm$ SE)[a]					
	Female		Male		Male and female	
	Exp.	Control	Exp.	Control	Exp.	Control
Intraspecific bioassays (vs. *B. intermedia*)						
Female	66.1[a]	33.9(± 5.9)[b]	72.8[a]	27.2(± 10.4)[b]	71.8[a]	28.2(± 11.4)[b]
Male	86.7[a]	13.4(± 2.7)[b]	85.8[a]	15(± 8.8)[b]	—	—
Female and male	79.5[a]	20.5(± 7.7)[b]	91.9[a]	8.1(± 4.0)[b]	72.7[a]	27.3(± 7.9)[b]
Interspecific bioassays (vs. *B. lasus*)						
Female	99.2[a]	0.8(± 0.8)[b]	97.1[a]	2.9(± 1.9)[b]	93.6[a]	6.4(± 5)[b]
Male	84.5[a]	15.5(± 10.9)[b]	95.8[a]	4.2(± 0)[b]	54.3[a]	46(± 15)[a]
Female and male	63.9[a]	36.1(± 15.1)[a]	53.1[a]	46.9(± 13.6)[a]	83.3[a]	16.7(± 11)[b]

[a] Each bioassay (combination of filter paper source and test parasitoids) was conducted with $N = 6$ replicates, and 25 parasitoids/replicate. Significant differences between experimental (exp.) and control are indicated by different letters and vice versa. The Student's t test was used at $P < 0.025$, $df = 10$.

TABLE 2. INTRASPECIFIC AND INTERSPECIFIC BIOASSAYS OF *B. lasus*-CONDITIONED FILTER PAPER FOR AGGREGATION RESPONSE

Source of conditioned filter paper: *B. lasus*.	Percent aggregated ($X \pm$ SE)[a]					
	Female		Male		Male and female	
	Exp.	Control	Exp.	Control	Exp.	Control
Intraspecific bioassays (vs. *B. lasus*)						
Female	93^{a}	7.0($\pm$ 4.5)^{b}	93^{a}	7($\pm$ 3.2)^{b}	95^{a}	5.0($\pm$ 3.3)^{b}
Male	93.6^{a}	6.4($\pm$ 4.5)^{b}	82.2^{a}	17.8($\pm$ 7.9)^{b}	—	—
Female and male	64.5^{a}	36.9($\pm$ 9.1)^{b}	87.9^{a}	12.1($\pm$ 9.8)^{b}	97.6^{a}	2.4($\pm$ 1.6)^{b}
Interspecific bioassays[b] (vs. *B. intermedia*)						
Female	17.4^{a}	82.6($\pm$ 1.7)^{b}	NR	NR	53.4^{a}	46.6($\pm$ 10.3)^{a}
Male	NR	NR	NR	NR	NR	NR
Female and male	NR	NR	NR	NR	NR	NR

[a] Each bioassay (combination of filter paper source and test parasitoids) was conducted with $N = 6$ replicates, and 25 parasitoids/replicate. Significant differences between experimental (exp.) and control are indicated by different letters and vice versa. The Student's t test was used at $P < 0.025$, $df = 10$.

[b] Less than 10% of parasitoids aggregated and is indicated by NR (no response).

TABLE 3. LABORATORY BIOASSAYS FOR AGGREGATION ACTIVITY OF *B. intermedia* TO COLUMN AND GAS-LIQUID CHROMATOGRAPHIC FRACTIONS, 2-HEXANONE, AND 3-HEXANONE[a]

Test material[b]	Aggregation (%)	
	Exp.	Control
Column chromatographic fraction		
1	81.7[a]	18.2 ± 4.9[b]
2	33.7[a]	66.2 ± 5.4[b]
GLC fraction	72.3[a]	27.6 ± 2.9[b]
3-hexanone	81.3[a]	18.7 ± 3.1[b]
2-hexanone	19.7[a]	80.2 ± 3.9[b]

[a] Assays were conducted with concentrations of 5–10 ng 3-hexanone, N = 12 replicates with 10 females or male and female parasitoids per bioassay. Significant differences are indicated by different letters using Student's t test at $P < 0.001$, $df = 22$.

[b] Column chromatographic fraction 1, indicates a 40% ether–hexane fraction; fraction 2 represents other fractions from adsorption column chromatography; and GLC fraction is a component taken at 4.4 min from a 10% OV-101 column.

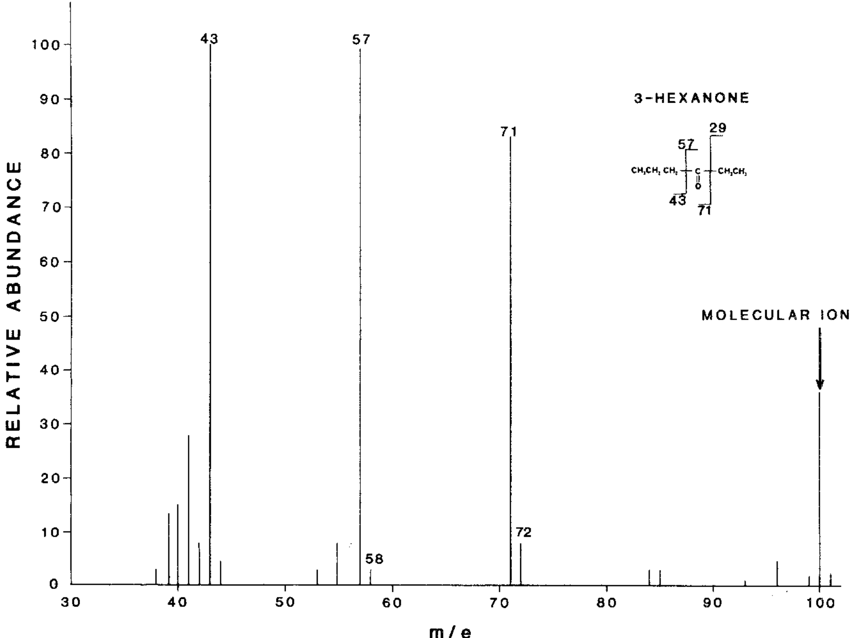

FIG. 1. Mass spectrum of the aggregation pheromone (3-hexanone) isolated from female and male plus female-conditioned filter paper of *B. intermedia*.

pheromone of *B. intermedia*. Another component suggests itself from the fact that, although *B. lasus* displays an interspecific response, it is not responsive to 3-hexanone.

The volatility of 3-hexanone (bp = 125°C) seems consistent with some aspects of the biology of *B. intermedia*. This species is polyphagous and capable of widespread dispersal, as evidenced by both sexes being strong fliers. In order to assemble at a particular site, a volatile pheromone would seem to be the best strategy. The evolution of an aggregation pheromone could have resulted from an increase in reproductive success by increasing the probability of mate location, as well as offering the possibility of mate choice.

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FIELD RESPONSE AND GAS-LIQUID CHROMATOGRAPH SEPARATION OF OPTICALLY ACTIVE SYNTHETIC AND NATURAL PHEROMONES IN TWO SYMPATRIC DIPRIONID SAWFLIES, *Neodiprion nanulus nanulus* and *Neodiprion sertifer* (HYMENOPTERA: DIPRIONIDAE)

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Abstract—The male red pine sawfly *Neodiprion nanulus nanulus* Schedl responds mainly to the (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-yl acetate (2*S*,3*S*,7*S*-A) in the field. No other isomer is as effective by itself or synergizes 2*S*,3*S*,7*S*-A in this species. For *Neodiprion sertifer* (Geoffroy), however, we confirmed our earlier report that males responded significantly to a 5:0.003 mixture of 2*S*,3*S*,7*S*-A and either 2*S*,3*R*,7*R*-A or 2*S*,3*R*,7*R*/*S*-A. The 2*S*,3*S*,7*S*-A isomer was separated from 2*S*,3*R*,7*R*-A by capillary gas-liquid chromatographic (GLC) analysis using Carbowax 20 M and DB-5 columns. The latter column also separated 2*S*,3*S*,7*S*-A from 2*S*,3*R*,7*S*-A. Only 2*S*,3*S*,7*S*-A was found in the natural pheromones of *N. n. nanulus* and *N. sertifer* by GLC analysis.

Key Words—Sawflies, *Neodiprion nanulus, Neodiprion* sertifier, Hymenoptera, Diprionidae, pheromones, chiral, chirality, 3,7-dimethylpentadecan-2-yl acetate.

INTRODUCTION

The native red pine sawfly, *Neodiprion nanulus nanulus* Schedl is an important defoliator of red pine (*Pinus resinosa* Ait) and jack pine (*P. banksiana* Lamb.)

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in the Lake States. Red pine is the predominant conifer being planted in this region. One complete defoliation of red pine by sawflies caused a 21% reduction in height growth and two successive defoliations reduced height growth as much as 64% (Kapler and Benjamin, 1960). Such defoliations are uncommon in the Michigan study area.

Monitoring tools for this pest at present consist of both scouting for the larval stages and estimating the injury levels. Jewett et al. (1976) isolated and identified diprionid sawfly sex pheromone as 3,7-dimethylpentadecan-2-ol (diprionol), the acetate or propionate ester of which was active in attracting males in the field. Kraemer et al. (1983) reported that the optical isomers (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-yl acetate (2*S*,3*S*,7*S*-A) and propionate (2*S*,3*S*,7*S*-P) as well as (2*S*,3*S*,7*R*)-3,7-dimethylpentadecan-2-yl acetate (2*S*,3*S*,7*R*-A) were highly active, but that 2*S*,3*S*,7*S*-A was significantly superior to others at lower concentrations. The 2*S*,3*S*,7*S*-A thus appeared to be the major pheromone component for *N. n. nanulus* as well as for *N. pinetum* (Norton) (Kraemer et al., 1979), and *N. lecontei* (Fitch) (Matsumura et al., 1979; Kraemer et al., 1981). However, in another sympatric species, *N. sertifer* (Geoffrey), where 2*S*,3*S*,7*S*-A is also the major pheromone component, its combination with small amount of (2*S*,3*R*,7*R*)-3,7-dimethylpentadecan-2-yl acetate (2*S*,3*R*,7*R*-A) greatly increases its field effectiveness (Kikukawa et al., 1983). Here we report results of field studies to examine whether such interaction of optical isomers also occurs with *N. n. nanulus*. We also report field confirmation of our earlier studies on *N. sertifer* and GLC analyses of the natural pheromones of both species.

METHODS AND MATERIALS

Field tests. The red pine sawfly trapping was carried out in Vogel Center, Sec. 32, Aetna Loop, Misaukee County, Michigan. Some trapping was also done at sec. 7, Manistee River, Garfield Township, Kalkaska County, and at Sec. 36, Higgins Lake, Old I-27, Beaver Township in Roscommon County, Michigan. The main experimental area, Vogel Center, is about 1.2 km^2 and is surrounded by farmlands such that within a 13-km radius only scattered plots of red pine could be found. Larval colonies of *N. n. nanulus* were collected from all sites in late spring and early summer of 1981, 1982, and 1983.

All field trapping of *N. sertifer* was at Rose Lake Wildlife Research Station, Sec. 33, Bath, and at Arboretum, Lansing, Ingham County, Michigan. Larval colonies were collected at Rose Lake from scotch pine, *Pinus sylvestris* L., in an area of 8 km^2.

Pherocon II traps were hung at a height of 1.5–2.5 m and spaced no closer than 15 m within and between trap rows. Weekly or biweekly observations were made, at which time the traps were rotated randomly within and between rows.

Pheromone components were prepared in 1 ml hexane and sealed in glass ampules. At the test site, the contents were dispensed onto a 4.0 to 5.0-cm dental cotton roll which was then positioned at the center on one side inside the Pherocon trap. A randomized block experimental design was used with at least three replicates of each treatment. Data were analyzed by analysis of variance and the means compared by Duncan's multiple-range test at the 5% level.

Laboratory Rearing of Larvae and Extraction of Natural Pheromone. Female sawflies from which pheromone was extracted were reared from larvae and cocoons collected at the test sites. Larvae were reared outdoors under shade near the greenhouse. The larvae were fed fresh foliage as necessary. Mature larvae formed cocoons among the grass in the cage. These cocoons, as well as the field-collected cocoons, were sexed according to size, the large cocoons being females. Cocoons were placed singly in gelatin capsules until adults emerged. Virgin females were placed in 3 × 5-cm vials contains strips of Teflon to simulate pine needles and left for a few days before being frozen. The natural pheromone was extracted as described for *N. sertifer* (Kikukawa et al., 1983).

Synthetic Pheromones. Two groups of chemists from Japan synthesized and supplied us with the optical isomers used. Mori's group synthesized the 2*S*,3*S*,7*S*-A and the corresponding alcohol (Mori et al. 1978). These materials contained some unknown percent of optical impurities, but are believed to have been less than 1%, the approximate limit of detection by PMR assay. Tai's group synthesized the 2*S*,3*R*,7*R*/*S*-A with a known 5% erythro contamination (Tai et al., 1978). The same group later synthesized 2*S*,3*S*,7*S*-A (unpublished) and 2*S*,3*R*,7*R*-A and 2*S*,3*R*,7*R*-AG (Kikukawa et al., 1982). In this instance the 2*S*,3*S*,7*S*-A and 2*S*,3*R*,7*R*-A contained less than 1% optical impurities but 2*S*,3*R*,7*R*-AG synthesized through a Grignard reaction was greater than 99.9% optically pure.

Preparative GLC Fraction Collection. A Varian 1700 preparative gas chromatography fitted with stainless steel column, 7.2 m and 3 mm ID, packed with 10% Carbowax 20 M on 80–100 mesh Chrom Q was used for fraction collection of acetate of diprionol. A 9:1 postcolumn splitter was used which directed 90% of the injected material for collection. The retention time of natural pheromone was determined first by injecting synthetic standard and then by coinjecting the standard with natural pheromone at column temperatures of 200° and 220°C.

Capillary GLC Analysis. A Varian 3700 capillary GLC equipped with two FID was used. Synthetic standards containing 1 mg/ml of 2*S*,2*S*,7*S*-A, and mixtures at ratios 1:2, 1:1, 3:2, and 2:1 of 2*S*,3*S*,7*S*A-/2*S*,3*R*,7*R*-A and 2*S*,3*S*,7*S*-A/2*S*,3*R*,7*S*-A were used. Natural extracts derived from the above purification were used. Concentration of natural pheromone was in the order of 5000 female equivalents (FE)/ml. Two fused silica capillary columns, one Carbowax 20 M, 30 m × 0.25 mm ID, and the other DB-5, 40 m × 0.25 mm ID (J. & W. Scientific Co., Folsom, California), were capable of resolving threo

diastereomers from erythro counterparts. Runs were made isothermally at 180°C and temperature programmed from 130 to 190°C at 4°C/min, with initial hold at 0 min, and final hold at 18 min giving a total analysis time for each injection of 32 min.

RESULTS

Field Tests. The 2*S*,3*S*,7*S*-A isomer alone was more active than any combinations of 2*S*,3*R*,7*R*/*S*-A or 2*S*,3*R*,7*R*-A as an attractant for *N. nanulus* (Table 1). At lower concentrations, 2*S*,3*R*,7*R*/*S*-A and 2*S*,3*R*,7*R*-A appeared to increase the effectiveness of the 2*S*,3*S*,7*S*-A isomer, but the increase was not statistically significant (Table 2). One possibility for this lack of synergistic effect could be that the effectiveness of the synergist may not be expressed at high sawfly populations as this particular test caught many males. Lower concentrations of 2*S*,3*S*,7*S*-A were used in 1983 to attract fewer males and thus bring on clear differences, if any, among the treatments. Additional data (Table 3) indicate that the traps baited with 2*S*,3*S*,7*S*-A alone attracted more males than did mixtures. Such results support our hypothesis that 2*S*,3*S*,7*S*-A alone is the major pheromone component of *N. n. nanulus*. These data also agree with those of Kraemer et al. (1983). However, since they found 2*S*,3*S*,7*R*/*S*-A as active as 2*S*,3*S*,7*S*-A, we have compared the two isomers in a side-by-side test in the field. With a total catch of 640 males with 2*S*,3*S*,7*S*-A-baited traps compared to 253 males with 2*S*,3*S*,7*R*/*S*-A-baited traps, we thus, proved the

TABLE 1. NUMBER OF MALE *N. nanulus nanulus* CAUGHT IN TRAPS BAITED WITH INCREASING CONCENTRATIONS OF TWO ISOMERS, 2*S*,3*R*,7*R*/*S*-A AND 2*S*,3*R*,7*R*-A ON 2*S*,3*S*,7*S*-A ISOMER[a]

Amount (μg/trap)	2*S*,3*S*,7*S*-A + 2*S*,3*R*,7*R*/*S*-A ($X \pm$ SE)[b]	2*S*,3*S*,7*S*-A + 2*S*,3*R*,7*R*-A ($X \pm$ SE)[c]
10/0	582.5 ± 9.1	98.0 ± 5.6a
8/2	92.5 ± 3.7	35.3 ± 3.0a
6/4	98.5 ± 4.5	35.7 ± 3.3a
4/6	107.5 ± 3.8	9.3 ± 1.6b
2/8	60.5 ± 3.5	18.7 ± 2.6b
0/10	69.5 ± 2.3	18.6 ± 2.5b

[a]Test was conducted in Roscommon County between September 4 and October 16, 1981.
[b]Mean of two replicates.
[c]Mean of three replicates. Means followed by same letter not significantly different at 5% level of Duncan's multiple-range test.

TABLE 2. NUMBER OF MALE *N. nanulus nanulus* CAUGHT IN TRAPS BAITED WITH INCREASING CONCENTRATIONS OF TWO ISOMERS 2*S*,3*R*,7*R*/*S*-A AND 2*S*,3*R*,7*R*-A ON 2*S*,3*S*,7*S*-A ISOMER[a]

Amount (μg/trap)	2*S*,3*S*,7*S*-A + 2*S*,3*R*,7*R*/*S*-A (X^b ± SE)[c]	2*S*,3*S*,7*S*-A + 2*S*,3*R*,7*R*-A (X^b ± SE)[d]
10/0	413.7 ± 10.2abc	47.0 ± 4.5a
10/0.1	383.3 ± 8.8abc	81.7 ± 5.3a
10/0.3	440.3 ± 9.5ab	87.0 ± 5.9a
10/1	524.3 ± 11.9a	50.7 ± 3.8a
10/3	64.5 ± 4.5c	18.3 ± 2.2a
10/10	261.3 ± 10.1abc	20.0 ± 2.2a
10/30		19.7 ± 2.4a

[a]The test was conducted at two locations in Michigan from September 4 to October 16, 1981.
[b]Mean of three replicates; means followed by same letter not significantly different at 5% by Duncan's multiple-range test.
[c]Tested at Vogel Center, Misaukee County, Michigan.
[d]Tested at Higgins Lake, Roscommon County, Michigan.

superiority of 2*S*,3*S*,7*S*-A isomer over 2*S*3*S*,7*R*/*S*-A and the importance of *S* on the 7-carbon in the major pheromone component.

Based on apparent injury level at three locations in Roscommon County, Michigan, and the level of larva population sampled on twenty 78-cm branches, three sites [Manistee (with low populations), 0.3 ± 0.7 SD/branch; Roscommon

TABLE 3. FIELD RESPONSE OF *N. nanulus nanulus* TO 2*S*,3*S*,7*S*-A AND MIXTURES OF 2*S*,3*S*,7*S*-A WITH SERIAL DILUTIONS OF 2*S*,3*R*,7*R*-A ISOMER[a]

2*S*, 3*S*, 7*S*-A/2*S*, 3*R*, 7*R*-A (μg/trap)	Catch/trap (mean ± SE)[b]
5/0	41.0 ± 2.9
5/0.001	16.3 ± 0.9
5/0.003	27.3 ± 1.9
5/0.01	16.3 ± 1.3
5/0.03	30.0 ± 2.1
5/1	18.3 ± 1.4
5/5	26.0 ± 2.0
5/30	17.7 ± 1.7
5/100	33.0 ± 3.0

[a]Test conducted at Vogel Center, Michigan, September 10 to October 7, 1983.
[b]Means of three replicates. Analysis of variance not significant at 5% level.

(with medium populations), 14.5 ± 10.9 SD/branch; and Vogel Center (with high populations), 28.05 ± 8.7 SD/branch] were chosen to allow comparison of the field effectiveness of the synthetic and natural pheromones. At all locations, trap catches increased with increasing concentration of the synthetic compound (Table 4). This agrees with the suggestion that an attractant with the correct optical configuration shows a dose-related response (Cardé et al., 1977). Traps baited with the natural pheromone, however, caught no adults at Manistee or Roscommon, apparently due to the low population numbers. No valid comparison between the synthetic and natural pheromone can be made with the data in Table 4 because the concentration of the synthetic was not low enough.

The effectiveness of the synthetic was tested side by side with the natural pheromone and the results are shown in Table 5. Based on capillary GLC analysis, one virgin female contains about 2 ng pheromone stored as alcohol. The response to the natural pheromone was not dose-related. Catches were generally lower at higher concentrations. There is the possibility that the extracts may contain impurities which, at the higher concentration, were inhibitory. In 1982, all the natural preparations were further purified after acetylation by passing them through one extra TLC system-preparative TLC $HF_{254+366}$ and developing

TABLE 4. COMPARISON OF FIELD EFFECTIVENESS OF SYNTHETIC AND NATURAL PHEROMONE OF *N. nanulus nanulus*[a]

Pheromone	Amount (μg/Trap)	Locations			Total	Mean/trap
		Roscommon	Manistee	Vogel		
Synthetic mixture: 2*S*,3*S*,7*S*-A + 2*S*,3*R*,7*R*/*S*-A (5:1)	3.00	162	22	664	848	282.7
	1.00	123	16	429	568	198.3
	0.30	29	5	124	158	52.7
	0.01	12	1	55	68	22.7
Natural pheromone (acetate)	1.00 FE[b]	0	0	6	6	2.0
	0.30 FE	0	0	3	3	0.7
	0.10 FE	0	0	4	4	1.3
	0.03 FE	0	0	18	18	6.0

[a] Test conducted at three locations in Michigan between September 5 and October 16, 1981.
[b] Female equivalent (one FE corresponds to roughly 2 ng of pheromone).

TABLE 5. COMPARISON OF FIELD EFFECTIVENESS OF SYNTHETIC AND NATIONAL PHEROMONE OF *N. nanulus nanulus*[a]

Pheromone	Amount (μg/trap)	Catch/trap (mean ± SE)[b]
Synthetic		
2*S*,3*S*,7*S*-A + 2*S*,3*R*,7*R*/*S*-A		
(5:1)	3.000	70.7 ± 2.9a
	1.000	33.0 ± 0.8b
	0.300	19.3 ± 1.7bc
	0.100	5.7 ± 0.8cd
	0.030	3.0 ± 0.8d
	0.010	7.0 ± 1.4cd
	0.003	3.0 ± 1.0d
	0.001	5.0 ± 1.2d
Control	0	0.0 ± 0.0d
Natural pheromone (acetate)	3.000 FE	3.0 ± 1.0d
	1.000 FE	1.7 ± 0.6d
	0.300 FE	15.7 ± 2.2cd
	0.100 FE	19.3 ± 2.2bc
	0.030 FE	11.3 ± 2.0cd
	0.003 FE	1.7 ± 0.9d

[a]Test conducted at Vogel Center, Michigan, September 16 to October 16, 1981. Three replicates randomized three times.
[b]Means followed by same letter not significantly different at 5% level.

with 20% ether in hexane as the mobile phase (Kikukawa et al., 1983). The synthetic 2*S*,3*S*,7*S*-A (Mori et al., 1978) was purified through a charcoal–celite column. The 2*S*,3*S*,7*S*-A prepared through different synthetic routes by Kikukawa et al. (1982) was also purified. The 2*S*,3*S*,7*S*-A from both sources indicate a threshold at 0.003 μg (Table 6). Both the synthetic and natural pheromones were comparable.

To determine indirectly the form in which natural pheromone is stored in the females, the ester and alcohol fractions of *N. n. nanulus* females were first purified by TLC and the latter fraction was acetylated and then tested for relative activity on the field (Table 7). To test whether unesterified diprionol itself might have any effect on effectiveness of synthetic pheromone, varying amounts of 2*S*,3*S*,7*S*-alcohol was added to a fixed amount of 2*S*,3*S*,7*S*-A isomer. It is clear from these data that the alcohol is inhibitory at least at higher doses (Table 8). The responses of males of *N. n. nanulus* and *N. sertifer* to different com-

TABLE 6. THRESHOLD OF SYNTHETIC PHEROMONE 2*S*,3*S*,7*S*-A ISOMER FROM TWO SOURCES AND COMPARISON WITH NATURAL PHEROMONE IN THE FIELD AGAINST *N. nanulus nanulus* AT VOGEL CENTER, MICHIGAN, SEPTEMBER 18 TO OCTOBER 17, 1982

Pheromone	Amount/trap (ng)	Trap catch in three replicates (mean[a] ± SE)	
		2*S*,3*S*,7*S*-A[b]	2*S*,3*S*,7*S*-A[c]
Synthetic	10.000	131.0 ± 2.4a	298.7 ± 2.7a
	5.000	93.0 ± 2.1b	195.0 ± 4.1b
	1.000	39.0 ± 1.9c	119.7 ± 3.5c
	0.300	37.7 ± 3.2c	26.0 ± 2.2d
	0.100	5.3 ± 1.2d	6.0 ± 0.8d
	0.030	0.7 ± 0.4d	2.3 ± 0.8d
	0.010	1.0 ± 0.6d	0.3 ± 0.4d
	0.003	0.7 ± 0.6d	1.0 ± 0.6d
	0.001	0.0 ± 0.0d	0.0 ± 0.0d
Control	0	0.0 ± 0.0d	0.0 ± 0.0d
Natural pheromone (acetate)	10.000 FE	1.7 ± 1.0d	0.3 ± 0.4d
	3.000 FE	1.0 ± 0.6d	0.0 ± 0.0d
	1.000 FE	1.7 ± 0.6d	2.3 ± 0.7d
	0.300 FE	1.0 ± 0.8d	0.3 ± 0.4d
	0.100 FE	0.0 ± 0.0d	0.3 ± 0.0d

[a] Means followed by same letter not significantly different at 5% level by Duncan's multiple-range test.
[b] From Mori et al (1978).
[c] From Kikukawa et al., 1982 (unpublished).

TABLE 7. FIELD EFFECTIVENESS OF TLC FRACTIONS OF ALCOHOL AND ESTER FROM FEMALE *N. nanulus nanulus*[a]

TLC fractions tested[b]	Female equivalents	Replicates			Total trap catch	Catch/trap (mean ± SE)
		A	B	C		
Ester	1	2	1	1	4	1.3 ± 0.6
Alcohol[c]	1	4	7	7	18	6.0 ± 1.0

[a] Test conducted at Vogel Center from September 16 through October 16, 1981.
[b] R_f values for each fraction in this TLC system was: alcohol fraction 0.07–0.30 (corresponding to band between cinnamyl alcohol and cinnamyl acetate), ester fraction 0.39–0.65 [corresponding to 10-(*E*)-dodecen-1-yl acetate].
[c] Acetylated before tested on the field.

TABLE 8. EFFECT OF 2*S*,3*S*,7*S*-DIPRIONOL ON FIELD EFFECTIVENESS OF 2*S*,3*S*,7*S*-A AGAINST *N. nanulus nanulus*[a]

2*S*,3*S*,7*S*-A/2*S*,3*S*,7*S*-OH (μg)	Catch/trap (mean $\pm$ SE)[b]
5/0.000	107.7 $\pm$ 3.7a
5/0.001	73.0 $\pm$ 3.5a
5/0.003	46.0 $\pm$ 3.6b
5/0.010	55.3 $\pm$ 2.4a
5/0.030	83.3 $\pm$ 2.1a
5/0.100	66.3 $\pm$ 1.6a
5/0.300	40.7 $\pm$ 2.3b
5/1.000	46.0 $\pm$ 3.9b
5/5.000	12.3 $\pm$ 1.8b
5/10.000	7.7 $\pm$ 1.3b

[a]Test conducted at Vogel Center, Michigan, September 18 to October 17, 1982.
[b]Mean catch of three replicates. Means followed by same letter may not be significantly different at 5% level by Duncan's multiple-range test.

binations of 2*S*,3*S*,7*S*-A and 2*S*,3*R*,7*R*-AG were different from each other (Table 9). As observed earlier (Table 2), the 2*S*,3*R*,7*R*-A isomer was neither synergistic nor inhibitory to the field effectiveness of the 2*S*,3*S*,7*S*-A isomer against *N. n. nanulus*. Males of *N. sertifer*, however, seemed to prefer the combination of 2*S*,3*S*,7*S*-A and 2*S*,3*R*,7*R*-A between the ratios of 5:0.001 and 5:0.01, although the figures were not statistically significant (Table 10).

To investigate the possible difference between *N. nanulus* populations

TABLE 9. RESPONSE OF MALES OF *N. nanulus nanulus* AND *N. sertifer* TO DIFFERENT COMBINATIONS OF OPTICAL ISOMERS OF 3,7-DIMETHYLPENTADECAN-2-yl ACETATE[a]

2*S*,3*S*,7*S*-A/2*S*,3*R*,7*R*-AG (μg/trap)	*N. nanulus nanulus*[b]	*N. sertifer*[b]
5/0.000	2	2
5/0.001	0	22
5/0.003	4	9
5/0.010	3	10
5/0.030	4	3
5/0.100	1	0
5/0.300	6	0
5/1.000	4	0
5/5.000	2	0

[a]Test conducted in a red pine stand at Vogel Center, Michigan, October 16-31, 1982.
[b]Total catch of three replicates.

TABLE 10. IMPORTANCE OF CONFIGURATIONS AT C-7 OF THREO ISOMERS ON EFFECTIVENESS OF TRAP CATCH OF MALE *N. sertifer* BY 2*S*,3*S*,7*S*-A[a]

Pheromone	Series A[b] Oct. 5–Nov. 5, 1981 (x ± SE)[c]	Series B[b] Sept. 22–Oct. 19, 1982 (x ± SE)[c]
2*S*,3*S*,7*S*-A/2*S*,3*R*,7*R/S*-A	4.0 ± 1.2	4.6 ± 1.2
2*S*,3*S*,7*S*-A/2*S/R*,3*R*,7*R*-A	5.6 ± 1.3	0.7 ± 0.6
2*S*,3*S*,7*S*-A/2*S*,3*R*,7*S*-A	2.6 ± 0.9	2.3 ± 0.8
2*S*,3*S*,7*S*-A/2*S*,3*R*,7*R*-A	4.3 ± 1.1	4.3 ± 1.3

[a]Test was conducted at Rose Lake October 5–November 5, 1981 and September 22–October 19, 1982.
[b]The result of catches by three sets of traps for each treatment.
[c]Analysis of variance insignificant and no significant difference among the means by Duncan's multiple-range test.

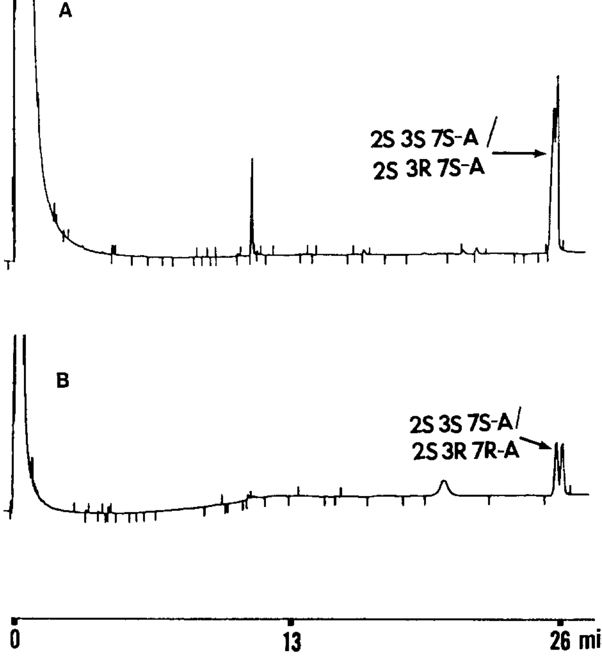

FIG. 1. Capillary GLC separation on DB-5 column temperature programmed 130–190°C at 4°/min. (A) 2*S*,3*S*,7*S*-A/2*S*,3*R*,7*S*-A mixture, 100 ng, (B) 2*S*,3*S*,7*S*-A/2*S*,3*R*,7*R*-A mixture, 50 ng.

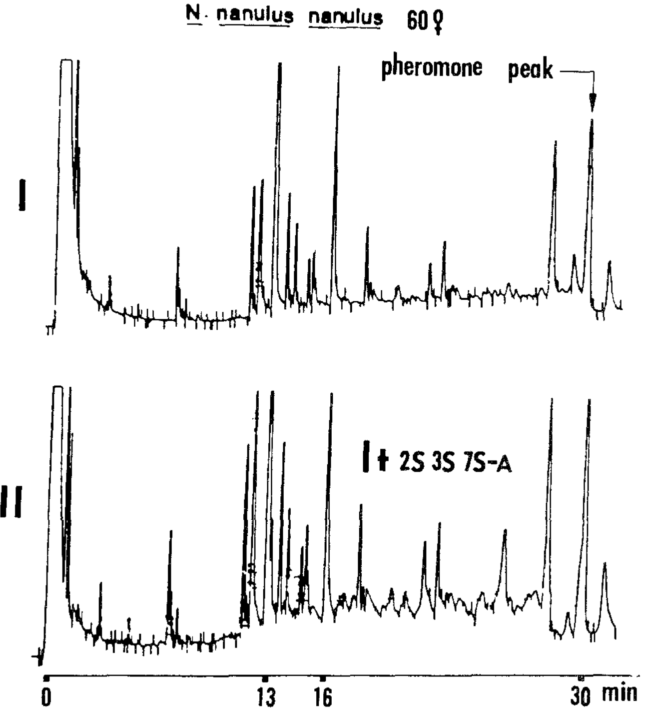

FIG. 2. (I) Capillary GLC on DB-5 recordings of acetylated natural pheromone of *N. nanulus nanulus*, 60 FE. (II) Capillary GLC recordings of acetylated natural pheromone of *N. nanulus nanulus* 60 FE, spiked with 100 ng 2*S*,3*S*,7*S*-A.

feeding on jack pines vs. red pines (Knerer and Atwood, 1973) traps baited with 2*S*,3*S*,7*S*-A and 2*S*,3*S*,7*R*/*S*-A were set in Vogel Center (red pine) and Houghton Lake (jack pine). In both locations 2*S*,3*S*,7*S*-A showed superior effectiveness over 2*S*,3*S*,7*R*/*S*-A in attracting males of *N. nanulus* (data not shown).

Capillary GLC Analysis. The two GC columns used for this analysis were capable of separating the 2*S*,3*S*,7*S*-A from the 2*S*,3*R*,7*R*-A. With the latter having a longer retention time, better resolution was obtained with the DB-5 column where the 2*S*,3*S*,7*S*-A was also separated from 2*S*,3*R*,7*S*-A (Figure 1). By matching the retention times of the standard synthetic isomers 2*S*,3*S*,7*S*-A, and 2*S*,3*R*,7*S*-A and 2*S*,3*R*,7*R*-A with the natural pheromone peaks, and by coinjection of 2*S*,3*S*,7*S*-A with the natural pheromones, it was observed that only the 2*S*,3*S*,7*S*-A isomer was detectable in *N. n. nanulus* and *N. sertifer* (Figures 2 and 3). It was determined, with the DB-5 column, that virgin females of *N. n. nanulus* and *N. sertifer* each contained 2 ng and 10 ng of pheromone, respectively.

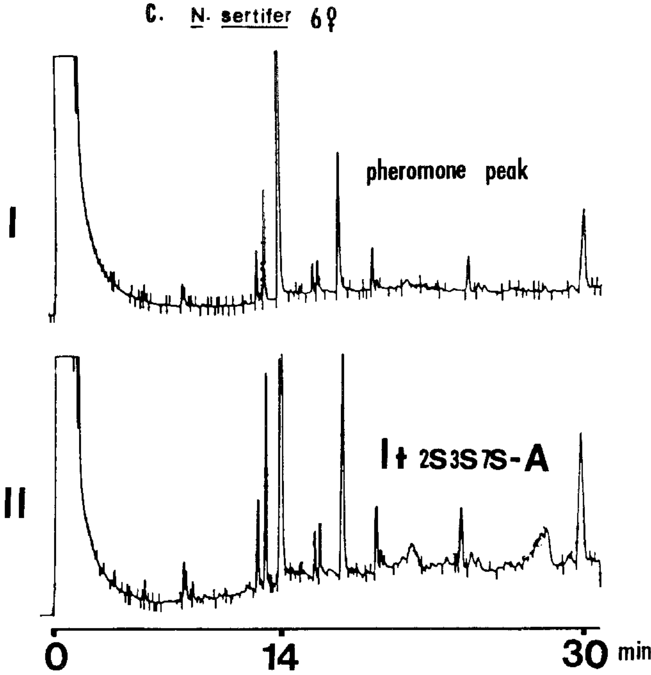

FIG. 3. (I) Capillary GLC on DB-5 recordings of acetylated natural pheromone of *N. sertifer*, 6 FE. Compare to Figure 2 for the locations of pheromones. (II) Capillary GLC recordings of acetylated natural pheromone of *N. sertifer*, 6 FE, spiked with 50 ng 2*S*,3*S*,7*S*-A.

DISCUSSION

It is clear from the current study results that 2*S*,3*S*,7*S*-A is the major pheromone component *N. n. nanulus*. The addition of 2*S*,3*R*,7*R*/*S*-A, 2*S*,3*R*,7*R*-A, and 2*S*,3*R*,7*S*-A was neither inhibitory nor synergistic to its effectiveness. The synthetic pheromone, either as 2*S*,3*S*,7*S*-A alone or as mixtures, was as effective as the natural pheromone extracted from females (Table 5).

By contrast the most effective synthetic preparation for *N. sertifer* was a mixture of 5:0.003 to 5:0.01 (μg/μg) of 2*S*,3*S*,7*S*-A to 2*S*,3*R*,7*R*-A. It must be emphasized here that adults of these two species emerge at the same time of the year (one generation per year) and that they are observed to distribute in similar areas in the Great Lakes region. Futhermore, there are two races of *N. nanulus*: one feeding on jack pine and the other on red pine. Therefore, these two species, at least the ones feeding on jack pine, must be regarded as truly sympatric. A natural question arising from the current study results is whether

the action of the small amount of synergist 2*S*,3*R*,7*R*-A on *N. sertifer* alone could offer a critical species differentiation between them.

In the area where these two species coexist (e.g., Vogel Center, Table 9) *N. sertifer* males were caught only to traps containing 0–0.03 μg synergist to 5 μg of 2*S*,3*S*,7*S*-A, whereas *N. nanulus* males were caught in all ranges of synergist concentrations. The results indicate that the males of the former species is much more sensitive to the presence of the synergist and thereby discriminatory to specific isomer ratio than the latter. Thus, such a species difference in pheromone recognition could play at least an important part in the species isolation for *N. sertifer*. How *N. nanulus* males recognize the females of their own species from others is not clear. It is possible that there are other mechanisms, particularly those involved in close-range behavioral signals, which play important roles in this regard. Much more work would be needed to clarify this point.

There is good agreement between the field studies and the GLC analyses of the natural pheromone in these two species. Assuming a 5 : 0.003 optimum blend of 2*S*,3*S*,7*S*-A to 2*S*,3*R*,7*R*-A obtained from field studies, the 36 pg of 2*S*,3*R*,7*R*-A present in the 6 FE pheromone of *N. sertifer* injected at the time was beyond the detection limit of the FID system used. This explains why only 2*S*,3*S*,7*S*-A was detected by GLC analysis for both *N. n. nanulus* and *N. sertifer*. Thus, despite the failure of reorganizing the synergist component in *N. sertifer* extract, the GLC analysis has clearly established for the first time the true chiral identity of the major pheromone for both species.

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EFFICACY OF PINE OIL AS REPELLENT TO WILDLIFE

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Abstract—Pine oil, a by-product of the pulp industry, is a feeding repellent to snowshoe hares and voles. In pen trials with snowshoe hares and field trials with voles, when given a choice between food in a pine oil-treated bowl and a control bowl, the animals fed preferentially from the control bowl. When the hares were presented with food only in a pine oil-treated bowl, two hares showed a reduced rate of food consumption and one hare did not feed at all. Pine oil contains monoterpenes which may inhibit microbial symbionts in the digestive tracts of hares and voles. The repellent action of pine oil is likely based on this interference of digestive processes, and it is of adaptive advantage for cecal digestors to avoid it. Pine oil has potential as a commercial repellent for snowshoe hares and voles.

Key Words—wildlife damage, snowshoe hare, *Lepus americanus*, Townsend vole, *Microtus townsendii*, pine oil, repellents, seedling protection.

INTRODUCTION

Wildlife can pose serious problems to intensive forest management, specifically to conifer regeneration programs. Feeding by hares and voles is responsible for substantial damage to seedlings in the Pacific Northwest (Black et al., 1979). Snowshoe hares, *Lepus americanus* Erxleben, damage seedlings by clipping the terminal and lateral shoots (Dimock, 1970) and saplings or large trees by debarking, which can result in girdling of trees (Sullivan, 1984). Volves, *Microtus* spp., debark stems and clip growing shoots of seedlings (Santamour et al., 1963; Green, 1978). Control measures can either eliminate the pest animals or protect trees from the pest. A strategy whereby the crop trees are made inaccessible or undesirable to wildlife is a more desirable approach given current

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attitudes to wildlife. Considerable attention has been focused on chemical repellents to protect seedlings. No currently available repellent has proved completely effective against wildlife feeding damage, and there is a continuiing search for new products.

Pine oil,[1] a by-product of the pulp industry, is an oily liquid containing a mixture of terpene alcohols and monoterpenes. These terpenes are present in naturally occurring, essential oils found in foliage resistance to animal damage. Pine oil has repellent properties to forest insects (Nijholt et al., 1981; Alfaro et al., 1984; Richmond, 1985). It has been used in Scotland as a deer repellent in seedling plantations (P.R.W. Sanders,[2] personal communication). These observations and reports suggest the potential of pine oil as a repellent for wildlife. Since it is extracted from pulp waste, it is both plentiful and accessible to the forest industry.

The overall aim of this research was to determine whether pine oil had repellent properties to snowshoe hares and voles and if future research is warranted.

METHODS AND MATERIALS

Effect of Pine Oil on Penned Snowshoe Hares. Three males snowshoe hares were kept separately in adjacent outdoor pens (5.0 × 1.5m). Each pen contained two covered feeding stations, 1.1 m apart. These hares were provided with lab pellets (Purina Rabbit Chow) and fresh food (apples and carrots) between 0800 and 1500 hr (PST) each day. Food was removed from the pens 2 hr prior to the start of a trial.

Feeding preference experiments were conducted overnight between 1700 and 0800 hr, because hares are more active and consume the bulk of their daily food in take during this time. Trials were conducted between October 15 and December 10, 1985. At each feeding station, each hare was given a bowl of food containing 100 g of lab pellets (increased to 150 g when consumption rose during cold winter months). The bowls were presented in a combination of treated–control or control–control. To create a vapor "barrier" around the food, pine oil was applied by drenching 1 m of pipe cleaner in 25 ml of pine oil. The pine oil wick was then wrapped in burlap, placed around the bowl, and secured with a rubber band. Thus the smell of pine oil could permeate the atmosphere around the food but the hare could not come into direct contact with it. Control bowls were presented with the burlap and pipe cleaner but no pine oil. At the end of the 15-hr trial period, the bowls were removed and the remaining food

[1] Manufactured by Northwest Petrochemical Corp., Anacortes, Washington 98221, under the commercial name Norpine-65.

[2] P.R.W. Sanders, Resident Silviculturalist, University of British Columbia Research Forest, Maple Ridge, B.C., Canada.

in each bowl weighed. Preference was determined by the relative amount of food consumed. To eliminate bias for a particular feeding station, the treated and control bowls were randomly positioned for each replicate.

Fourteen-hour, no-choice experiments began at 1700 and ended at 0700 hr. Each hare was given a single pine oil-treated or control bowl containing 50 g of lab pellets. The food was replaced every 2 hr. Each time, the food remaining in the bowl was weighed and the amount of food consumed during the 2 hr period was calculated.

Effect of Pine Oil on Voles in the Field. Experiments were conducted at a field in Richmond, B.C. (approximately 1 km north of Vancouver International Airport) inhabited by Townsend voles, *Microtus townsendii* Bachman. The field was covered with deep grass and a few shrubs. Thirty-one feeding stations, at least 10 m apart, were established near vole runways over a 1-hectare area. Each feeding station consisted of two open-ended milk cartons pegged side by side to the ground. They were baited with rolled oats in small glass dishes placed in the centers of the cartons to ensure protection from weather and foraging birds. The field was prebaited for five days to accustom the voles to oats.

During the experiment, dishes containing 5 g of rolled oats were placed in each milk carton. At each feeding station, there was a choice between a dish surrounded by a ring of pipe cleaner soaked in pine oil in one carton and a dish surrounded by a ring of clean pipe cleaner in the remaining carton. The experiment ran for 72 hr, from February 10–13 and 18–21, 1986, after which the food remaining in the dishes was collected and weighed. The presence or absence of droppings in dishes and cartons was noted at each feeding station.

RESULTS

Snowshoe Hares. When given a choice, all three hares consumed significantly more food from the control bowl than from the pine oil-treated bowl (Table 1). Two of the hares fed almost exclusively from the control bowl, while hare 1 ate some food from the pine oil-treated bowl in all eight replicates. In one replicate, it ate more than half of its nightly intake from the treated bowl. This variation in individual reaction by snowshoe hares was also observed in experiments testing predator odors as repellents (T.P. Sullivan,[3] personal communication). When the hares were given a choice between two control bowls, there was no differences in the amount of food consumed from each bowl (Table 2).

The choice experiments indicate that pine oil has significant repellency resulting from olfactory stimuli. The vapor barrier around the bowl apparently deterred the hares from feeding. The hares did not appear to become habituated

[3] T.P. Sullivan, Director, Institute for Applied Mammal Research, Langley, British Columbia.

TABLE 1. MEAN FOOD CONSUMPTION BY PENNED SNOWSHOE HARES GIVEN A CHOICE BETWEEN FOOD IN CONTROL OR PINE OIL-TREATED BOWLS

Hare	No. of replicates	Weight (g) of food consumed in 15 hr ($\bar{X} \pm$ SE)[a]	
		Control bowl	Treated bowl
1	8	71.6 ± 8.2	14.7 ± 5.1
2	7	65.8 ± 10.4	1.8 ± 1.6
3	6	98.5 ± 9.7	0.0 ± 0.0

[a] Difference between consumption from treated and control significant in all cases, Student's *t* test, $P \leq 0.05$.

to the smell of pine oil, since there was little difference in results between the start of the experiments in October and their termination in December.

For the no-choice experiments, the nightly feeding patterns for each hare under the control and treatment regimes are shown in Figure 1. For all three hares, the mean total food consumption was greater from the control than the treated bowl (Table 3). Different hares had different feeding patterns when presented only with food in a pine oil-treated bowl. Hares 1 and 2 fed from the pine oil-treated bowl in the absence of alternative food, while hare 3 did not feed at all from the pine oil-treated bowl (Table 4). Sinclair et al. (1982) reported a one- to four-day "hunger strike" in snowshoe hares presented with

TABLE 2. MEAN FOOD CONSUMPTION BY PENNED SNOWSHOE HARES GIVEN A CHOICE BETWEEN FOOD IN TWO CONTROL BOWLS

Hare	No. of replicates	Weight (g) of food consumed in 15 hr ($\bar{X} \pm$ SE)[a]	
		Control bowl	Control bowl
1	9	51.0 ± 10.2	37.9 ± 6.0
2	7	29.5 ± 13.1	23.1 ± 6.7
3	6	48.9 ± 13.7	41.3 ± 14.5

[a] No significant difference in food consumption from two control bowls in all cases, Student's *t* test, $P > 0.05$.

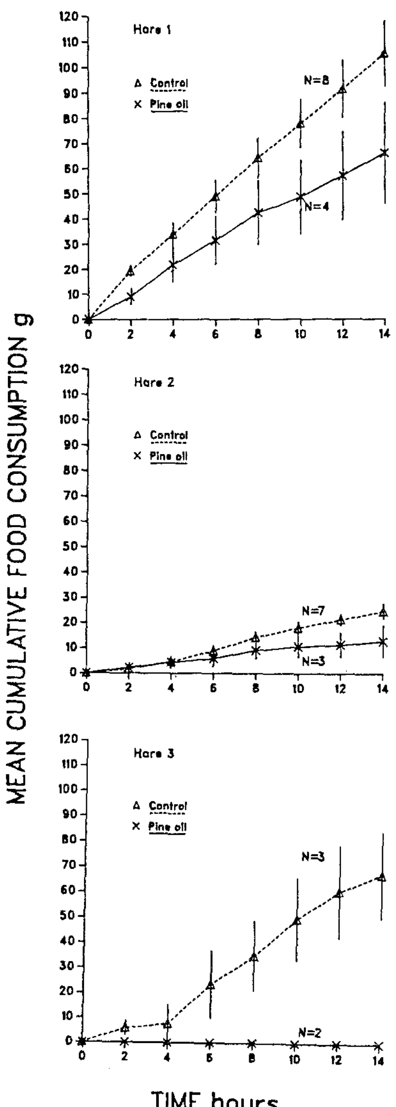

FIG. 1. Mean cumulative food consumption by three hares when given either a control food bowl or a food bowl treated with pine oil for a 14-hr feeding period. SE is shown by vertical bars.

TABLE 3. MEAN FOOD CONSUMPTION BY PENNED SNOWSHOE HARES GIVEN PINE OIL-TREATED BOWL OR CONTROL BOWL

	No. of replicates		Total weight (g) of food consumed in 14 hr ($\bar{X} \pm$ SE)[a]	
Hare	Control	Treated	Control	Treated
1	8	4	105.5 ± 12.9	66.3 ± 20.2
2	7	3	24.7 ± 3.0	12.7 ± 6.2
3	3	2	67.6 ± 17.1	0.0 ± 0.0

[a] Significant differences in food consumed from control and treated bowls occurred for all three hares, Wilcoxon signed-ranks test, $P \leq 0.05$.

low-quality food, after which the animals resumed feeding. The duration of our experiment was insufficient to observe such behavior by hare 3.

Pine oil did not prevent feeding by two hares, although it did reduce their rate of intake. If such feeding behavior occurred in natural populations, pine oil may not prevent feeding on treated plants. If a "feeding strike" occurred, followed by a resumption of normal feeding behavior (Sinclair et al., 1982), the repellent effects on pine oil on animals such as hare 3 might be of short duration. In most field situations, however, alternative foods exist, suggesting that pine oil might have considerable practical potential for use against snowshoe hares.

Voles. When feces were present in a carton, food had always been removed. Data for six feeding stations were removed from the first experiment and four from the second because of the lack of fecal pellets and absence of feeding at treated and control dishes. These data were removed because lack of

TABLE 4. MEAN WEIGHT OF FOOD CONSUMED BY VOLES FROM CONTROL AND PINE OIL-TREATED DISHES FOR TWO FIELD EXPERIMENTS IN RICHMOND, B.C. FIVE g OF ROLLED OATS WERE IN EACH DISH AND LEFT FOR THREE DAYS.

		(Food consumed g, ($\bar{X} \pm$ SE)[a]	
Experiment	No. of replicates	Control dishes	Treated dishes
1	25	4.4 ± 0.2	1.6 ± 0.4
2	27	4.8 ± 0.1	0.9 ± 0.3

[a] Difference between consumption from treated and control dishes significant in both experiments, Student's t test, $P \leq 0.05$.

pellets likely indicates that voles did not encounter the feeding stations. At the remaining stations, voles fed from 52 of 52 control dishes and only 19 of 52 pine oil-treated dishes ($\chi^2 = 15.34$, $df = 1$, $P \leq 0.05$). There was also significantly more (3.7 times) food taken from the control than the pine oil-treated dishes (Table 4).

At 33 stations there were no droppings in the cartons containing pine oil and no food was removed, suggesting that in these cases the pine oil odor was sufficiently repellent to prevent the voles from even entering the cartons. However, at 19 stations, voles had entered the cartons and fed from the pine oil-treated dishes, taking an average of 3.4 ± 0.4 g of food. The apparent lack of pine oil repellency at these stations could be due to differences in effective concentration of pine oil or to individual variation in response to pine oil, a phenomenon also observed with snowshoe hares. At all of these 19 stations, however, the 5 g of food in the control cartons had been completely consumed. This suggests that voles may have fed from treated dishes only after food in control cartons was completely consumed. If this was the case, it indicates the need for alternative food sources when using pine oil as a feeding repellent.

At 20 stations, all of the food from the control cartons was removed but the pine oil-treated food remained untouched. This may be due to less feeding at those stations or a result of the individual variation in response of voles to pine oil. Overall, pine oil seems to be an effective repellent to voles in the presence of alternative food.

DISCUSSION

Some strains of Douglas fir are more resistant to deer and snowshoe hare damage than others, and this resistance has a genetic basis (Dimock et al., 1976; Silen and Dimock, 1978). The difference between damage-resistant clones and susceptible clones of Douglas fir is based on the essential oil (terpene derivatives obtained from plant material by steam distillation) content of the foliage, and the levels of volatile terpenes emitted by these oils (Radwen, 1978; Radwan and Ellis, 1975). More than 40 essential oils have been identified in Douglas-fir needles. It is generally believed that these oils serve a defensive purpose and deter herbivores from feeding on the foliage (Freeland and Janzen, 1974). The natural resistance of deciduous woody plants and conifers to browsing by snowshoe hares and voles has been attributed to the presence of defense compounds (Von Althuen, 1971; Bryant, 1981) and, in particular, oxygenated monoterpenes in conifers (Bryant and Kuropat, 1980). Oxygenated monoterpenes in Douglas-fir foliage have an inhibitory effect on microbial activity (Oh et al., 1967). Pine oil contains high levels of terpene volatiles, including some oxygenated monoterpenes such as α-terpineol and limnonene (Nijholt, 1980) with known antimicrobial properties (Connolly et al., 1980). The antimicrobial action of these monoterpenes may be detrimental to herbivores.

Snowshoe hares and voles are cecal digestors and are dependent on microorganisms for efficient food digestion (Robbins, 1983). The consumption of essential oils containing microbial inhibitors may disrupt the digestive process (Bryant and Kuropat, 1980). A herbivore should be able to detect physiologically detrimental defensive chemicals, or indicator chemicals, in a plant before eating it (Levin, 1976). Detection of the characteristic odor, of essential oils may, therefore, be of adaptive advantage to cecal and ruminant digestors for food selection. The use of pine oil as a repellent may exploit any ability herbivores have to recognize metabolically costly compounds by olfaction.

Foraging theory predicts that an animal will always try to maximize its rate of energy intake within the constraints of risk of predation (Krebs, 1978). The use of a chemical repellent aims to reduce or mask the quality of a high-value foraging patch or food item. The animal will mistake desirable food for food that is energetically expensive to digest and will search for an alternative food source with a ''higher'' energy value. When such an alternative food source is available, a repellent should be very effective in protecting trees (Green, 1978). In the absence of such a food source, however, the animal may be forced to eat the apparently ''low-value'' food. The field trials with voles are consistent with this hypothesis.

Despite the preliminary nature of our experiments, our results suggest a possible commercial future for pine oil as a wildlife repellent. Preliminary tests showed that it is phytotoxic to seedlings when applied topically, and an alternative method of application, such as in slow release devices, could be feasible. Our initial success with pine oil as a repellent justifies a continuation of its testing as a potentially marketable product. Field experiments with hares and voles should be performed using seedlings and wild populations of animals. Phytotoxicity tests should be conducted on different species of trees at different ages and developmental stages with different modes of application.

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CHEMICAL BASIS FOR ASYMMETRIC MATING ISOLATION BETWEEN STRAINS OF SCREWWORM FLY, *Cochliomyia hominivorax*

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Abstract—Laboratory mating tests and bioassays for a contact, mating-stimulant pheromone were conducted within and between two strains of the screwworm fly, *Cochliomyia hominivorax*. No strain or sex difference in pheromone activity occurred at emergence. However, the pheromone activity of females increased with age in one strain but declined in the other. Activity also declined in males of both strains but more steeply than in the females. Thus, sexual dimorphism in pheromone activity developed with age in both strains but to differing degrees. The decline in female pheromone activity was partly compensated for within that strain by a higher male responsiveness to pheromone. Female pheromone activity and mating success were positively correlated. Virgin females and those inseminated 24–48 hr previously were equally stimulatory. It appears that the strain differences arose from selection for reduced pheromone activity during laboratory colonization.

Key Words—Screwworm fly, *Cochliomyia hominivorax*, Diptera, Calliphoridae, sex pheromone, mating-stimulant pheromone, mating behavior.

INTRODUCTION

Female-produced sex pheromones located on the cuticle and acting on contact or at short range occur in many Diptera (see Howard and Blomquist, 1982). Most of these pheromones are hydrocarbons, usually alkenes or branched alkanes with chain lengths of 23 (C_{23}) to 37 (C_{37}) carbons. Some function as short-range attractants, such as the C_{23} alkene, (Z)-9-tricosene, of the housefly, *Musca domestica* (Carlson et al., 1971). Others, such as the C_{35} and C_{37} methyl branched alkanes that are present in tsetse flies (*Glossina* spp.), are not signif-

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icantly volatile and function as contact aphrodisiacs (Langley et al., 1975; Carlson et al., 1984).

In the screwworm fly, *Cochliomyia hominivorax* (Coquerel), chemically mediated attraction of males to females is not known, but male copulatory behavior is released upon contact with a chemical stimulus or set of stimuli produced by the female (Mackley and Broce, 1981; Hammack, 1986). However, heterogeneity apparently exists among screwworm strains with respect to this pheromone system. Female pheromone activity estimated from male copulatory attempts directed at dead females in intrastrain tests increased significantly with age after emergence in one strain but declined in another (Hammack, 1986). The decline occurred in a long-colonized strain and began before females were old enough to mate. These features and the report by Pomonis and Mackley (1985) that cuticular lipid profiles change with colonization in female but not male screwworm flies led to the hypothesis that the declining pattern is the result of adaptation to mixed-sex holding conditions in the laboratory, where high pheromone activity might prove detrimental to female survival. Regardless of origin, sexually receptive females differing in pheromone activity could prove useful for evaluating the respective roles of female behavior and chemical stimuli in elicting male courtship. Strain heterogeneity in the pheromone system, if accompanied by mating isolation between strains, may also be relevant to screwworm eradication programs that depend on the ability of mass-reared sterile males to mate competitively with wild populations.

The purposes of this study were to (1) confirm that contrasting relationships between female age and sex pheromone activity occur in different screwworm strains; (2) determine whether or not mating affects female pheromone activity in a strain showing an age-dependent increase in pheromone activity; (3) determine when strain differences in the pheromone activity of females occur and if these are compensated for by strain differences in the responsiveness of males to a pheromone stimulus; and (4) conduct interstrain mating tests to determine the relationship between female pheromone activity and mating success.

METHODS AND MATERIALS

Insects. The screwworm strains used here were the 009 and FC-96 (hereafter FC). The 009 strain, established from multiple egg masses collected in Texas, had been colonized in the laboratory for > 100 generations. It had previously failed to show an age-dependent increase in the ability of dead females to stimulate male copulatory attempts (Hammack, 1986). The FC strain originated from a single egg mass collected in Chiapas, Mexico, and was tested after 4–26 generations in the laboratory.

Larvae were reared on a beef-based diet, and adults held on a 12:12 light–

dark photoperiod at 25 ± 1°C with access to corn syrup and water (Hammack, 1984). Breeding females of the 009 strain were held in mixed-sex colonies, whereas those of the FC strain were held without males except for the few minutes required to achieve insemination. Experiments were conducted with virgin flies 6–8 days old, unless stated otherwise. Virgins were obtained by separating the sexes within 24 hr of emergence, using CO_2 anesthesia. Flies older than 24 hr were not anesthetized when handled.

Pheromone Extraction. Extracts of cuticular components were prepared by washing 20 females for 15 sec in 1 ml of *n*-hexane (Hammack, 1986). The brief wash was retained because longer extractions in preliminary tests either had no effect on the recovery of pheromone activity (1 min) or reduced it (5 min). Extracts were concentrated in a stream of N_2 and stored up to eight days at ca. −70°C before testing.

Pheromone Bioassays. Bioassays determined whether or not test males attempted copulation with decoy insects when the males were paired with decoys for 1 min. The decoys were either whole insects fresh-killed by freezing at −20°C for 30 min (untreated decoys) or hexane-washed males treated with female extract (or solvent). The males killed by rinsing in three 0.5-ml aliquots of hexane were air dried and then treated, unless stated otherwise, with 1.0 female equivalent (FE) of extract in 5 μl of hexane.

Some decoys were mounted for assay using pins as in previous tests (Hammack, 1986), and these are identified in Tables 2 and 4. However, most were mounted with the ventral side applied to the small end of No. 6 corks via nylon thread tightened against the dorsal surface of the neck. The thread was taped to opposite sides of the cork where test males could not touch. Each cork with attached decoy was fitted in a glass test tube (15 × 150 mm) that contained one test male. Inversion of the tube forced contact between the test and decoy insects. The change in mounting method was made to avoid puncturing the body surface of decoys after loose decoys previously punctured with pins proved less stimulatory than unpunctured controls (Hammack, unpublished). Except for the altered method of mounting most decoys and the use of solvent-washed males in place of females to test pheromone extracts, bioassay procedures were as described previously (Hammack, 1986). Males were substituted here because solvent washing might not remove from females all sex-specific cues contributing to the release of male copulatory behavior.

Solvent-washed males treated with 5 μl of hexane were assayed to determine control responses. Washed males treated with female extract were assayed simultaneously to check responsiveness of the test males. Within strains, each of five decoys per treatment was tested with 10 males.

Matings were observed at four days of age among mixed-sex pairs of the FC strain, after establishing in preliminary trials that mating frequencies were identical whether determined visually or by detection of sperm in spermathecae. The inseminated females and matched virgins were extracted separately 24 and

48 ± 4 hr after mating. For each interval, responses of FC males to extracts of mated and virgin females were compared in bioassays.

Pairwise comparisons of pheromone activities were made using test males 6–8 days old and the following untreated dead decoys: (1) 0 vs. 6- to 8-day-old females within strains, (2) males vs. females at 0 and 6–8 days within strains, (3) 0 vs. 6- to 8-day-old FC males using 009 test males, and (4) FC vs. 009 females at 0 and 6–8 days using males of each strain. Six- to eight-day-old FC and 009 females were also compared by assaying their extracts with males of each strain. Finally, the responsiveness of 009 and FC males was compared using both 0 day and sexually mature female decoys of each strain. All 0 day insects were killed within 4 hr of emergence.

Females of the 009 strain that had all emerged during the same photophase interval were frozen at the following ages postemergence: 3 ± 2 hr and 1, 2, 3, 4, 6, and 8 days ± 2 hr. They were stored at ca. −70°C up to 25 days and then extracted. Extracts (one per age group) were prepared simultaneously and each tested on three decoys in intrastrain assays. The extraction and assay procedures were repeated twice.

Data were analyzed using Wilcoxon's signed-rank test or Friedman's nonparametric test for the randomized complete block design (Xr^2 statistic distributed approximately as X^2) (Steel and Torrie, 1960). Each decoy tested with 10 individually exposed males was considered a replicate.

Mating Tests. Two tests were done in which males were given the opportunity to mate with live unrestrained females. Both involved four crosses set up simultaneously: the two intrastrain and two reciprocal interstrain ones. The crosses in the first test were made by placing 20 virgins of each sex in cages (ca. 28 × 18 × 23 cm) and then isolating the females after 24 hr. Those with sperm in their spermathecae were considered mated. Mating frequencies from eight replicates per cross were analyzed using Friedman's test for the randomized complete block design.

For the second test, the sexes were combined in single pairs and forced into contact for 1 min in glass shell vials (25 × 95 mm) stoppered with foam rubber plugs. A new plug was used for 10 successive replicates and discarded. Observations were made of the number of pairs (1) in which males attempted copulation with the female and (2) that were in copula within 1 min. Insects of both sexes were tested once and discarded. One hundred twenty pairs were observed per cross using 6- to 8-day-old 009 and 12- to 14-day-old FC flies. The test was repeated using only 6- to 8-day-old insects.

RESULTS

Pheromone Bioassays. Within the FC strain, no males (0/50) attempted copulation with hexane-washed males treated only with solvent, although 84% (42/50) responded to those treated with 1.0 FE of extract. None of the 009

males responded to solvent (0/50), although 44% (22/50) did so to 1.0 FE of 009 extract. Thus, control decoys were not stimulatory within either strain.

Insemination had no effect on the pheromone activity of females of the FC strain when they were extracted for testing 24–48 hr after mating (Table 1). Data obtained at 24 and 48 hr within mating treatments were similar and have been pooled in Table 1.

Sex and strain differences in the ability of untreated decoys to stimulate male copulatory attempts were absent at 0 days (Table 2, tests 1–4). Between 0 and 6–8 days, the stimulatory ability of FC-strain females increased significantly (Table 2, test 5), whereas that of 009-strain females declined significantly (Table 2, test 6). In this respect, 009 females resembled males of both strains (Table 2; tests 2, 7, 9), although the decline was steeper among males because sexual dimorphism in pheromone activity was evident in both strains when they were tested at 6–8 days of age (Table 2; tests 8, 9). Sexually mature female decoys of the FC strain stimulated significantly more copulatory responses than those of the 009 strain, regardless of whether test males were of the FC or 009 strain (Table 2; tests 10, 11). These differences also occurred when extracts of FC and 009 females were assayed (Table 3), leaving no doubt that sex pheromone was the stimulus responsible for the greater activity of mature FC females.

The percentage of 009 males responding to extracts of 009 females varied significantly with female age and declined from 94% at 0 days to 66% at 8 days ($Xr^2 = 13.4$, $df = 6$, $P < 0.05$). This pattern is contrasted in Figure 1 with those age-dependent changes in pheromone activity already inferred for FC-strain females and males of both strains. This was accomplished by plotting all pheromone activities relative to that of insects 0 days old when sex and strain differences were lacking. The pattern of 009 females was clearly unique but, in its decline with age, more closely resembled that of FC and 009 males than that of FC females.

TABLE I. EFFECT OF MATING ON SEX PHEROMONE ACTIVITY OF FEMALE SCREWWORM FLIES OF FC STRAIN EXTRACTED 24–48 HR POSTMATING

Dose (FE)	Mean male response (%)[a]	
	Virgin females	Mated females
1.0	93	86[b]
0.5	86	80[b]
0.25	61	64[b]

[a] $N = 10$ decoys per treatment and dose, each tested with ten 6- to 8-day-old males of the FC strain.
[b] Responses did not vary between mated and virgin treatments within doses ($P > 0.05$, Wilcoxon's signed-rank test). Responses did vary with dose after pooling data across treatments ($P < 0.001$, Friedman's test).

TABLE 2. EFFECTS OF AGE, SEX, AND STRAIN ON ABILITY OF DEAD DECOY INSECTS TO STIMULATE COPULATORY ATTEMPTS IN MALES OF TWO STRAINS OF SCREWWORM FLY

	Type of decoy				Mean male response (%)[b]			
Test	Age (days)[a]	Sex	Strain	Strain of test male	Pin mount		Thread mount	
1	0	F	FC	FC	14	NS[c]	13	NS
	0	M	FC	FC	8		19	
2	0	F	009	009	90	NS	98	NS
	0	M	009	009	87		97	
3	0	F	FC	FC	6	NS	10	NS
	0	F	009	FC	4		23	
4	0	F	FC	009	77	NS	100	NS
	0	F	009	009	66		95	
5	0	F	FC	FC	13	**[c]		
	6–8	F	FC	FC	66			
6	0	F	009	009	80	**		
	6–8	F	009	009	44			
7	0	M	FC	009	77	**	100	**
	6–8	M	FC	009	0		1	
8	6–8	F	FC	FC	71	**	93	**
	6–8	M	FC	FC	0		0	
9	6–8	F	009	009	61	**	74	**
	6–8	M	009	009	0		1	
10	6–8	F	FC	FC			91	**
	6–8	F	009	FC			2	
11	6–8	F	FC	009			96	**
	6–8	F	009	009			51	

[a] 0-day-old insects were killed within 4 hr of emergence.
[b] $N = 10$ decoys per type and mounting method, each tested with ten 6- to 8-day-old males.
[c] NS, not significant at 0.05 probability level. ** $P < 0.01$, referring to the difference within tests and mounting methods between members of a pair (Wilcoxon's signed-rank test).

Although sexually mature females of the FC strain had more pheromone activity than 009 females, the data in Table 2 suggest that males of the 009 strain were more responsive to a pheromone stimulus than FC males. This higher 009 responsiveness was demonstrable in paired comparisons and did not depend on the strain or age of females used to supply the pheromone stimulus (Table 4).

TABLE 3. EFFECT OF SCREWWORM STRAIN ON SEX PHEROMONE ACTIVITY OF FEMALES 6-8 DAYS OLD

Strain supplying 1.0 FE of female extract	Strain of test male	Mean male response (%)[a]
FC	FC	93 **[b]
009	FC	2
FC	009	95 **
009	009	29

[a] $N = 15$ decoys per strain combination, each tested with ten 6- to 8-day-old males.
[b] $**P < 0.01$, referring to the difference within strains of males between strains of females (Wilcoxon's signed-rank test).

Mating Tests. When the sexes were combined for 24 hr, significant differences in mating frequencies occurred among the four test crosses involving FC and 009 strains (Figure 2, 24-hr test) ($Xr^2 = 12.2$, $df = 3$, $P < 0.01$). However, deletion from the statistical analysis of the cross involving 009 females and FC males abolished significance ($Xr^2 = 2.4$, $df = 2$, $P > 0.25$). Thus, FC females were readily mated by males of both strains, whereas 009 females were readily mated only by 009 males.

A similar mating pattern was obtained for the four crosses when insects were observed for 1 min in mixed-sex pairs (Figure 2, 1-min test). More im-

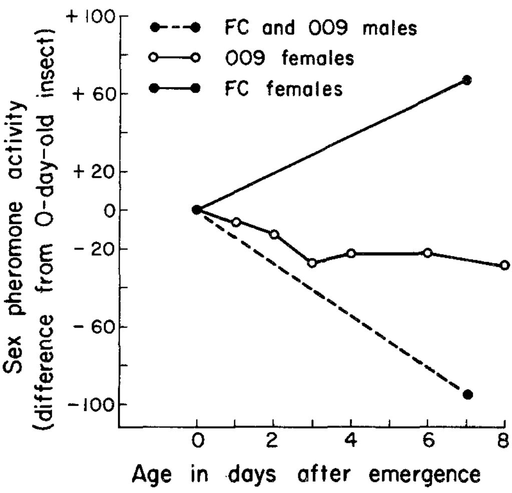

FIG. 1. Effects of age, sex, and strain on the sex pheromone activity of screwworm flies.

TABLE 4. EFFECT OF SCREWWORM STRAIN ON RESPONSIVENESS OF MALES TO SEX PHEROMONE STIMULUS

Test	Type of female decoy[a] Age[b] (days)	Type of female decoy[a] Strain	Strain of test male[c]	Mean male response (%)[d]
1	0	FC	FC	2 **[e]
	0	FC	009	94
2	0	009	FC	0 **
	0	009	009	80
3	9–10	FC	FC	50 **
	9–10	FC	009	100
4	6–8	009	FC	0 **
	6–8	009	009	58

[a] Pin method of mounting decoys.
[b] 0-day-old females were killed within 4 hr of emergence.
[c] Males were 6–8 days old except for 9- to 10-day-old FC males used in test 3.
[d] $N = 10$ decoys per type, each tested with 10 males.
[e] $**P < 0.01$, referring to the difference within tests between members of a pair (Wilcoxon's signed-rank test).

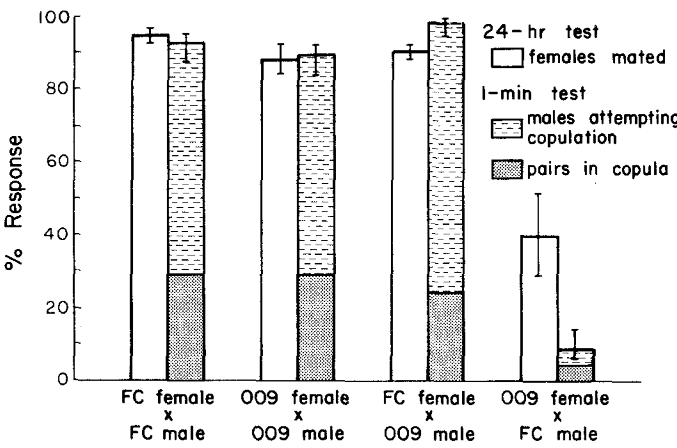

FIG. 2. Mating behaviors in intra- and interstrain crosses involving 009 and FC strains of the screwworm fly. Vertical bars represent standard errors of the mean in the 24-hr test and 95% binomial confidence intervals in the 1-min test.

portantly, the low mating frequency in the cross involving 009 females and FC males occurred because few males even attempted copulation (Figure 2, 1-min test). Data generated by 6- to 8- and 12- to 14-day-old FC insects are pooled in Figure 2. The two age groups produced homogeneous results, except that older FC females, whether paired with FC or 009 males, appeared more receptive to mating than younger ones.

DISCUSSION

The results confirmed earlier data implicating a female-produced sex pheromone in the release of male copulatory behavior in the screwworm fly (Mackley and Broce, 1981; Hammack, 1986) and more convincingly demonstrated that this stimulus is required for mating.

The contrasting relationships between female age and ability to stimulate copulation that were observed here in the 009 and FC strains, and previously in the Aricruz and DE-9 strains (Hammack, 1986), could be attributed to pheromone differences because whole females and extract of females both produced similar response patterns. Thus, the pheromone activity of females increased with age after emergence in FC and DE-9 strains but remained unchanged or declined in Aricruz and 009 strains. Interpretation of these differing ontological patterns was intially complicated by the strong responses obtained to newly emerged insects within the latter two strains. However, the interstrain comparisons done here indicate that these were due in the 009 strain to a high male responsiveness to pheromone and not to any difference between strains in the pheromone activity of newly emerged insects. The higher responsiveness of 009 males was demonstrable using pheromone from either strain and partly compensated for the lower sex pheromone activity of sexually mature females of the 009 as compared with the FC strain. This difference between mature females was demonstrable using males of either strain. The simplest explanation is that the strains differed quantitatively in the production and reception of either pheromone(s) or else in compounds interfering with responses to pheromone; however, qualitative differences were also certainly possible.

The pheromone activities obtained here and in previous behavioral tests (Hammack, 1986) correlate well with the nonpolar cuticular lipid profiles published for the screwworm fly by Pomonis and Mackley (1985). The profiles show no sex or strain differences at emergence and no strain differences among males; however, sexual dimorphism occurs after the age of 48 hr, as do differences among strains in the cuticular lipid patterns of females. These differences among females involve a shift toward lipids with shorter chain lengths as duration of laboratory colonization increases.

Interstrain variation with respect to aphrodisiac production by females as well as its reception by males is well documented in several *Drosophila* spp.

(Luyten, 1982; Jallon, 1984; Antony et al., 1985). In addition, like 009 and Aricruz strains of the screwworm fly, teneral *D. melanogaster* of both sexes or their extracts often stimulate as much male sexual activity as older females do, or even more (Cook and Cook, 1975; Jallon and Hotta, 1979; Jallon, 1984). However, even though its function is unclear, courtship of the young occurs to varying degrees in both wild and laboratory strains and is considered a normal component of *D. melanogaster* sexual behavior (McRobert and Tompkins, 1983).

The results here showed a positive correlation between the sex pheromone activity of screwworm females and their mating success when strain differences in the responsiveness of males to a pheromone stimulus were also taken into consideration. Thus, significant mating depression was only observed when females with the lower pheromone activity (009 strain) were paired with males showing the lower responsiveness to pheromone (FC strain). The failure of most FC males to even attempt copulation with 009 females indicates that the mating depression likely occurred because of an inappropriate pheromone stimulus. In addition, the sexual isolation was asymmetric in that no depression occurred in the reciprocal cross involving females of the more stimulatory strain (FC) paired with males of the more responsive one (009). Asymmetric mating patterns have received considerable attention from those interested in *Drosophila* speciation, but hypotheses as to how such asymmetries might reflect phylogeny vary (see Robertson, 1983, for discussion and references).

Richardson et al. (1982) postulated strong reproductive isolation among screwworm field populations, but subsequent cytogenetic and morphological studies have failed to uncover interspecific-level variation among strains from different habitats or localities (see Dev et al., 1986, and LaChance and Whitten, 1986, for discussion and references). Mating preference studies supported the latter, except that some mating isolation frequently occurred when newly colonized males were offered females from different long-colonized strains (C.J. Whitten, personal communication). The generality of such a pattern lends support to the hypothesis that selection during colonization led to the pheromone and mating differences observed here between FC and 009 strains.

The present tests showed no reduction in the pheromone activity of FC females within 24–48 hr after mating. Therefore, the standard method of maintaining screwworm colonies in mixed-sex groups until oviposition is stimulated would subject the monogamous female to repeated male copulatory attempts. This in turn might favor survival and reproduction of those females least stimulatory to males (Baumhover, 1965). Should mechanisms to rapidly deactivate pheromone not be available once high levels have been established, then such pressure might lead, during colonization, to selection for low pheromone activity from the time of emergence as seen here in females of 009 strain. While obviously speculative, this scenario envisions a heightened male responsiveness to pheromone to be secondary to selection for reduced female pheromone pro-

duction in an insect that nevertheless requires a chemical stimulus to release male copulatory behavior.

Even if the above scenario proves demonstrable during mass rearing of the screwworm, its consequences for population control via the sterile-insect-release method would still require clarification. The data provided no evidence that males of a long-colonized strain like the 009 would be unable to recognize or readily mate wild females; nevertheless, such males may be handicapped in their ability to recognize and terminate inappropriate interactions. For example, the observation that sexual dimorphism in pheromone activity was less marked in the 009 than the FC strain suggests potential difficulties with sex discrimination in the 009 strain. Indeed, this may account for the occurrence of copulatory behavior in all-male groups of many laboratory strains such as the 009 (Mackley and Broce, 1981), but not others such as the FC (Hammack, unpublished). Strikes were common among FC males, but homosexual interactions were terminated without the release of copulatory behavior. Colonization might also affect the ability of males to discriminate among species, but the role of the screwworm pheromone in species recognition remains to be investigated.

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REPEATABILITY OF PHEROMONE EMISSIONS FROM INDIVIDUAL FEMALE ERMINE MOTHS *Yponomeuta padellus* and *Yponomeuta rorellus*[1]

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Abstract—The repeatability of the composition of pheromone emitted by individual female moths (*Yponomeuta*) was determined by gas chromatographic analysis. An improved technique for repeated collections of airborne volatiles from the same moth was developed. The procedure did not harm the females and allowed precise quantification of 0.05 ng of pheromone components in 30-min collections. Individual females of *Y. padellus* ($N = 10$) and *Y. rorellus* ($N = 5$) were sampled six and eight days after emergence. *Y. padellus* released, on average, 4.8 ng/30 min of tetradecyl acetate, (*E*)-11-tetradecenyl acetate, (*Z*)-11-tetradecenyl acetate, hexadecyl acetate, (*Z*)-9-hexadecenyl acetate, and (*Z*)-11-hexadecenyl acetate in the average ratio 445:38:100:494:35:421. The amount of (*E*)-11-tetradecenyl acetate relative to (*Z*)-11-tetradecenyl acetate was less variable [coefficient of variation (CV) = 15%] than the relative amounts of the other components (CV = 46–61%). The repeatability of the relative amounts of compounds released by individual females was high for all components ($r = 0.82$–0.90). The average emission of tetradecyl acetate, tetradecanol, hexadecyl acetate, and hexadecanol by *Y. rorellus* was 8.3 ng/30 min (ratio 100:8:10:2). The repeatability was low for tetradecanol ($r = 0.51$) but higher for hexadecyl acetate ($r = 0.87$) and hexadecanol ($r = 0.89$). The low interindividual variance for the (*E*)-11-/(*Z*)-11-tetradecenyl acetate ratio in *Y. padellus* might be due to its importance for reproductive isolation, i.e., it is under strong selection pressure.

Key Words—Lepidoptera, Yponomeutidae, ermine moth, sex pheromone, airborne volatiles, repeatability, reproductive isolation, pheromone.

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INTRODUCTION

The pheromone production of individual moths has been shown to vary within populations of several species (Klun and Maini, 1979; Miller and Roelofs, 1980; Haynes et al., 1984; Löfstedt et al., 1985; for a review see Schlyter and Birgersson, 1986). There is both a quantitative variation in the absolute amount of pheromone produced and a variation in the relative amounts of different compounds in the pheromone blend. The total phenotypic variance in a population can be partitioned into variation within and between individuals. If the latter component constitutes a major proportion of the total variance, this indicates that there is a constant pheromone production in individual females. The constancy of a trait can be measured as its repeatability. This quantity sets an upper limit to the fraction of the variance of that trait which is heritable, i.e., to its heritability (Falconer, 1960). A population's potential for evolutionary change is limited by the amount of heritable variation present; hence knowledge about these quantities are necessary for our understanding of the evolutionary processes molding sex pheromone communication.

The present study was undertaken to determine the day-to-day constancy of pheromone blend emission by individual female *Yponomeuta padellus* and *Y. rorellus*. The pheromone gland of *Y. padellus* contains seven acetates of which at least (Z)-9-tetradecenyl acetate (Z9-14:OAc), (*E*)-11-tetradecenyl acetate (*E*11-14:OAc), (Z)-11-tetradecenyl acetate (Z11-14:OAc), and (Z)-11-hexadecenyl acetate (Z11-16:OAc) are important for the attraction of males (Löfstedt and Van Der Pers, 1985; Löfstedt, unpublished). The pheromone gland of *Y. rorellus* contains no unsaturated acetates or alcohols and high attractivity is achieved with tetradecyl acetate as a single component (Löfstedt et al., 1986). The repeatability of pheromone emission in these species was determined by repeated collections of airborne volatiles from individual calling females.

METHODS AND MATERIALS

Insects. Insects were collected from the field as pupae or late-instar larvae. *Y. padellus* originated from several places in the province of Skåne, southern Sweden; *Y. rorellus* was collected in the Netherlands, close to Leiden, and mailed to our laboratory as pupae. The sexes were separated and the females emerged at 23°C under a reversed light–dark 16:8 photoperiod. The adults were maintained under the same conditions and fed a 5% sucrose water solution. Most females displayed their pheromone glands in a typical calling posture at the beginning of the photophase four to five days after emergence.

Collection of Airborne Volatiles from Calling Females. To allow the repeated collection of pheromone from individual females, it was essential to

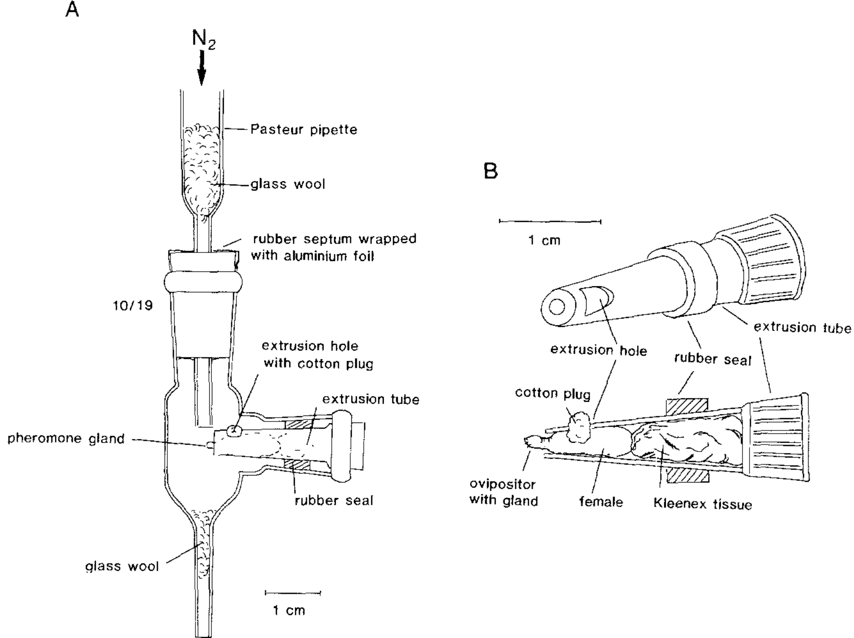

FIG. 1. (A) Device for collection of airborne volatiles from female moths, modified from Baker et al. (1981). A stream of purified nitrogen (ca. 50 ml/min) is directed to the exposed pheromone gland with a Pasteur pipet. The linear velocity at the outlet of the pipet is approximately 0.6 m/sec. (B) Expanded view of the extrusion tube with an inserted female.

employ a technique that left the females alive and in good condition after sampling. A modified version of the collection device developed by Baker et al. (1981) fulfilled this requirement (Figure 1). A calling female was placed in the extrusion tube (Figure 1B) made from a disposable plastic pipet tip (Labsystems Oy, Finland). A small piece of Kleenex tissue was inserted behind the head of the female. Then a small ball of paper was pressed into the extrusion hole, gently squeezing the female abdomen, so that the ovipositor with the sex pheromone gland was protuded. The extrusion tube with the female was placed in the holder and nitrogen, filtered through a molecular sieve and glass wool, was introduced. The carrier gas (50 ml/min) was directed towards the gland with a Pasteur pipet. The linear velocity at the outlet of the pipet, 1–2 mm from the gland, was 0.6 m/sec. Airborne volatiles were collected on glass wool during 30 min and subsequently eluted with 75 μl of hexane. The solution was condensed to 5–10 μl with a gentle stream of nitrogen in a pointed 4-cm-long × 0.4-mm-ID glass vial. All glass ware was rinsed with distilled acetone and conditioned in an oven at 300°C for at least 2 hr before use.

After collection, the female moth was immediately released in a 250-ml plastic cup, covered with a nylon screen, and kept at the same conditions as described above. The second collection was obtained two days later. Only calling females were used for the collections.

Determination of Method Error. Small pieces of rubber (3–5 mm^3) were cut out of red rubber septa (A.H. Thomas Company, Philadelphia, Pennsylvania). The rubber pieces were extracted by hexane and acetone and dried before impregnation with synthetic pheromone components. For impregnation, 10 rubber pieces were placed in a vial to absorb 100 μl of hexane containing 380 μg 14:OAc, 4 μg *Z*9–14:OAc, 45 μg *E*11–14:OAc, 100 μg *Z*11–14:OAc, 3820 μg 16:OAc, 307 μg *Z*9–16:OAc, and 2234 μg *Z*11–16:OAc. The dispensers were conditioned 2 hr at room temperature before use. Formulated in this way, one dispenser released a blend of pheromone components similar, in quantity and proportions, to the emission of an average *Y. padellus* female as determined from 30-min collections of airborne volatiles in the above-mentioned device. The overall method error was estimated by analysis of 10 airborne collections from synthetic dispensers. The precision of the GC procedure was determined by repetitive analysis of a standard solution containing one "average female *Y. padellus*-equivalent" per 3 μl hexane.

Gas-Chromatography. The concentrated solution obtained from washing of the glass wool was injected splitless (split valve opened 0.5 min after injection) on a Hewlett Packard 5880 capillary gas chromatograph equipped with a flame ionization detector. The injector temperature was 250°C and the detector temperature 275°C. The column temperature was maintained at 80°C for 2 min following injection and then programmed at 10°C/min to 230°C. A 30-m × 0.25-mm-ID Supelcowax 10 fused silica column (Supelco Inc.) was used for the separations, and hydrogen (H_2) carrier gas was supplied at 40 cm/sec linear velocity. Peak heights were used for quantification, as these provided more precise measures of standard solutions than peak areas did.

Statistical Analysis. The total phenotypic variance in the relative amounts of pheromone components (*Z*11–14:OAc = 100 by definition) was partitioned into variance within individuals and variance between individuals using a one-way analysis of variance. The repeatability (r) was calculated as r = variance between individuals/(variance between individuals + variance within individuals) (Falconer, 1960).

RESULTS

Method Error. Gas chromatographic analysis of airborne volatiles from a rubber dispenser ($N = 10$) revealed the precision of the overall method. The mean relative amounts of the respective compounds and their coefficients of variation (within parentheses) were: 14:OAc, 350.7 (2.3%); *E*11–14:OAc,

45.5 (2.8%); Z11-14:OAc, 100 (0, by definition); 16:OAc, 360.1 (7.6%); Z9-16:OAc, 50.5 (8.3%); and Z11-16:OAc, 287.3 (6.9%). The amount of Z9-14:OAc was below the limit of detection. The coefficients of variation for the 16-carbon acetates were higher than for the 14-carbon acetates. This is probably because all amounts are relative to a 14-carbon acetate. The variance in a ratio usually increases with differences in volatility between the two compounds, because of irreproducible discrimination in the split/splitless gas-chromatographic injector. Repeated analysis of a standard mixture on the GC confirmed this interpretation. The coefficients of variation of the relative amounts were 14:OAc, 2.6%; *E*11-14:OAc, 2.3%; Z11-14:OAc (0, by definition); 16:OAc, 4.8%; Z9-16:OAc, 6.3%; and Z11-16:OAc, 4.8%. Thus gas-chromatographic parameters explain most of the difference in precision between 14-carbon and 16- carbon acetates.

Repeated Airborne Collections from Individual Y. padellus *and* Y. rorellus

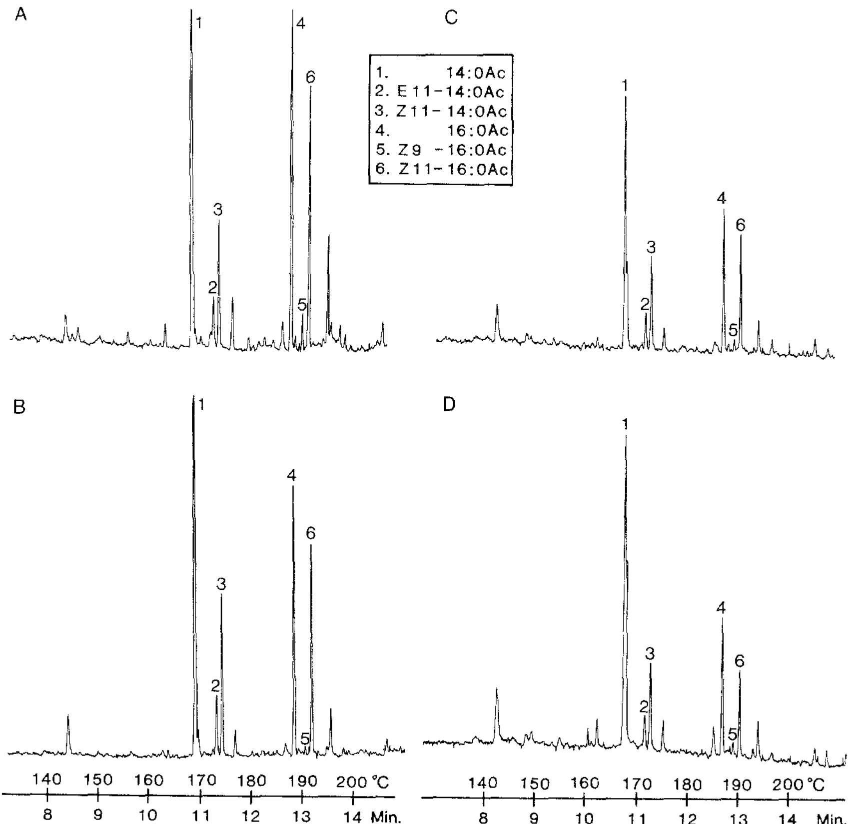

FIG. 2. Gas chromatograms of airborne volatiles collected from the same female *Y. padellus* at her sixth (A), eighth (B), tenth (C), and twelfth (D) day after emergence.

TABLE 1. PHEROMONE EMISSION FROM INDIVIDUAL *Yponomeuta padellus* FEMALES

Female	Age (days)	Amount relative to Z11-14:OAc						Total amount (ng/30 min)
		14:OAc	*E*11-14:OAc	*Z*11-14:OAc[a]	16:OAc	*Z*9-16:OAc	*Z*11-16:OAc	
I	6	556	33.0	100.0	517	33.3	367	8.1
	8	397	35.1	100.0	105	—	92	4.7
	10	474	38.8	100.0	655	49.0	414	7.3
	12	449	32.8	100.0	318	26.2	174	4.6
II	6	315	31.5	100.0	275	29.0	202	0.7
	8	304	36.5	100.0	169	10.0	130	1.3
	10	279	39.1	100.0	159	14.9	124	1.6
	12	356	35.7	100.0	160	13.1	101	2.4
III	6	576	40.5	100.0	501	65.8	347	8.4
	8	589	42.9	100.0	573	30.6	435	16.7
IV	6	661	41.1	100.0	193	25.0	120	3.9
	8	484	40.8	100.0	107	—	86	4.0
V	6	141	30.0	100.0	310	18.0	426	4.1
	8	188	33.0	100.0	304	—	204	1.0

VI	6	787	48.9	100.0	966	70.2	343	4.3
	8	541	52.9	100.0	931	86.3	355	4.6
VII	6	342	38.5	100.0	389	18.8	349	6.9
	8	270	35.0	100.0	350	35.0	220	0.8
VIII	6	349	44.4	100.0	691	53.3	560	4.1
	8	371	39.2	100.0	414	58.5	296	3.7
IX	6	238	40.0	100.0	792	21.6	638	6.3
	8	414	34.0	100.0	1075	45.5	573	5.0
X	6	502	35.4	100.0	304	10.4	123	8.8
	8	480	27.3	100.0	186	15.9	240	2.5
$\bar{X} \pm SD$		446 ± 203	38.3 ± 5.9	100.0	494 ± 252	34.5 ± 21.1	421 ± 222	4.8 ± 3.5
CV (%)		46	15	—	51	61	53	
Repeatability (r)		0.90	0.88	—	0.91	0.83	0.82	

[a] Equals 100 by definition.

Females. Volatiles collected from *Y. padellus* females generally contained significant amounts of 14:OAc, *E*11–14:OAc, *Z*11–14:OAc, 16:OAc, *Z*9–16:OAc, and *Z*11–16:OAc (Figure 2). However, *Z*9–14:OAc was usually below the limit of detection. The total release rate varied between 0.7 and 16.7 ng/30 min (Table 1). However, some of this variation in absolute amounts might be due to the handling of the sample, as no internal standard was used. The quantitatively dominating constituents were 14:OAc, 16:OAc, and *Z*11-16:OAc. Upon comparison of the coefficients of variation for the different components, it appeared that the amount of *E*11–14:OAc relative to *Z*11–14:OAc was more stable (CV = 15%) than the amounts of the other compounds (CV = 46–61%) (Table 1). Most of the variation was due to between-female variation, whereas the individual females produced rather constant ratios (Table 1, Figure 2). For instance female II released low relative amounts of the 16-acetates at all four sampling occasions compared to the population means. Female IV released a high relative amount of 14:OAc, while the 16-acetates where substantially below the respective mean. Female V released low amounts of the 14-acetates. Both females VI and IX released high amounts of 16:OAc, but differed with respect to *Z*11–16:OAc and *E*11–14:OAc; female IX released a high relative amount of *Z*11–16:OAc, whereas VI was characterized by a high relative amount of *E*11–14:OAc.

The positive correlations between the composition of the pheromone emitted by individual females on their sixth and eighth day, respectively, also show the production of female-specific blends (Figure 3). The repeatability varied between 0.82 and 0.90 for the five compounds (Table 2). In the airborne collections from *Y. rorellus*, 14:OAc was the dominating constituent, as was reported elsewhere (Löfstedt et al., 1986). The total amount of 14:OAc, 14:OH, 16:OAc, and 16:OH released varied between 2.7 and 16.0 ng/30 min (Table 2). The amounts of 14:OH, 16:OAc, and 16:OH relative to 14:OAc (= 100) was 10 or lower, and the coefficients of variation were 41–50%. Only a small number of insects were available for the study, but based on five individuals, the repeatability was high for the proportions of 16:OAc and 16:OH and lower for 14:OH.

DISCUSSION

The determination of the repeatability of pheromone production is an important step in a research program on the heritability of pheromone production. The technique used by us for collection of airborne volatiles from calling females allowed two or more collections from the same female. The females were usually in such a good shape after the collections that it should be possible to mate them afterwards. Thus, the procedure is an attractive alternative to the technique used by Du et al. (1984) in their selection for abberant pheromone production in the red-banded leafroller moth. They picked only a part of the

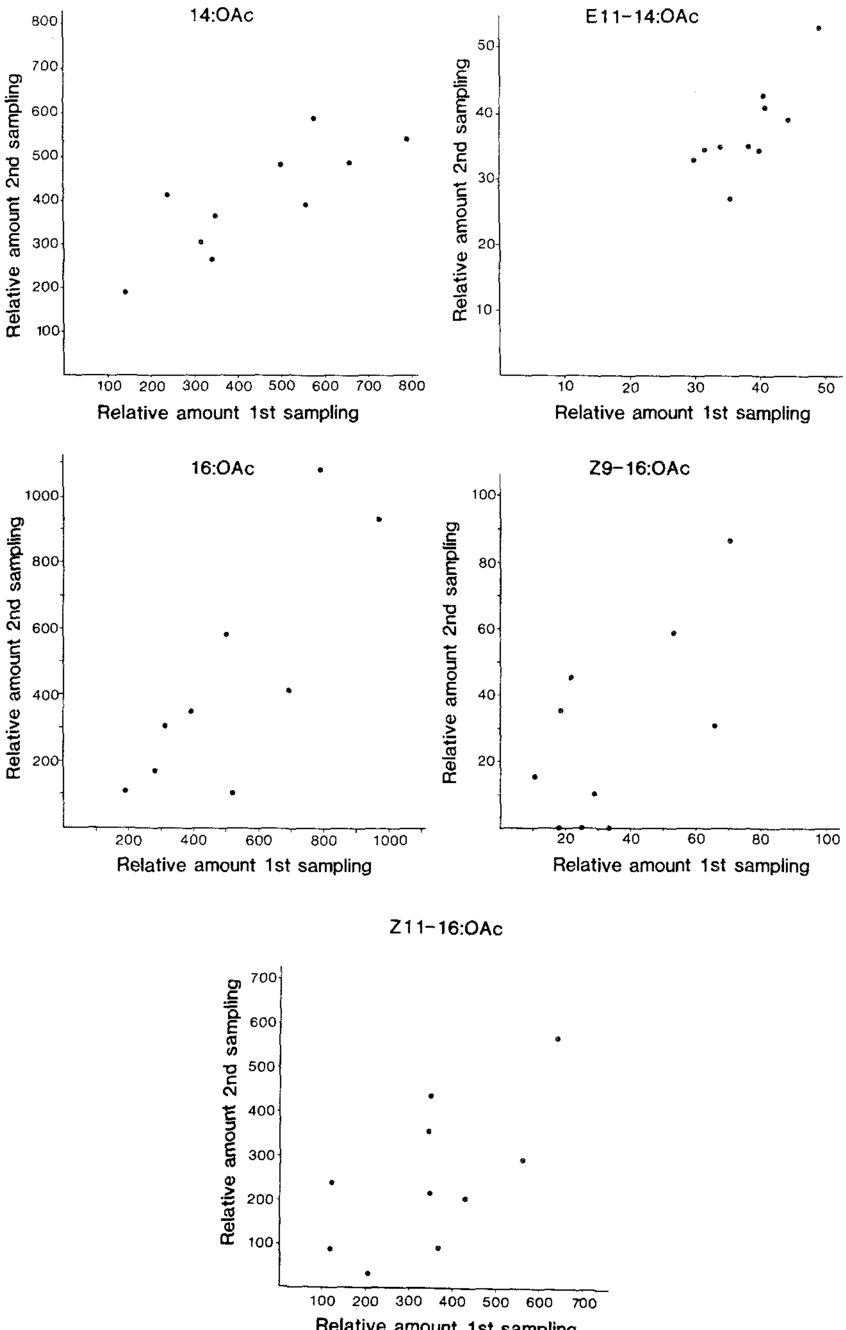

FIG. 3. Relative amount of five pheromone components (Z11-14:OAc = 100) in first and second airborne collection from 10 individual *Y. padellus* females.

TABLE 2. PHEROMONE EMISSION FROM INDIVIDUAL *Yponomeuta rorellus* FEMALES

Female	Age (days)	Amount relative to Z11-14:OAc				Total amount (ng/30 min)
		14:OAc[a]	14:OH	16:OAc	16:OH	
I	6	100	6.6	7.7	1.9	2.7
	8	100	3.3	7.4	1.3	6.5
II	6	100	4.3	5.8	1.0	11.8
	8	100	3.8	7.2	1.2	12.4
III	6	100	6.8	6.2	1.7	6.1
	8	100	4.6	7.6	1.6	6.2
IV	6	100	7.6	14.7	2.6	8.6
	8	100	5.5	17.7	4.3	8.1
V	6	100	12.6	17.1	3.9	4.1
	8	100	5.5	10.2	3.5	16.0
$\bar{X} \pm$ SD		100	7.6 ± 3.1	10.3 ± 5.2	2.2 ± 1.1	8.3 ± 4.1
CV (%)			41	50	50	
Repeatability (r)			0.51	0.87	0.89	

[a] Equals 100 by definition.

pheromone gland for analysis to allow subsequent mating of the female. Such a technique is only applicable to high-producing moth species with easily accesible glands, whereas collection of airborne volatiles should be feasible with any moth species releasing reasonable amounts of pheromone. We were successful with female moths releasing less than 1 ng/30 min.

Measurements of the heritability of pheromone blends are rare thus far. Collins and Cardé (1985) estimated the heritability of the ratio between (Z, E)- and (Z, Z)-hexadecadienyl acetate isomers in the pink bollworm moth to be 0.35 from a breeding experiment. Working with *Argyrotaenia velutinana*, Roelofs et al. (1987) found a similar heritability for the Z11- to E11-14:OAc ratio when only females were selected. However, mating of selected females with selected males yielded a heritability twice as high (0.85), because males also contribute to the pheromone production of their daughters. This higher figure agrees with the level of repeatability observed in *Yponomeuta*. Thus *A. velutinana* seems to realize the maximum heritability that could be expected in our moths.

Ratios between Z and E isomers are generally believed to be more important for reproductive isolation than other ratios in the pheromone blend (see for instance Cardé and Baker, 1984). The results of our study seem to support this idea. The interindividual coefficient of variation for the Z/E ratio was lower

than for any of the other ratios measured. In contrast, the repeatability was equally high for all of the compounds. Thus an individual female seems to be able to control all of the ratios, not only the one between the geometric isomers, but selection for reproductive isolation might have reduced the interindividual variance in the *Z*/*E* ratio.

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COMPARISON OF ELECTROANTENNOGRAMS FROM FEMALE AND MALE CABBAGE LOOPER MOTHS (*Trichoplusia ni*) OF DIFFERENT AGES AND FOR VARIOUS PHEROMONE CONCENTRATIONS

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Abstract—The electroantennogram (EAG) responses of both male and female *Trichoplusia ni* to two components of the female-released sex pheromone were determined over a range of concentrations of chemical and age of insect. The EAG was at a peak at a dose of 100 μg for both dodecyl acetate and (*Z*)-7-dodecenyl acetate (*Z*7–12:OAc) for both sexes. The EAG responses varied with age of the insect. Responsiveness to both components was at a maximum at three days postemergence for males followed by a fairly rapid senescence, and for females it peaked at three days followed by a rapid senescence for *Z*7–12:OAc but not for dodcecyl acetate.

Key Words—*Trichoplusia ni*, Lepidoptera, Noctuidae, (*Z*)-7-dodecenyl acetate, dodecyl acetate, pheromone, electroantennogram, age, maturation.

INTRODUCTION

Electroantennograms (EAGs) have frequently been used to show that both male and female insects can perceive many diverse odors through their antennae (Boeckh et al., 1965; Schneider, 1962; Schneider and Steinbrecht, 1968) including, in many Lepidoptera, male scent-brush secretions (Light and Birch, 1979; Seabrook et al., 1979). However, in the great majority of moths studied

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to date, only the male antennae give EAG responses to female-produced sex pheromones (Light and Birch, 1979). In at least some of these species, the female antennae lack the sensilla trichodea associated with perception of the female-produced sex pheromone by males (Boeckh et al., 1965).

In contrast, in a few lepidopteran species such as the spruce budworm *Choristoneura fumiferana* and the cabbage looper *Trichoplusia ni*, in which there is little antennal dimorphism (Albert and Seabrook, 1973; Jefferson et al., 1970), the sex pheromone emitted by the female is detected by females of the same species (Palaniswamy and Seabrook, 1978; Ross et al., 1979; Grant, 1970; Light and Birch, 1979) and can influence the behavior of conspecific females (Palaniswamy and Seabrook, 1978; Birch, 1977). The EAGs from female spruce budworm antennae from moths of different ages and for various pheromone concentrations have been studied (Ross et al., 1979).

The major component of the pheromone produced by female cabbage loopers was first identified as (Z)-7-dodecenyl aceate (Z7–12 : OAc) (Berger, 1966). A second component, dodecyl acetate (12 : OAc) comprises 9.6% by weight of the total pheromone extracted from glands (Bjostad et al., 1980). In the present study, the effect of age and the concentration of the two major components of the pheromone on EAG response in male and female *T. ni* was investigated.

METHODS AND MATERIALS

A colony of *Trichoplusia ni* was maintained in the laboratory using a method similar to that described by Shorey and Gaston (1964). All stages were maintained at a temperature of 25°C ± 2°C. They were sexed as pupae and thereafter kept separately by sex.

The EAG recording system used in this study was similar to that described by Roelofs and Comeau (1971) with the exception that a unity gain voltage follower was used in place of the 100 × amplifer. The recording electrode was made from a Pasteur pipet containing a silver–silver chloride wire electrode and filled with Pringle's Ringer (containing 156 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, and 22 mM glucose at pH 6.8). The preparation was grounded through a third electrode to a common ground via the saline pool in which the proximal end of the antenna was placed.

Antennae were exposed to test compounds in 1-sec puffs. The compounds were carried in ultra-zero-grade bottled air (Matheson Co.) at a flow rate of 150 ml/min. The odor-delivery system was similar to that described by Albert et al. (1974). An odor-evacuation system was set up immediately adjacent to the preparation in order to prevent a possible build-up of pheromone near the antenna. This provided a flow of clean air at a velocity of approximately 1 cm/sec over the preparation between releases of test chemicals.

The 12:OAc and Z7–12:OAc (both 99+ % pure by GLC) were dissolved in ether at concentrations ranging from 10^{-5} to 10^{+3} μg per 50 μl of solvent. Fifty-microliter aliquots of the test solution were applied to 1.5 × 1.5-cm pieces of Whatman No. 1 filter paper and evaporated to dryness at room temperature. The treated filter papers were placed in glass tubes (6 mm inside diameter × 65 mm long), which were then wrapped in Parafilm until used later that same day. The control stimulus was a similar piece of filter paper, treated with 50 μl of ether and evaporated to dryness. Each stimulus source was used no more than three times.

Each antennal preparation was initially exposed to a control stimulus. This was followed by an experimental stimulus after a 3-min recovery period. It is known that in *T. ni* essentially complete recovery of antennal sensitivity to pheromone occurs within 30–60 sec at the flow rates and concentrations used (Payne et al., 1970; Mayer, 1973; Light and Birch, 1979). EAG responses to both control and pheromone stimuli were photographed directly from the oscilloscope screen on Polaroid film, and amplitude measurements were made from the resulting prints. The stimulus EAG peak voltage was calculated by subtraction of the control voltage from the voltage generated by a given compound. As there was no apparent reduction in the EAG voltage during the 1-sec test, a more sophisticated treatment of the data (Roelofs and Comeau, 1971) was not necessary.

The EAG saturation concentration, the concentration of odorant molecules beyond which there is no further increase in EAG amplitude (Boeckh, 1969), for each sex was found from a dose–response curve obtained from exposing antennae from 3-day-old insects to one of a series of concentrations for each chemical and determining the corrected stimulus EAG voltage. By this age, male *T. ni* are known to respond maximally to sex pheromone, and the percentage of males mating has reached a peak (Shorey et al., 1968b). The saturation concentration was then used as the stimulus for antennae from moths of both sexes ranging in age from 1 day postemergence to 7 or 8 days postemergence. Three replicates were carried out for each test.

RESULTS

The EAG dose–response curves for the tested compounds for both males and females are shown in Figures 1 and 2. The saturation concentration was reached at a dose of 100 μg in both sexes and for both pheromone components. At this saturation concentration, the response of the male antenna to the principal pheromone component, Z7–12:OAc, was more than three times that of the female antenna. At lower doses, the difference in magnitude of response between male and female antennae, although reduced (Figure 1), remained significant ($P < 0.01$, Wilcoxon signed-ranks test for matched pairs). As the con-

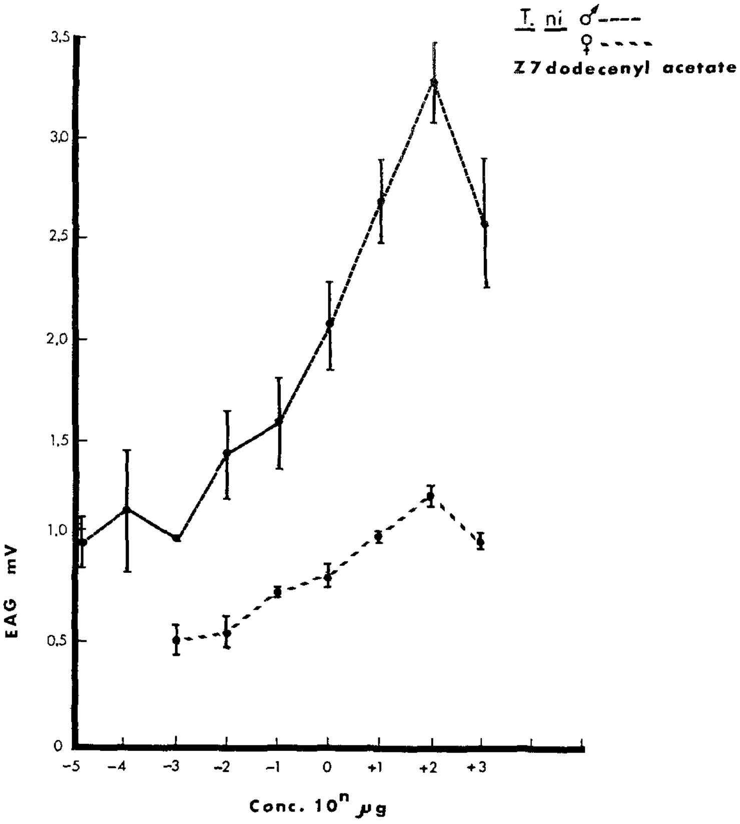

FIG. 1. Dose-response curves for 3-day-old male and female *T. ni* exposed to various concentrations of (Z)-7-dodecenyl acetate. Means are ±SEM. At doses between 0.1 and 100 μg, the regression for male response is $\hat{Y} = 2.14 + 0.570X$, $r^2 = 0.832$ and for female the regression is $\hat{Y} = 0.868 + 0.164X$, $r^2 = 0.878$.

centration of Z7–12:OAc was increased, the increase in the mean amplitude of the EAG became significant at 0.1 μg for both males and females [$P < 0.05$, Student-Newman-Keuls (SNK) test for comparison of differences among means]. In both sexes the EAG amplitude increased as concentration was increased from 0.1 to 100 μg ($r^2 = 0.832$ for male response, $r^2 = 0.878$ for female response).

In the dose-response curves for 12:OAc, male and female sensitivities are most similar (1.4 mV vs. 1.3 mV) at or near the saturation concentration, but at lower doses, females are much less sensitive than males ($P < 0.025$, Wilcoxon signed-ranks test for matched pairs) (Figure 2).

When the EAG responses of the female antennae to each component are compared, the two curves are quite similar in shape, and the peak EAG ampli-

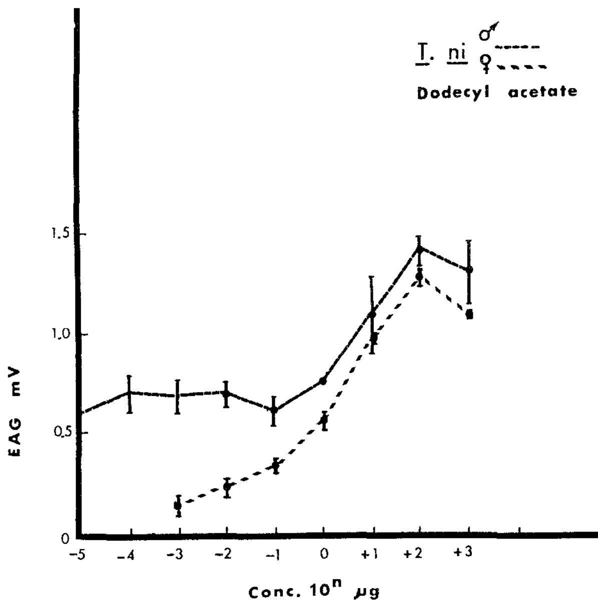

FIG. 2. Dose-response curves for 3-day-old male and female *T. ni* exposed to various concentrations of dodecyl aceate. Means are ±SEM. At doses between 0.1 and 100 μg, the regression for male response is $\hat{Y} = 0.842 + 0.274X$, $r^2 = 0.775$, and for females the regression is $\hat{Y} = 0.628 + 0.324X$, $r^2 = 0.973$.

tudes are approximately the same (1.2 mV and 1.3 mV), although at low stimulus concentrations, the female appears to be less sensitive to 12:OAc than to Z7-12:OAc.

In contrast, over all concentration ranges, the amplitude of EAGs from male antennae is two or three times larger for Z7-12:OAc than for 12:OAc. The changes in the mean EAG amplitudes of male responses to increasing doses of dodecyl acetate were not significantly different at concentrations below 10 μg ($P < 0.05$, SNK test). For females, significant increases in mean EAG responses began above 0.01 μg of 12:OAc.

The sensitivity of both male and female antennae to both pheromone components differs with age (Figures 3 and 4). In male antennae, at three days after emergence, the magnitude of response to both compounds reaches a peak significantly higher than the magnitude on day 1 ($P < 0.05$, SNK test). In females the response to Z7-12:OAc also reaches a peak on day 3 which is significantly higher than the response on either the preceding or the following day ($P < 0.05$, SNK test), but there does not appear to be a clear peak in sensitivity to 12:OAc related to age. The temporal pattern of response obtained for the maturation and senescence of the response to both chemicals is similar in males, but not in females. Senescence of the response after day 3 was fairly rapid until

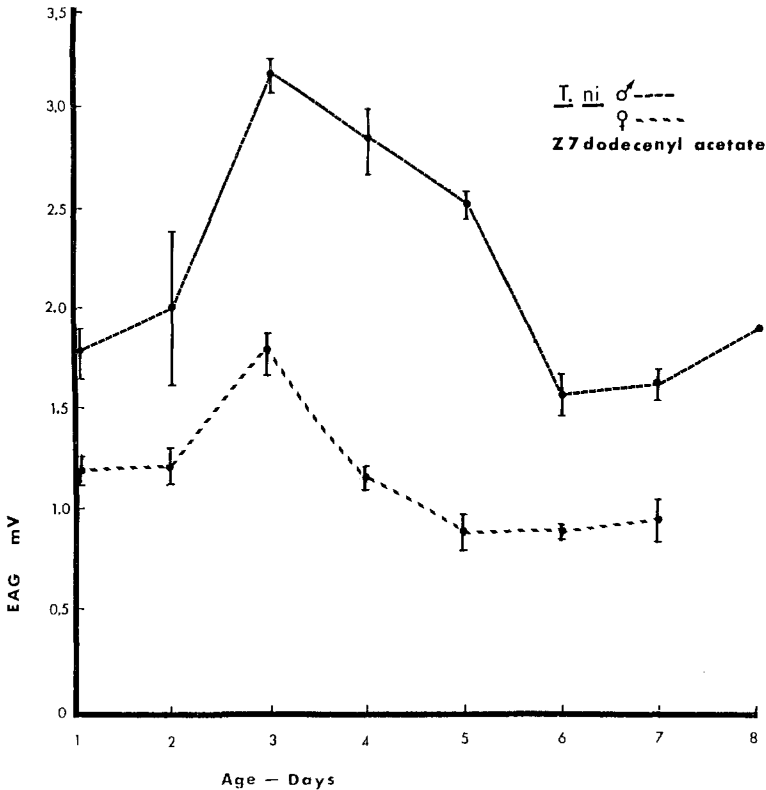

FIG. 3. Effects of age on the antennal response of male and female *T. ni* to a 100-μg source of (Z)-7-dodecenyl acetate. Means are ±SEM.

day 5 or 6, except for females to 12:OAc. The EAG voltage dropped to half of its peak value by six days postemergence for the response of males to both components and the response of females to Z7–12:OAc. Female responses to 12:OAc senesced more slowly. At ages of 6 days or over, there was no further senescence of the response.

DISCUSSION

Many aspects of the responses, both behavioral and electrophysiological, of male *Trichoplusia ni* to pheromones have been extensively studied and are probably better known than for any other species of moth. However, the responses of the females to their own sex pheromones have received much less attention. The shape of the dose–response curve obtained over the range of 10^{-3} to 10^{+2} μg of Z7–12:OAc is similar to that obtained by Mayer (1973) (Figure

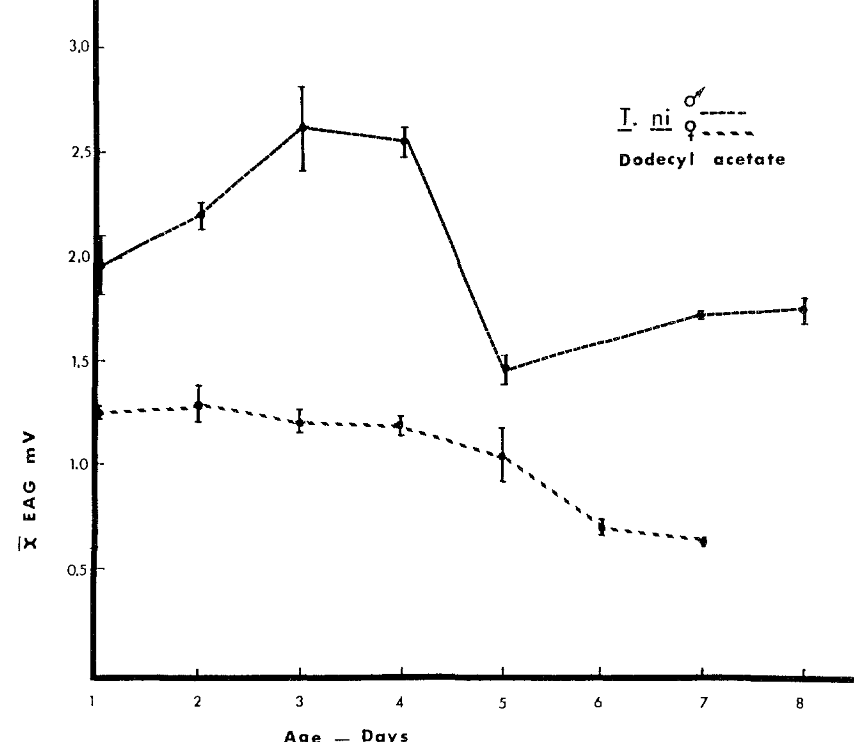

FIG. 4. Effects of age on the antennal response of male and female moths to a 100-μg source of dodecyl acetate. Means are ±SEM.

1). The dose-response curve for male *T. ni* to Z7-12:OAc reported by Alford and Hammond (1982) covered a wider range of doses and showed a similar trend, except that they did not detect a decrease in EAG amplitude at concentrations higher than 10^{+2}. On the other hand, Payne et al. (1970) and Grant (1970) did detect a reduction in male EAG amplitude at higher concentrations of Z7-12:OAc.

Mayer (1973) estimated that the EAG in response to a stimulus of dodecyl acetate indicated a sensitivity roughly 500 times less than that to looplure. The data presented in Figure 2 confirm that the EAG response of male *T. ni* to 12:OAc is lower in amplitude at all concentrations tested than the EAG produced by Z7-12:OAc by about the same amount found by Grant (1970).

The trend of the dose-response curve would indicate that male *T. ni* moths should be unable to detect a change in concentration at low concentrations of 12:OAc. This does not appear to be true of females or of Z7-12:OAc for either males or females. At a level of stimulation close to the peak of the dose-re-

sponse curve, both males and females could detect a concentration gradient, since in this range responses change with a change in stimulus concentration (Figures 1 and 2). Females are known to respond to pheromone lures (both Z7–12:OAc and virgin females) in this species (Birch, 1977), and EAG recordings have been obtained previously from female antennae exposed to Z7–12:OAc (Grant, 1970; Light and Birch, 1979). The amplitude of the female EAG response to a 2-μg standard dose of Z7–12:OAc has been found to be only 25% that of the male (Light and Birch, 1979). Although we did not use a stimulus of 2 μg, at a dose of 1 μg of Z7–12:OAc the female response was approximately 40% of the male EAG (0.82 ± 0.06 mV vs. 2.08 ± 0.22 mV), and the curves for male and female had the same general shape (Figure 1).

In the spruce budworm, the pheromone dose–response curves for males and females also have the same shape, although these curves are more obviously sigmoid (Ross et al., 1979) than those of *T. ni* to Z7–12:OAc. However, in the budworm, the difference in amplitude of the EAG between the sexes is less pronounced and disappears entirely at concentrations between 10^{-5} and 10^{-1} μg of pheromone (Ross et al., 1979).

Antennal response of *T. ni* to both pheromone components changes with the age of both females and males. In males the response to both compounds increases initially to reach a peak at three days after eclosion, remains at a high level for two or three days, then declines sharply to levels below those found on day 1 before leveling off (Figures 3 and 4). The pattern between age and mating frequency of males shows some similarities to this pattern in that mating frequency peaks between three and four days of age, and then begins to decline slightly (Shorey et al., 1968a). Shorey et al. (1968b) found that the age at which 50% of males responded behaviorally to pheromone at a dose of 10^{-3} female equivalents was 2 days, approximately the time sexual maturity was reached, but response levels remained high thereafter (Shorey and Gaston, 1964; Payne et al., 1970). Payne et al. (1970) also found that newly emerged males responded behaviorally only to high concentrations of Z7–12:OAc and responsiveness to lower concentration increases rapidly through the age of 2.5 days.

The pattern of male response over time is quite different from that reported for the spruce budworm, which showed only small and irregular changes with age (Ross et al., 1979). It bears some resemblance to the pattern of maturation and senescence in EAGs from male *Pseudaletia unipunctata* in response to the male-produced scent brush odors (Seabrook et al., 1979), but the changes in amplitude are less dramatic.

The response from female antennae to the two components over time showed different patterns. Sensitivity to Z7–12:OAc peaked at the age of 3 days, as it did in males (Figure 3), followed by a decline. The shape of the curve and the timing of the peak are quite similar to the relationship found between age and mating frequency (Shorey et al., 1968a). The peak is close to

the time of egg maturation (Shorey et al., 1968a). A similar pattern of maturation followed by senescence in female response to pheromone has been reported in spruce budworm (Ross et al., 1979) and in *P. unipunctata* (Seabrook et al., 1979).

In contrast, the sensitivity of the female antenna to 12:OAc appeared to remain fairly constant for several days, after which it began to decline slowly (Figure 4).

This difference in the pattern of maturation and senescence of the EAG response to the two pheromone components implies that the cells sensitive to Z7-12:OAc and to 12:OAc mature and senesce at different rates. This could be due to an increase followed by a decrease in the number of available acceptors, which in turn could be under hormonal control, as has been suggested for the spruce budworm (Palaniswamy and Seabrook, 1985). Such a hormone could act on different cells differentially. If the argument that at saturation concentration all available acceptor sites are occupied is correct, and if the two pheromone components are occupying the same acceptors, then one would expect the EAG response to each component to mature and senesce at the same rate over time. Since this does not happen in the case of the female EAGs, we assume that different acceptors are used to bind Z7-12:OAc and 12:OAc.

For both components of the pheromone, the sensitivity of females at all ages appears to be considerably less than that of males. This was also true in the spruce budworm at high concentration (Ross et al., 1979). In the case of the budworm, it was suggested to be due to the fact that female antennae have one third to one half the number of sensilla trichodea found on male antennae (Albert and Seabrook, 1973). In males, the sensilla trichodea are known to be the pheromone receptors (Albert et al., 1974). This explanation may also hold for *T. ni*, as there is some sexual dimorphism between male and female antennae based on the relative numbers of the sensillae trichodea (Jefferson et al., 1970). However, there also is a difference in the ability of the antennae to enzymatically degrade Z7-12:OAc, with male antennae being capable of degrading the pheromone component to an alcohol twice as fast as the female (Mayer, 1975). It is possible that this could also contribute to the observed response differences.

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MONOTERPENE METABOLISM IN FEMALE MOUNTAIN PINE BEETLES, *Dendroctonus ponderosae* HOPKINS,[1] ATTACKING PONDEROSA PINE[2]

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Abstract—Abdominal volatiles of female mountain pine beetles, *Dendroctonus ponderosae* Hopkins, fed in ponderosa pine, *Pinus ponderosa* Dougl. ex Laws, and in lodgepole pine, *P. contorta* var. *latifolia* Engelmann, were analyzed by gas chromatography and coupled gas chromatography-mass spectrometry and were found to comprise host oleoresin components and beetle-produced alliylic alcohols, aldehydes, and ketones derived from host monoterpenes. Neither *exo*- and *endo*-brevicomin nor frontalin were detected. Three metabolic pathways are proposed to account for the distribution of beetle-produced monoterpene alcohols. The first pathway involves hydroxylation of monoterpene substrates on allylic methyl groups which are *E* to a methylene or vinyl group. This oxidation pathway is indiscriminate with respect to substrate and probably functions to detoxify monoterpenes. A second pathway, which hydroxylates the *endo*-cyclic methylene *E* to a vinyl methyl group of bicyclic monoterpenes to give almost exclusively the *trans* alcohol, is hypothesized to be involved in pheromone production. A third detoxification pathway involves anti-Markovnikov addition of water to the *exo*-cyclic double bond of β-phellandrene to give predominantly *trans*-2-p-menthen-7-ol.

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Key Words—*Dendroctonus ponderosae*, mountain pine beetle, Coleoptera, Scolytidae, monoterpene metabolism, monoterpene alcohols, pheromones, allylic hydroxylation, anti-Markovnikov hydration, *Pinus ponderosa*.

INTRODUCTION

During the early phases of attack by bark beetles, living pine trees exude oleoresin in which the predominant volatile components are monoterpenes. To survive in galleries filled with volatile, toxic monoterpenes (Smith, 1965, 1966), these beetles must possess effective mechanisms for monoterpene metabolism. The primary products of monoterpene metabolism in pine bark beetles, especially in the genera *Ips* and *Dendroctonus*, are the corresponding allylic alcohols which may undergo rearrangement, hydrogenation, or further oxidation (White et al., 1980; Francke and Vité, 1983). It was initially proposed that these insects possessed enzymatic systems which detoxify monoterpenes by nonspecific oxidation of allylic positions (Hughes, 1973a; Renwick and Hughes, 1975). There is, however, evidence that if the oxidation products are pheromones, the process can be highly stereo- and enantioselective (Renwick et al., 1976a; Fish et al., 1979; Klimetzek and Francke, 1980; Byers, 1983).

The mountain pine beetle, *Dendroctonus ponderosae* Hopkins, successfully colonizes and kills millions of lodgepole pines (*Pinus contorta* var. *latifolia* Engelmann) and ponderosa pines (*Pinus ponderosa* Dougl. ex Laws.) each year in western North America. Such success is evidently due in part to the ability of the pioneer female beetles to metabolize host monoterpenes efficiently without discrimination and to produce aggregation pheromones which induce mass attack.

The volatile constituents of the oleoresin of these pines are acyclic, monocyclic, and bicyclic monoterpenes (Smith, 1965, 1966). Emergent female *D. ponderosae* are devoid of volatile, monoterpene-derived attractants (Pitman and Vité, 1969; Hughes, 1973b); they produce these compounds only after attacking fresh host trees. The biological activity of several of the female-produced compounds has been investigated in the laboratory (Conn 1981) and in the field (Conn et al., 1983; Borden et al., 1983, 1986; Libbey et al., 1985).

Despite the growing knowledge of volatiles produced by *D. ponderosae*, the hypothesis that these beetles efficiently metabolize a wide range of monoterpenes in detoxification reactions has not been rigorously tested. Our major objective was to investigate in detail monoterpene metabolism in female *D. ponderosae* during the initial stage of gallery construction in ponderosa and lodgepole pines.

METHODS AND MATERIALS

Instrumental Methods. A Hewlett-Packard 5830 gas chromatograph equipped with an 18835B capillary inlet system and a flame-ionization detector

was employed for analyses by gas–liquid partition chromatography (GC). Micropreparative GC was carried out with a Varian 1200 instrument fitted with a 10:1 effluent splitter, a thermal gradient collector (Brownlee and Silverstein, 1968), and a 3.05-m × 3.18-mm-OD stainless steel column packed with 10% SP-1000 on Chromosorb W AW (100/120) (Supelco, Inc., Bellefonte, Pennsylvania). For both chromatographs, injection port and detector temperatures were 260° and 275°C, respectively.

A Hewlett-Packard 5985B GC/MS/DS was employed for coupled gas chromatography–mass spectrometry (GC-MS). Glass (0.25 mm ID) or fused silica (0.32 mm ID) open-tubular columns (30 m) coated with SP-1000 or Carbowax 20 M (J & W Scientific, Inc., Rancho Cordova, California) were used. The injection port, Pt-Ir transfer line, and ion source were maintained at 260°, 240°, and 200°C, respectively. Helium was the carrier gas for GC and GC-MS.

A Varian 5000 liquid chromatograph was utilized for high-performance liquid chromatography (HPLC). Column effluent was monitored at 220 nm with a Varian Series 634S UV-visible spectrophotometer fitted with microvolume flow cells.

Reference Compounds for Gas Chromatography and Mass Spectrometry. Components were identified by comparison of their mass spectra (GC-MS) with those of authentic samples or published reference spectra. Commercially available compounds were; *exo-* and *endo*-brevicomin and (+)-3-carene (Albany International, Columbus, Ohio), α-pinene, and (+)-*p*-mentha-1,8-dien-10-ol (Aldrich Chemical Co., Milwaukee, Wisconsin), *p*-mentha-1,8-dien-7-ol (perilla alcohol) (CA Aromatics Co., Floral Park, New York), carvone, β-citronellal, geraniol (Eastman Organic Chemicals, Rochester, New York), acetophenone and 2-phenylethanol (Matheson Coleman & Bell, East Rutherford, New Jersey), *p*-isopropylbenzaldehyde, *p*-isopropylbenzyl alcohol (Pfaltz and Bower, Inc., Stamford, Connecticut) and β-myrcene (Sigma Chemical Co., St. Louis, Missouri).

Compounds generously donated were: ipsdienol and *cis*-verbenol (L.E. Browne, University of California, Berkeley, California); *cis-* and *trans*-2-*p*-menthen-7-ol, *p*-mentha-2,4(8)-dien-9-ol and *p*-mentha-2,4(8)-dien-10-ol (F. Camps, Instituto de Quimica Bio-organica, Barcelona, Spain); 3-caren-9-ol (T. Ishida, Hiroshima Institute of Technology, Hiroshima, Japan); (−)-β-phellandrene and (+)-*trans*-verbenol (B.J. Kane, SCM Organic Chemicals, Jacksonville, Florida); *p*-mentha-1,8-dien-4-ol, *p*-mentha-1,4(8)-dien-9-ol, and *p*-mentha-1,4(8)-dien-10-ol (E. Klein, Dragoco, Holzminden, Federal Republic of Germany); *m*-mentha-4,6-dien-8-ol and *p*-mentha-1,5-dien-8-ol (J.A.A. Renwick, Boyce Thompson Institute for Plant Research, Ithaca, New York); *trans*-pinocarveol (L.C. Ryker, Oregon State University, Corvallis, Oregon); frontalin (K.N. Slessor, Department of Chemistry, Simon Fraser University); and geranyl acetate (J.W. Wong, Department of Chemistry, Simon Fraser University).

Compounds synthesized by methods in the literature were: 2-caren-10-al (Büchi et al., 1969), 3-caren-10-al and 3-caren-10-ol (Gollnick et al., 1965; Gollnick and Schade, 1966a), 3-caren-5-one (Boyle et al., 1971; Burns et al., 1968), *p*-mentha-1,3-dien-7-al (Kayahara et al., 1968), *p*-mentha-1,4-dien-7-ol (Varo and Heinz, 1970a, b), (*E*)-2-methyl-6-methylene-octa-2,7-dienol (*E*-myrcenol) (Büchi and Wüest, 1967), and *cis*-3-pinen-2-ol (Whitham, 1961). 2-Caren-10-ol, 3-caren-5-ols, β-citronellol, *p*-mentha-1,3-dienol, and 1-phenylethanol were prepared by reduction of the parent aldehyde or ketone with lithium aluminum hydride in ether or sodium borohydride in ethanol. Perilla aldehyde and verbenone were prepared by oxidation of the precursor alcohols with MnO_2 and Jones reagent, respectively.

Hydroboration of (−)-β-phellandrene with borane–tetrahydrofuran complex (Aldrich Chemical) gave, after oxidative work-up, extraction, and vacuum distillation, a 1:3 mixture of *cis*- and *trans*-2-*p*-menthen-7-ols in 56% yield.

3-Thujen-10-ol. Steam distillation–continuous extraction (Flath and Forrey, 1977) of the macerated (Waring blender) foliage (2 kg) from a *Juniperus* sp. yielded 52 g of crude leaf oil. From this, a monoterpene fraction (21.7 g), containing approx. 45% sabinene, was obtained by distillation at water aspirator pressure. Redistillation of this material through a Nester-Faust annular Teflon spinning band column gave 6.1 g of sabinene with a purity >99%. Since photosensitized oxygenation of sabinene gave none of the reported hydroperoxide photoproduct (Dässler, 1959), sabinene was isomerized with potassium *t*-butoxide in dimethyl sulfoxide (Acharya et al., 1969) to a mixture of α-thujene (88%), sabinene (9%), and unidentified compounds (3%). Selenium dioxide oxidation of α-thujene (Catalan and Retamar, 1972) gave 3-thujen-10-al, in a complex mixture, which was reduced directly with sodium borohydride in ethanol. A pure sample of 3-thujen-10-ol was isolated by preparative GC from the distilled product. NMR (Varian A56/60A) (CCL_4 + 1% TMS): δ 0.005 (m, 1H, C-6 H_{endo}), 0.78 (dd, J = 4 Hz, J = 7.3 Hz, 1H, C-6 H_{exo}), 0.84 and 0.89 (d, J = 7.3 Hz, 6H, C-8 H and C-9 H), ca. 1.5 (m, 2H, C-5 H and C-7 H), 2.21 and 2.41 (AB quartet, J_{AB} = 17.9 Hz, 2H, C-2 H_{exo}, C-2 H_{endo}), 4.1 (s, 2H, C-10 H), 5.16 (s, 1H, C-3 H).

Collection and Maintenance of D. ponderosae. Beetles were allowed to emerge from caged lodgepole or ponderosa pine logs cut from infested trees obtained from interior British Columbia. Emergent beetles were collected periodically and kept at 2–4°C in jars containing moist dental cotton wicks and tissue paper.

Frass Production and Isolation of Abdomens from D. ponderosae. Female beetles were placed into entrance holes preformed in ponderosa (or in one case lodgepole) pine logs. The longer portion of a gelatin capsule was secured over each entrance hole with a loop of masking tape. After 24 hr the capsules were removed and the frass collected. Beetles were chipped from the bark and held over ice until dissection. Freezing of intact insects without or under solvent

resulted in disappearance of all volatiles upon later extraction (unpublished data). Therefore, abdomens were removed and immediately immersed in distilled pentane contained in a vial set in Dry Ice. No more than 100 abdomens were placed in each vial since in numbers more than 100, the frozen abdomens could not be crushed thoroughly. Frass and whole or crushed abdomens under pentane and frass were stored at −44°C.

Isolation of Abdominal Volatiles of Female D. ponderosae *by Codistillation with Water.* A pentane slurry of 530 crushed abdomens from female beetles fed 24 hr in ponderosa pine was placed in a 100-ml round-bottomed flask to which was added approx. 50 ml of distilled water. The magnetically stirred contents of the flask were heated to boiling, and the distillate was collected under a layer of chilled (~5°C) pentane. After approx. 35 ml of water had distilled, 30 ml of distilled water was added to the flask, and the distillation was continued until approx. 15 ml of water remained. The water and pentane layers were separated, and the aqueous layer extracted twice with pentane. The pentane extracts were dried (Na_2SO_4), filtered, and concentrated by slow distillation of solvent through a Vigreux column to a small volume (2–3 ml). The concentrated solution was made up to 5.3 ml (100 beetle equivalents/ml). A 1-ml aliquot was concentrated to approx. 10 μl for analysis by GC-MS.

Isolation of Abdominal Volatiles by Steam Distillation–Continuous Extraction. The volatiles of approx. 1000 abdomens from female beetles feeding in ponderosa pine were isolated by steam distillation–continuous extraction (SD-CE) (Godefroot et al., 1981, 1982). Aliquots (150–200 μl) of the pentane extract (1 ml) were employed for GC-MS analyses.

Isolation of Abdominal Volatiles of Female D. ponderosae *at Reduced Pressure.* The pentane extract of approx. 500 abdomens from females fed 24 hr in ponderosa pine was vacuum-distilled (0.001 mm) in a one-plate distillation apparatus (Varo and Heinz, 1970a). The cold finger of the apparatus was cooled with liquid nitrogen and the volatiles were collected for 1 hr. The cold finger was rinsed with acetone to remove the distillate. The solution was dried (Na_2SO_4) and concentrated under a stream of nitrogen to approx. 100 μl for analyses by GC and GC-MS.

Isolation of Primary Terpene Alcohols from Frass Volatiles. Steam was passed through 10 g of frass produced by female beetles feeding in ponderosa pine until 750 ml of distillate had collected under a layer of chilled pentane. The water layer was extracted with pentane (3 × 100 ml). The combined extracts were dried ($Na_2SO_4 + MgSO_4$), filtered, and concentrated by distillation. The final volume of the extract was made up to 10 ml of which 5 ml was concentrated to approx. 50 μl by evaporation under a stream of nitrogen. The concentrate was separated by HPLC on a 30-cm × 4-mm-ID MicroPak Si-10 column with hexane–isopropyl alcohol (99:1, v/v) as eluent (2 ml/min) to give three fractions: fraction 1, 0–10 min; fraction 2, 10–12 min; and fraction 3, 12–20 min. All fractions were concentrated to 1 ml under a stream of nitrogen.

Ponderosa Pine Phloem Oil. Steam was passed through 400 g of ponderosa pine phloem tissue (cut into small pieces) until 1200 ml distillate was collected. The distillate was extracted with pentane (3 × 100 ml). The combined extracts were dried ($MgSO_4$), filtered, and concentrated to ~1 ml by distillation of solvent through a Dufton column. Residual solvent was removed under a stream of nitrogen to give 220 mg of crude oil (0.055% of wet weight).

RESULTS

Abdominal Volatiles of Female D. ponderosae *Fed in Ponderosa Pine.* Typical total ion chromatograms of volatiles obtained from crushed abdomens of female beetles fed on ponderosa pine are shown in Figures 1 and 2. One sample (Figure 1) was obtained by steam distillation and subsequent pentane extraction of the distillate. A second sample (Figure 2) was obtained by steam distillation–continuous extraction. The terpene alcohol regions of the two samples reveal many of the same components, but differ markedly in the relative proportions of the components. In these two and several other samples, *trans*-verbenol (Scheme 1, 2t),[6] 3-caren-10-ol (Scheme 2, 10), and perilla alcohol (Scheme 9, 63) were the dominant terpene alcohols. *cis*-Verbenol (2c) was usually present as a minor component.

The only carbonyl compounds identified in these samples were verbenone (4), 3-caren-10-al (12), 3-caren-5-one (13), and perilla aldehyde (65). There was some evidence that metabolism of allylic alcohols to carbonyl compounds was dependent on substrate availability. For example, perilla aldehyde (65) was not detected in Figure 1, where perilla alcohol (63) was present in low relative amounts. In Figure 2, 63 was a major peak and 65 was present. Similar arguments apply to 10 and12. Verbenone (4) always accompanied 2t.

Acetophenone (18), 1-phenylethanol (19), and 2-phenylethanol (20) (Figure 3) were present in variable amounts in all samples. Neither *exo*- or *endo*-brevicomin (21 or 22) nor frontalin (23) was detected in female beetle volatiles. Compounds 18–23 were not present in the host phloem, although we found the commonly occurring natural products 24–29 (Figure 4) of phloem oil.

Verbenone (4) and *m*-mentha-4,6-dien-8-ol (11) coeluted under the analytical conditions of Figure 1. The mass spectrum of the latter was indistinguishable from that of *p*-mentha-1,5-dien-8-ol (6). Complete resolution of 4, 6, and 11 was achieved on the fused silica column (Figure 2) on which 12 eluted as a shoulder on 6. *trans*-Pinocarveol (16) was apparently the only beetle-de-

[6]The chiral structures shown in Schemes 1–3 and 5 are based upon the predominant enantiomers of α-pinene [(−)-1], 3-carene [(+)-7], β-pinene [(−)-14] and β-phellandrene [(−)-34] occurring in the oleoresins of ponderosa and lodgepole pines of the Pacific Northwest (Mirov, 1961). We did not independently determine the chiralities of the beetle-produced compounds or host monoterpenes.

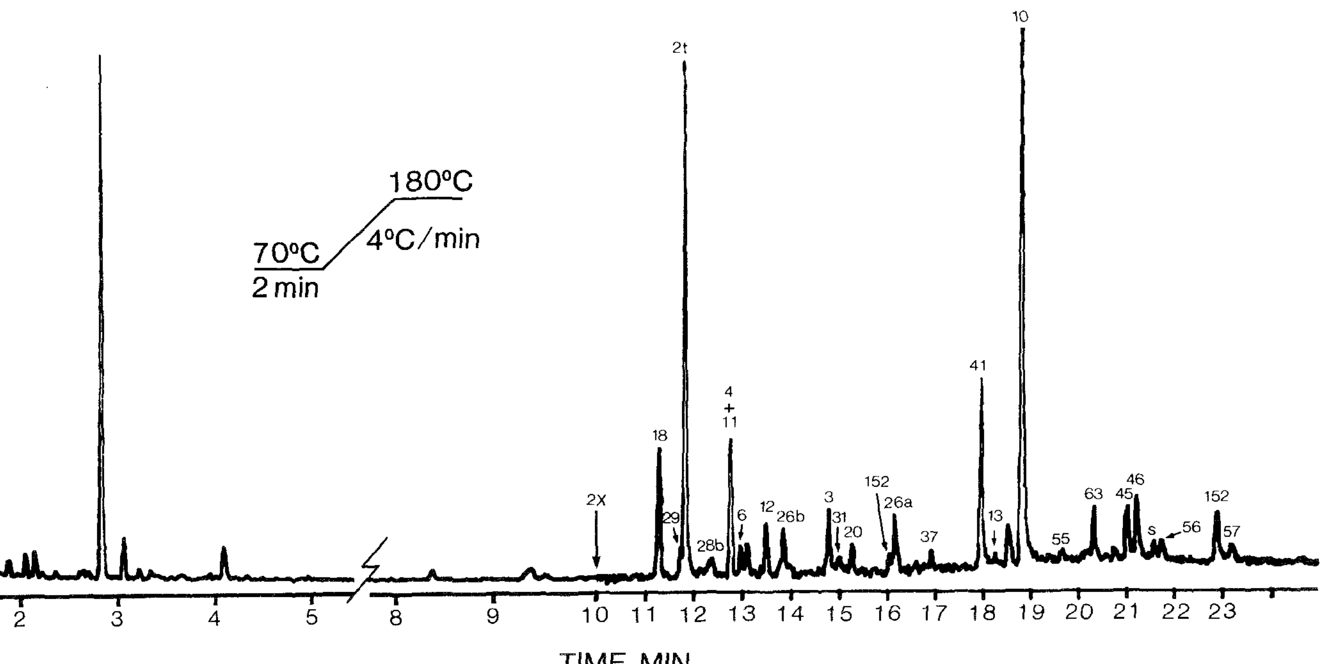


FIG. 1. Total ion chromatogram of the abdominal volatiles from female *D. ponderosae* fed 24 hr in ponderosa pine. Volatiles were isolated by codistillation-extraction and analyzed on a 30-m × 0.25-mm-ID borosilicate glass column coated with SP-1000. Note vertical scale expansion of 2× at R_t = 10 min. Intensities of peaks with R_t > 10 in are normalized on constituent 2t. See Table I for peak assignments. Molecular weights of unknown peaks are indicated by 150, 152, or 154. s = unknown sequiterpene.

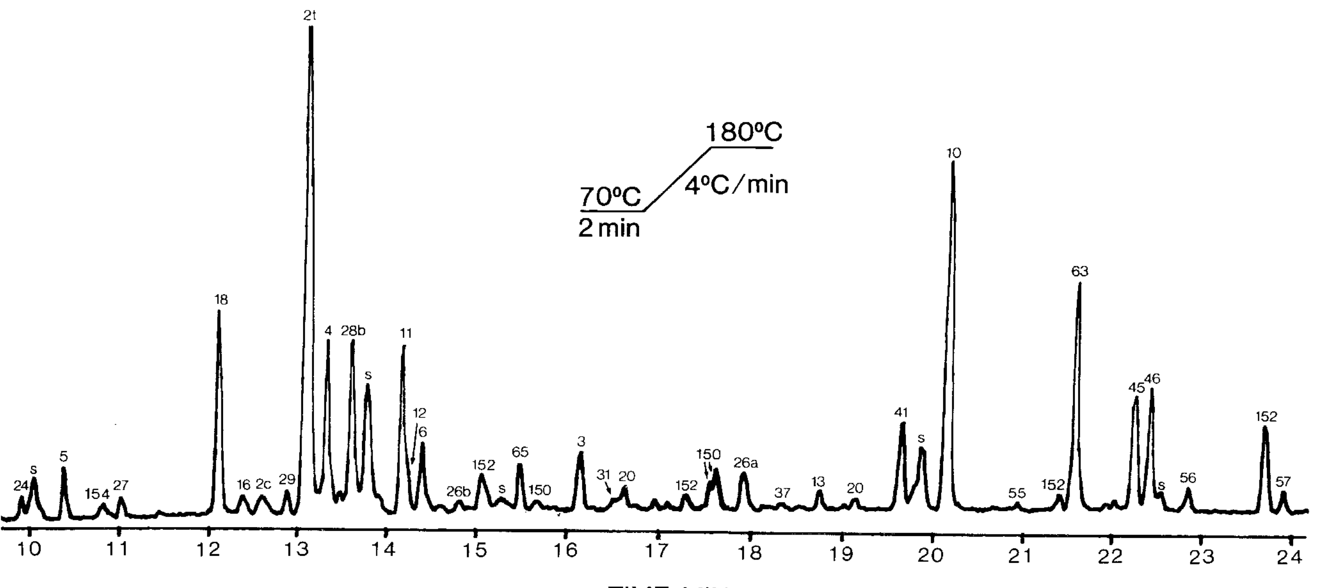
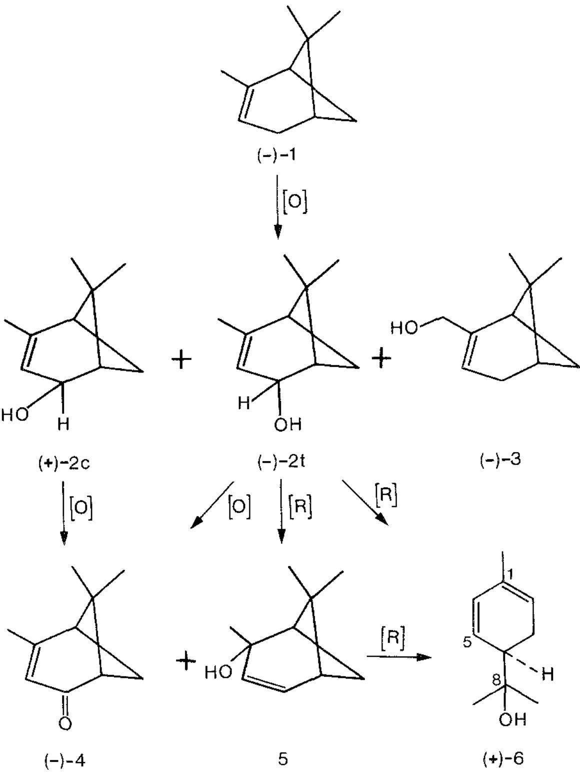


FIG. 2. Terpene alcohol region of the total ion chromatogram of the abdominal volatiles from female *D. ponderosae* fed 24 hr in ponderosa pine. Volatiles were isolated by steam distillation–continuous extraction and analyzed on a 30-m × 0.32-mm-ID fused silica column coated with SP-1000. Intensities of peaks are normalized on constituent 2t. See Table 1 for peak assignments and legend to Figure 1.



SCHEME 1. Allylic oxidation and rearrangement products derived from (−)-α-pinene (1).

rived metabolite of β-pinene (14) encountered (Scheme 3).

3-Thujen-10-ol (Scheme 4, 31, and Figures 1 and 2) was the only confirmed metabolite of α-thujene (30), a trace component of ponderosa pine oleoresin (Smith, 1977). The mass spectra of peaks at R_t = 15.8 min (mol wt = 150) and R_t = 17.3 min (mol wt = 152) in Figure 2 were quite similar to the published spectra of 3-thujen-2-one (umbellulone, 33) and *trans*-3-thujen-2-ol (*trans*-umbellulol, 32t) (Wheeler and Shonowo, 1974). While the R_t of the earlier eluting peak was appropriate for 33, that of the second peak seemed too long for 32t. Until authentic samples of 32 and 33 are available for measurement of their mass spectra and R_t's, the status of these oxidation products of α-thujene (30) remains undetermined.

The compound eluting directly after 26a in Figures 1 and 2 could be identified only as a 2-*p*-menthen-7-ol (37) (Scheme 5) since authentic samples of the *cis* and *trans* isomers were unavailable at the time the spectrum was recorded for R_t measurements. Although 37 was present only in small amounts

10 3 1 5 8 9 (+)-7

10 3 1 5 7 8 9 OH 67

HO 66

OH + + HO

OH

8 9 (+)-10

[R] [O,R] [O]

H 1 H 8 4 OH O H OH

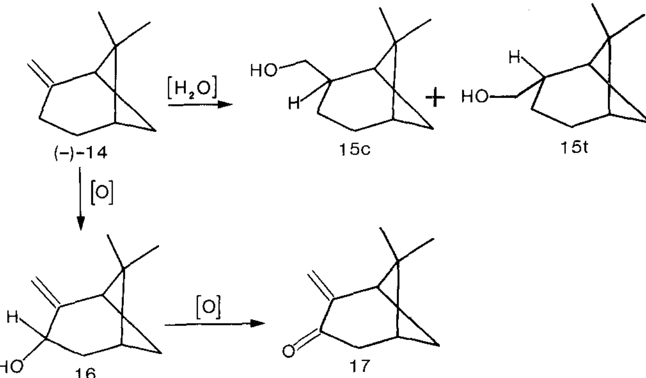
(-)-6 (-)-11 (-)-12

+

O

(-)-13

SCHEME 2. Allylic oxidation and rearrangement products derived from (+)-3-carene (7).



SCHEME 3. Allylic oxidation and hydration products derived from (−)-β-pinene (14).

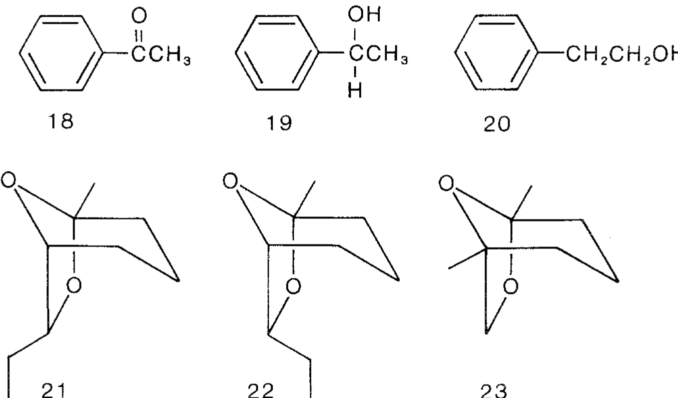


FIG. 3. Nonterpenoid compounds present in *D. ponderosae*: 18–20 in females; 21–23 in males.

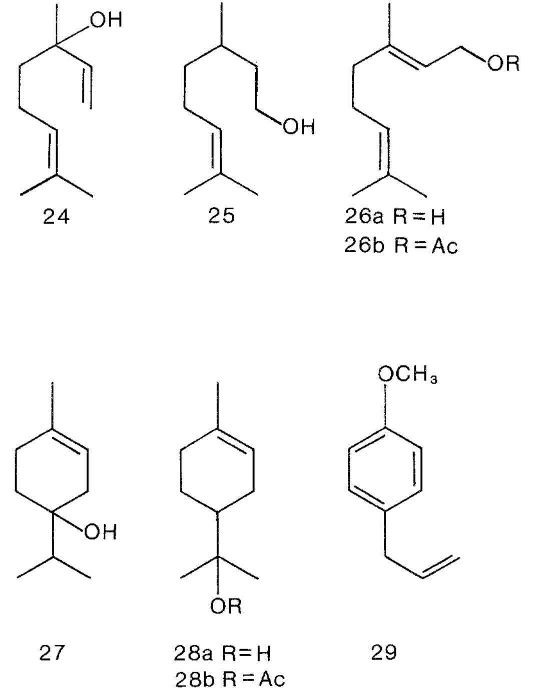


FIG. 4. Some compound occurring in ponderosa pine phloem oil.

TABLE 1. COMPOUNDS IDENTIFIED IN VOLATILES OF FEMALE *D. ponderosae* IN LODGEPOLE AND PONDEROSA PINE AND METABOLIC PATHWAYS PROPOSED FOR THEIR FORMATION.

Category and Compound	Scheme or Figure and Compound No.	Metabolic pathway
Metabolites of α-pinene (1)	Scheme 1	
cis-Verbenol[a]	2c	MP-II
trans-Verbenol[a]	2t	MP-II
Myrtenol[a,b]	3	MP-I
Verbenone[a]	4	Oxidation of 2
cis-Pinen-2-ol[a]	5	Rearrangment of 2
p-Mentha-1,5-dien-8-ol[a]	6	Rearrangment of 2 or 8
Metabolites of 3-carene (7)	Scheme 2	
3-Carene-10-ol	10	MP-I
m-Mentha-4,6-dien-8-ol	11	Rearrangment of 9
3-Caren-10-one	12	Oxidation of 10
3-Caren-5-one	13	Oxidation of 9
3-Caren-9-ol	67	MP-I
Metabolite of β-pinene (14)	Scheme 3	
trans-Pinocarveol[a]	16	MP-I (?)
Aromatic compounds	Figure 3	
Acetophenone	18	
1-Phenyethanol	19	Reduction of 18
2-Phenylethanol	20	
Compounds from host oleoresin	Figure 4	
Linalool[a,c]	24	
β-Citronellol	25	
Geraniol	26a	
Geranyl acetate	26b	
Terpinen-4-ol	27	
α-Terpineol	28a	
α-Terpinyl acetate[c]	28b	
Estragole[c]	29	
Metabolite of α-thujene (30)	Scheme 4	
3-Thujen-10-ol	31	
Metabolite of β-phellandrene (34)	Scheme 5	
2-*p*-Menthen-7-ol	37	MP-III
Metabolite of myrcene (38)	Scheme 6	
(*E*)-Myrcenol	41	MP-I
Metabolites of terpinolene (44)	Scheme 7	
p-Mentha-1,4(8)-dien-9-ol	45	MP-I
p-Mentha-1,4(8)-dien-10-ol	46	MP-I
p-Mentha-1,4(8)-dien-7-ol	47	MP-I
Metabolites of α-phellandrene (51), α-terpinene (52), and γ-terpinene (53)	Scheme 8	
p-Mentha-1,5-dien-7-ol[b]	54	MP-I

TABLE 1. (*Continued*).

Category and Compound	Scheme or Figure and Compound No.	Metabolic pathway
p-Mentha-1,3-dien-7-ol	55	MP-I
p-Mentha-1,4-dien-7-ol	56	Dehydrogenation of 54–56
Cuminyl alcohol	57	
Metabolites of limonene (58)		
p-Mentha-1,8-dien-10-ol	62	MP-I
Perilla alcohol	63	MP-I
Perilla aldehyde	65	Oxidation of 63

[a] Detected by Libbey et al. (1985).
[b] Reference mass spectrum: von Sydow et al. (1970).
[c] Reference mass spectrum: Jennings and Shibamoto (1980).

in female beetles fed in ponderosa pine, it was a major constituent of a female from lodgepole pine (Figure 4).

(*E*)-Myrcenol (41) was the only metabolite of myrcene (38) found in female beetle volatiles (Scheme 6). Although no authentic sample of the known *Z*-isomer (40) (Granger et al, 1972) was available, analysis of synthetic (*E*)-myrcenol (41) by GC-MS did reveal the presence of a minor component (presumably 40, $E:Z = 98:2$) with an identical mass spectrum which eluted slightly before 41. Examination of Figures 1 and 2 in the region between geraniol and (*E*)-myrcenol (41) in these preparations failed to detect *Z*-myrcenol (40).

Of the possible primary alcohols derivable from allylic oxidation of terpinolene (Scheme 7, 44), only dienols 45 and 46 were confirmed in female

SCHEME 4. Allylic oxidation products derived from (+)-α-thujene (30).

SCHEME 5. Allylic oxidation and hydration products of (−)-β-phellandrene (34).

volatiles. The mass spectrum and gas and liquid chromatographic properties of the component eluting at R_t = 22.9 min in Figure 1 and at R_t = 23.8 min in Figure 2 suggested that it was *p*-mentha-1,4(8)-dien-7-ol (47). Its mass spectrum was similar to that of 45, and the compound was produced in an amount comparable to 45 and 46. Perilla alcohol (63), 45, 46, and 47 were the most prominent of the beetle-produced primary *p*-menthadienols, while those derived from α-phellandrene (51), α-terpinene (52), and γ-terpenine (53) were present in low amounts or absent (Scheme 8). Other possible products of limonene, the

SCHEME 6. Allylic oxidation and hydration products of myrcene (38).

SCHEME 7. Primary allylic alcohols derived from terpinolene (44) and isoterpinolene (48).

carveols (61), *p*-mentha-1,8-dien-4-ol (60), isopiperitenols (64), and 59 were not detected by GC-MS (Scheme 9).

Steam distillation would have caused rearrangement of *cis*- and *trans*-3-caren-5-ols (9c and 9t) to *m*-mentha-4,6-dien-8-ol (11) (Gollnick and Schade, 1966b). Therefore, the volatile constituents were removed from female beetles (fed on ponderosa pine) under reduced pressure and condensed on a liquid N_2-filled cold finger. The concentrated condensate was analyzed by GC and GC-MS. The terpene alcohol region of the chromatograms was similar to Figure 2 and was devoid of 9t. Detection of 9c was not possible due to overlap with peaks eluting after *trans*-verbenol (2t).

Precursors of 1,5-dienol (6) might have been the verbenols (2) or 3-caren-2-ols (8) (Schemes 1 and 2). Absence of carveols (61) suggests that oxidation of 3-carene at C-2 probably did not occur in the beetles (Renwick and Hughes, 1975). Rearrangement of the verbenols (2) directly to 6 or via *cis*-3-pinen-2-ol (5) upon steam distillation or in the beetles (Renwick and Hughes, 1975) also accounts for the formation of 6.

Primary Alcohol Fraction from Female Frass Volatiles. Since a sesquiterpene eluting near 19.9 min interfered with measurement of the mass spectra of several metabolites eluting between components 41 and 10 (Figure 2), the primary terpene alcohols from steam-distilled female frass volatiles were isolated by HPLC (fraction 3) and analyzed by GC-MS (Figure 5, Table I). Although their mass spectra were not recorded, on the basis of retention times structures of *trans*-2-*p*-menthen-7-ol (37t), *p*-mentha-1,5-dien-7-ol (54), *p*-mentha-1,3 dien-7-ol (55), and *p*-mentha-1,8-dien-10-ol (62) were assigned to the small

51 52 53

[O]

54 55 56

-2H

57

SCHEME 8. Primary allylic alcohols derived from α-phellandrene (51), α-terpinene (52), and γ-terpinene (53). 57 = *p*-isopropylbenzyl (cuminyl) alcohol.

peaks as shown in Figure 5 (see Schemes 5, 8, and 9). Comparison of Figure 5 to Figures 1 and 2 reveals that many of the primary terpene alcohols found in the volatiles of female beetles were also present in the frass. In particular, there were substantially greater quantities of host geraniol (26a), 3-caren-5-one (13), and perilla alcohol (63), and a lesser amount of 3-caren-10-ol (10). As mentioned above, only (*E*)-myrcenol (41) was detected in female volatiles, and there is no suggestion in Figure 5 of the Z-isomer (40). Neither component possessing a molecular weight of 154 and eluting near 17.2 min and 19.1 min in Figure 5 was γ-geraniol (43), nor were the mass spectra of these components suggestive of an acyclic structure such as 42. 3-Caren-9-ol (Scheme 2, 67) was identified by comparison of its mass spectrum to that of a specimen isolated

SCHEME 9. Allylic oxidation and hydration products of limonene (58).

from the neutral metabolites of (+)-3-carene administered to rabbits (Ishida et al., 1977, 1981). 2-Caren-10-ol (66) was not detected in frass or beetle volatiles. Analysis of ponderosa pine phloem oil by GC-MS revealed a complete absence primary monoterpene alcohols eluting beyond geraniol (Figure 4). Hence, peaks in this region were produced by female beetles.

Abdominal Volatiles of Female Beetle Fed in Lodgepole Pine. Extraction of a single female beetle fed in lodgepole pine for 24 hr (Conn, 1981) was sufficient to show the presence of *trans*-2-*p*-menthen-7-ol (37t) and *p*-mentha-1,5-dien-7-ol (54) in the terpene alcohol region (Figure 6). Analysis of the sample on a different column indicated a *trans–cis* ratio of 8 : 1. Oxidation of 37 to an aldehyde and oxidation of β-phellandrene at C-6 to yabunikkeols (35) (Fujita et al., 1970) did not occur to any appreciable extent. The areas of unidentified peaks were < 10% of that of 54. Due to poor resolution and the presence of numerous sesquiterpenes, fewer peaks in Figure 6 could be identified (Table 1), than in Figures 1 and 2. The numerous sesquiterpenes eluting before and after *trans*-verbenol precluded detection of any 2-*p*-menthenols (36).

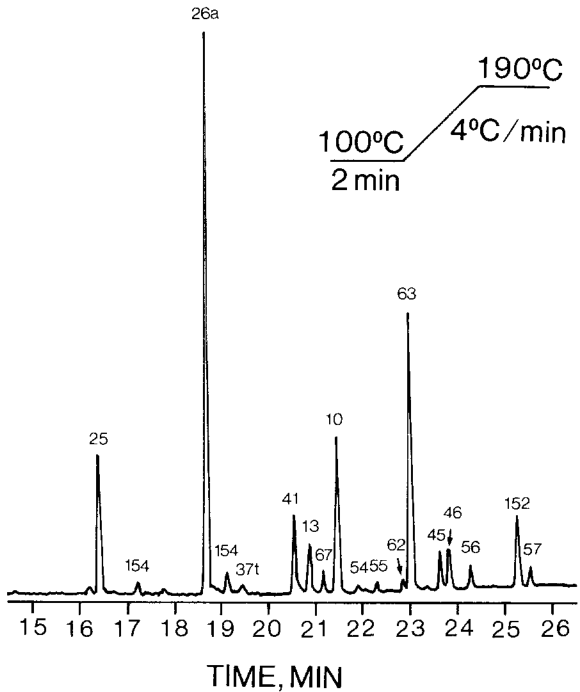


FIG. 5. Total ion chromatogram of the primary terpene alcohols isolated from steam-distilled female frass. See legend of Figure 1 for description of column and Table 1 for peak assignments. Molecular weights of unknown peaks are indicated by 152 or 154.

DISCUSSION

The discovery of acetophenone (18) and 1-phenylethanol (19) in the abdomens of feeding female *D. ponderosae* (Table 1) constitute the first record of these compounds in insects, as they have not previously been reported (Bradshaw, 1984). Renwick et al. (1976b) found 2-phenylethanol (20) in *Ips* and *Dendroctonus* spp. The absence of the brevicomins (21, 22) and frontalin (23) is in agreement with Ryker and Libbey (1982) and Libbey et al. (1985). Female *D. ponderosae* are atypical among other *Dendroctonus* which usually contain one of these bicyclic ketal pheromones upon emergence (Borden, 1985).

Allylic Alcohols: Precursor–Product Relationships. Monoterpenes are traditionally classified as acyclic, monocyclic, and bicyclic. Myrcene (38), the only prominent acyclic monoterpene in pine oleoresin, was exclusively oxidized by female *D. ponderosae* in ponderosa and lodgepole pine logs to (*E*)-myrcenol (41). However, female *D. ponderosae* exposed to myrcene vapors produced both (*E*)- and (*Z*)-myrcenol (*E*:*Z* = 49:1) (Scheme 6), while males produced approximately equal amounts of (*S*)-(+)-ipsdienol (97% ee) and (*E*) and (*Z*)-myrcenol (*E*:*Z* = 9:1) (Hunt et al., 1986). Male and female *D. brevicomis*

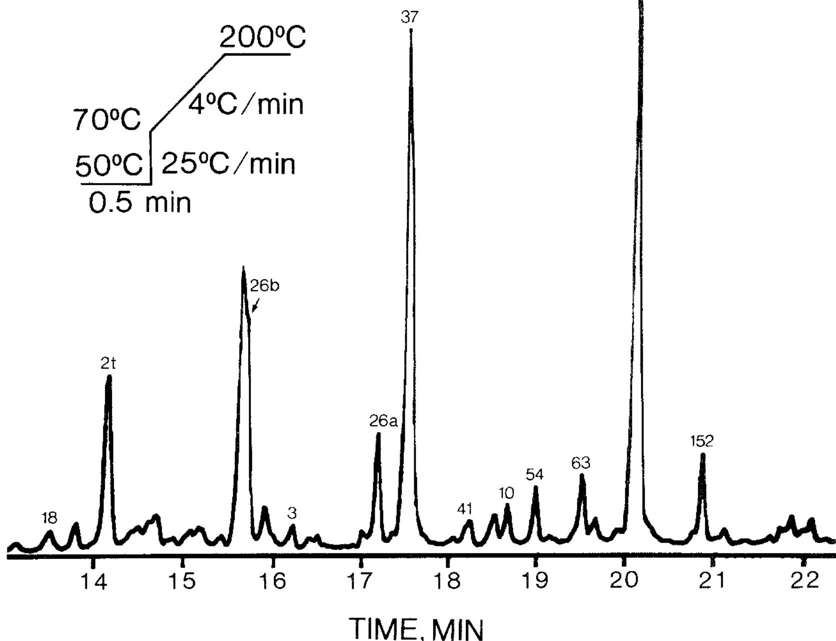


FIG. 6.. Terpene alcohol region of the total ion chromatogram of the abdominal volatiles extracted from a single female *D. ponderosae* fed 24 hr in lodgepole pine. See legend of Figure 2 for description of column. Splitless injection was used for sample introduction. See Table 1 for peak assignments and legend to Figure 1.

also convert myrcene to (*E*)-myrcenol (Renwick et al., 1976c),[7] while males produce *S*-(+)-ipdienol (Byers, 1982). Thus in these cases of myrcene oxidation to primary alcohols by *Dendroctonus* spp., the process is specific for the *E* methyl. Oxidation of the C-1 methyl group and C-6 of 1-methyl-1-cyclohexene (71) in male *D. frontalis* (Renwick and Hughes, 1975) occurred in approximately the same ratio (10 : 1) as oxidation of the (*E*)- and (*Z*)-methyl groups of myrcene by male *D. ponderosae*.

Monocyclic terpenes terpinolene (44) and limonene (58), α-phellandrene (51), and the isomeric terpinenes 52 and 53 were all oxidized at the allylic methyl groups to give primary alcohols. As for myrcene, the allylic methyls of these monocyclics which were *E* to a vinyl methylene group were oxidized.

[7] Although Renwick et al. (1976c) undoubtedly identified correctly the beetle-produced compound as the *E* isomer (41), the structure was drawn as the *Z* isomer (40). We attribute this error to an improper transcription of the structure given by Büchi and Wüest (1967). Granger et al. (1972) isolated from the essential oils of certain chemical races of *Thymus vulgaris* L., (*Z*)-myrcenol (40) and showed by NMR spectroscopy that it was the geometrical isomer of synthetic (*E*)-myrcenol (41).

Female *D. pondersoae* evidentally do not possess the ability to oxidize the *endo*-cyclic allylic methylenes of these monoterpenes, since anticipated metabolites such as 61 and 64 were not detected.

Primary allylic alcohols resulting from the oxidation of allylic methyl *E* to a ring methylene are encountered for α-pinene, 3-carene, and α-thujene. The relative proportions of the alcohols myrtenol (22), 3-carene-10-ol (10), and 3-thujene-10-ol (31) are close to the proportion of the monoterpene precursors in host volatiles.

The only prominent secondary alcohol in female volatiles is *trans*-verbenol (2t). We assume that verbenone (4), 5, and 6, are secondarily derived from *trans*-verbenol. Thus the oxidation of a α-pinene at the *endo*-cyclic allylic methylene occurs with the same high stereoselectivity as in other female *Dendroctonus* spp. (Hughes, 1973a; Byers, 1983).

Metabolic Pathways for Allylic Alcohol Formation. To account for the allylic oxidations (White et al., 1980; Francke and Vité, 1983) which would produce the distribution of allylic terpene alcohols found in the female volatiles, we propose two metabolic pathways.

Pathway MP-I (Figure 7) oxidizes any allylic methyl group which is *E* to

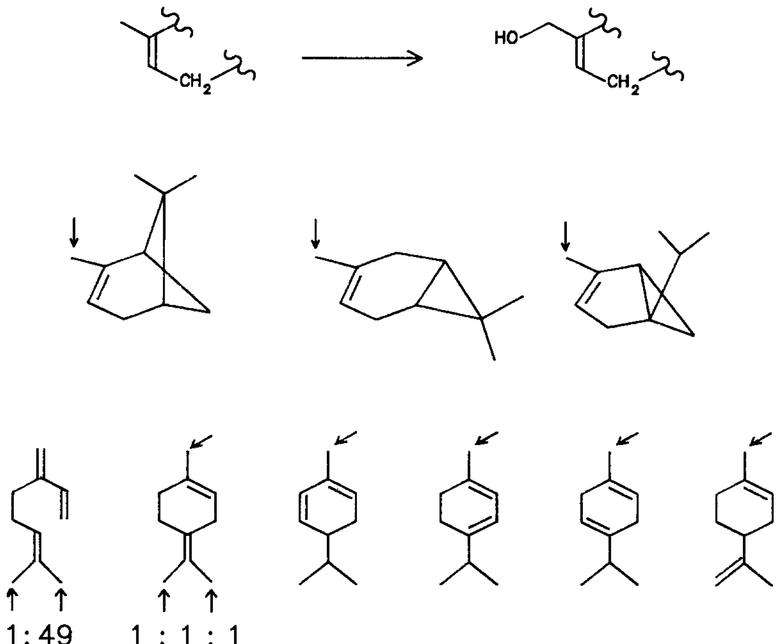


FIG. 7. Regiospecific oxidation (MP-I) of monoterpenes to primary alcohols in female *D. ponderosae.*

an allylic methylene or vinyl group (e.g., 52 → 55). It does not discriminate between different methyl groups in the same substrate as long as they possess the requisite geometry with respect to the olefin (e.g., 44 → 45:46:47, 1:1:1). Furthermore, MP-1 does not differentiate between substrates (1, 7, 30, 38, 44, 51–53, 59). The formation of 3-caren-9-ol (67), a minor metabolite of 3-carene (7), involves oxidation having the same stereochemical relationship to the carene cyclopropane as the methyl of myrcene oxidized during conversion to *E*-myrcenol has relative to the myrcene double bond. We hypothesize that both oxidations are mediated by the same system.

No discrimination between the enantiomers of α-pinene was found for oxidation to myrtenol (3) in *I. paraconfusus* (Renwick et al., 1976a). By analogy, MP-I would not discriminate between enantiomers of chiral substrates. Thus the board substrate specificity of MP-I and conversion of substrates to primary allylic alcohols, which can be oxidized to more polar metabolites, ideally suit this pathway for the detoxification of monoterpenes.

MP-II (Figure 8) mediates the oxidation of *endo*-cyclic allylic methylenes *E* to a vinyl methyl of bicyclic substrates. This pathway is responsible for the conversion of α-pinene (1) to *trans*-verbenol (2t), 3-carene (7) to 3-caren-5-ol (9t detected as 11), and possibly α-thujene (30) to 3-thujen-2-ol (32t). The greater amount of 2t compared to 3 in the volatiles of female *D. ponderosae* indicates that the relative rate of conversion of a substrate to product alcohol by MP-II is considerably greater than that of MP-I.

We suggest that MP-II is selective for enantiomers of bicyclic substrates which are structurally related to (−)-a-pinene (1). This suggestion is based on the observation that the *trans*-verbenol (2t) to myrtenol (3) ratio is large, whereas the *m*-mentha-4,6-dien-8-ol (11) to 3-caren-10-ol (10) ratio is small. Since the MP-I system responsible for formation of 3 and 10 operates on each of its

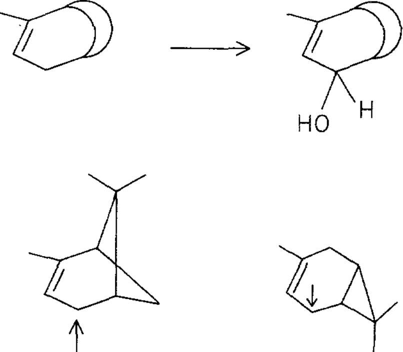


FIG. 8. Stereospecific oxidation (MP-II) of bicyclic monoterpenes to *trans* alcohols in female *D. ponderosae*.

substrates (in this case α-pinene and 3-carene) with equal facility, one would expect the ratio of 2t : 3 and 11 : 10 to be similar if α-pinene and 3-carene were oxidized at equal rates by MP-II to 2t and 11, respectively. The ratio of oxidation products observed suggests that 2t is produced by MP-II in much greater efficiency from α-pinene than is 11 from 3-carene. This would be expected if (−)-α-pinene were the preferred enantiomer of α-pinene for MP-II.

As shown in Figure 8 the stereochemical difference between (−)-1 and (+)-7 is that the *gem*-dimethyl group is above and below the plane of the double bond, respectively. The skeleton of (+)-1 is actually closely related to (+)-7. Thus, if oxidation of (+)-1 by MP-II is not as facile as oxidation of (−)-1, then oxidation of (+)-7 by this system should also be slow compared to oxidation of (−)-7. Since (+)-7 is the predominant enantiomer in ponderosa pine oleoresin (Mirov, 1961) and the proportion of MP-II-derived product from this substrate is small with respect to MP-I-derived 10, we infer that MP-II is specific for the (−)-enantiomer of α-pinene (1). Thus MP-II is enantioselective and is probably of little or no importance in monoterpene detoxification; however, it would be of importance in the production of (−)-2t, the major female-produced aggregation pheromone in *D. ponderosae* (Libbey et al., 1985; Borden et al., 1986).

Several studies have examined the stereochemistry of the conversion of α-pinene to *trans*-verbenol by *Dendroctonus* spp. Plummer et al. (1976) found that natural 2t isolated from the Porapak Q-trapped volatiles produced by female *D. frontalis* boring in short leaf pine logs for 96 hr was a 60 : 40 mixture of (+)-2t and (−)-2t, but they did not determine the enantiomeric composition of the host α-pinene. It is thus not possible to assess whether preferential oxidation of enantiomers occurred. Byers (1983) exposed male and female *D. brevicomis* to (+)-α-pinene (87.4% ee) and (−)-α-pinene (79.4%ee). Both sexes produced (+)-*trans*-verbenol (62% ee) from the (+)-*α-pinene, (−)-trans*-verbenol (53-54% ee) from (−)-α-pinene, and little *cis*-verbenol from either α-pinene.[8] While *D.brevicomis* and *D. frontalis* convert either enantiomer of α-pinene to *trans*-verbenol, the allylic oxidation of C-4 of chiral α-pinenes in *I. paraconfusus* proceeds by stereospecific replacement in the *pro*-(4*S*) hydrogen with hydroxyl. The process is amazingly insensitive to the steric effect of the bulky *gem*-dimethyl group since beetles exposed to (±)-α-pinene produced a 1 : 1 mixture of *cis*- and *trans*-verbenols (Renwick et al., 1976a). *I. amitinus* and *I. typographus* also metabolize the isomers of a α-pinene in a similar fashion (Klimetzek and Francke, 1980).

The metabolism of 3-carene by *I. paraconfusus* and *I. pini* is probably similar to that of α-pinene. The unstable 3-caren-5-ols (9) were not detected but rearranged in vivo to *m*-dienol 11, which was actually isolated by GC from

[8] A rotation of 168.75° (Schulz and Doll, 1944) was used for calculating the enantiomeric excess (ee) of the *trans*-verbenols produced by male and female *D. brevicomis*.

the hindguts of *I. pini* (Renwick et al. 1976c). The in vivo instability of 9 is somewhat puzzling since alcohol 9t was synthesized by Gollnick and Schade (1966b). Renwick et al. (1976c) proposed that ring opening occurred during the oxidation of 7. That 9 can survive for at least some time is suggested by the detection of its oxidation product 3-caren-5-one (13). Trace amounts of organic acids in the insect gut may catalyze the rearrangement of 9 to 11. We failed to detect 9 even when female volatiles were isolated at reduced pressure from pentane extracts of crushed abdomens by condensation on a liquid nitrogen-filled cold finger.

Metabolism of β-Phellandrene. Neither MP-I nor MP-II can metabolize β-pinene or β-phellandrene. In ponderosa pine, β-phellandrene is a minor compound. However, in lodgepole pine, female *D. ponderosae* encounter up to 70% (−)-β-phellandrene (34) in the oleoresin monoterpene fraction (Mirov, 1961; Smith, 1964). MP-III is specific for conversion of (34) to *trans*-2-p-menthen-7-ol (37) by anti-Markovnikov hydration of the *exo* double bond (Scheme 5). Being deficient in its ability to oxidize the allylic position α to an *exo* double bond (e.g., 14 → 16 and 58 → 62), *D. ponderosae* has apparently develop this hydration process for detoxification of β-phellandrene. Although β-pinene, myrcene, and limonene are abundant in lodgepole and ponderosa pines (Smith, 1964, 1977), hydration of these terpenes to the corresponding primary alcohols does not occur to detectable levels in female *D. ponderosae*.

It is of interest that male *Polygraphus poligraphus* (L.) contain *trans*-4-thujanol (69) and *cis*-4-thujanol (70) apparently produced by the Markovnikov hydration of sabinene (68) or α-thujene (30) (Scheme 10) (Francke and Vité, 1983). The hydration of sabinene would be mechanistically similar to that of β-phellandrene by MP-III.

Metabolic Pathways and Host-Insect Ineraction. Pine bark beetles may have adapted initially to their hosts by development of metabolic pathways that detoxify monoterpenes and later by exploiting the resultant metabolites as pheromones (Hughes, 1973a). The results of our study are consistent with this hypothesis. They have led to three proposed metabolic pathways that are adapted in *D. ponderosae* to overcome the host's chemical defense system.

OR [H_2O] + OH OH

68 30 69 70

SCHEME 10. Hydration products of sabinene (68) or α-thujene (30).

MP-I oxidizes most monoterpenes commonly occurring in the host and is apparently the general pathway for terpene detoxificiation. MP-III appears to be specific for detoxification of β-phellandrene. It would permit *D. ponderosae* to colonize lodgepole pine or similar species with high β-phellandrene content.

The relatively high rate of conversion of α-pinene to *trans*-verbenol by MP-II suggests that it is a specialized pathway utilized by female beetles to produce pheromone. Thus it is a critical process in initiating the complex series of semiochemical-mediated events (Borden et al., 1986) that result in the successful colonization of a living host tree.

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SOLUTION VOLUME AND SEED NUMBER: Often Overlooked Factors in Allelopathic Bioassays

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Abstract—Cucumber seeds were germinated under various combinations of solution volume and seed number with a range of ferulic acid concentrations. At each concentration, radicle growth decreased as the relative amount of ferulic acid available per seed increased from x (25 seeds/5 ml) to $5x$ (5 seeds/5 ml) to $19x$ (25 seeds/95 ml). With 2.0 mM ferulic acid in buffered solution, radicle lengths after 48 hr ranged from 71 to 47% of control. The amount of ferulic acid remaining in 2.0 mM solution after 48 hr was directly proportional to the amount initially available per seed, and ranged from 9 to 91%. Solution volume and seed number also significantly affected inhibition by vanillic acid, caffeic acid, and juglone. With 0.1 mM juglone, radicle lengths after 48 hr were 88% of control with 25 seeds/5 ml, 68% with 5 seeds/5 ml, and 56% with 25 seeds/90 ml. The data demonstrated that lower phytotoxin concentrations can produce equivalent or greater inhibitory effects than higher concentrations when the amount available per seed for uptake is greater. Equivalent inhibition of radicle growth was observed with 1.0 mM (5 seeds/5 ml) and 2.0 mM (25 seeds/5 ml) ferulic acid. Available literature on herbicides indicates that similar effects occur in greenhouse and field studies.

Key Words—Ferulic acid, vanillic acid, caffeic acid, juglone, cucumber, *Cucumis sativus*, allelopathy, competition, interference, seed bioassays.

INTRODUCTION

Allelopathy is a factor in determining vegetation patterns and community structure and has important implications for agriculture and forest management (Rice, 1984). Various bioassay techniques have been used to assess the biological activity of suspected phytotoxins (Stowe, 1979; Putnam and Duke, 1978;

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Einhellig et al., 1985). A common method is to test plant extracts or pure compounds for inhibition of seed germination or radicle elongation. Factors known to affect the results of such bioassays include high osmotic pressures (Anderson and Loucks, 1966; Bell, 1974; Stowe, 1979) and pH (Reynolds, 1975; Blum et al., 1984, 1985).

Ferulic acid is one of several simple phenolic acids with demonstrated phytotoxic activity. Conditions for germination and radicle elongation bioassays with ferulic acid have varied substantially. Guenzi and McCalla (1966) used 10 wheat (*Triticum aestivum,* L.) seeds in 6 ml solution; Lodhi (1975), 30 seeds each of little bluestem (*Andropogon scoparius*) and brome grass in an unspecified volume; Lodhi (1979), 25 seeds each of *Salsola kali* L. and radish (*Raphanus sativus*), thinned to 10 seedlings at 24 hr, in an unspecified volume; Liebl and Worsham (1983), 15 seeds of morning glory (*Ipomoea lacunosa* L.) and 10 each of corn (*Zea mays* L.) and soybean (*Glycine max* L.) in 10 ml solution; Blum et al. (1984), 25 seeds of cucumber in 5–7 ml solution; and Cameron and Julian (1980), 2 seedlings of lettuce in 2 ml solution. Similar variability of experimental conditions is seen in the literature for other compounds.

Blum et al. (1984) showed that the concentration of ferulic acid in a bioassay with cucumber declined approximately 80% (at high concentrations) to 100% (at low concentrations) during the 48 hr of the bioassay. This was due to a combination of plant uptake and microbial breakdown. The decrease in phytotoxin concentration over time suggested to us that solution volume and seed number may be of crucial importance in allelopathic bioassays. We hypothesized that the degree of inhibition caused by an allelopathic compound will depend upon both phytotoxin concentration and the total amount of phytotoxin available for uptake. Since solution volume and seed number determine the amount of phytotoxin available per seed, greater toxicity will be observed at a given concentration if there are fewer seeds per dish or a large solution volume. Lower phytotoxin concentrations may actually produce greater inhibitory effects than higher ones, if the total amount available per plant for uptake is greater. The experiments reported here were conducted to test these hypotheses and assess the magnitude of these effects in radicle elongation bioassays.

METHODS AND MATERIALS

Source of Materials. Cucumis sativus cv. Early Green Cluster seeds were purchased from Wyatt Quarles Seed Company, Raleigh, North Carolina. Ferulic acid, caffeic acid, vanillic acid, juglone and MES [2-(*N*-morpholino)ethanesulfonic acid] were obtained from Sigma Chemical Company, St. Louis, Missouri.

Experimental Design and Methods. Seeds were placed in plastic Petri dishes

(100 × 15 mm or 100 × 25 mm) which contained Whatman No. 1 filter paper and specified volumes of the test solution. The seed number–solution volume combinations were 25 seeds/5 ml, 5 seeds/5 ml and 25 seeds/90 or 95 ml. At a given concentration, the relative amount of phytotoxin available per seed ranged from x (25 seeds/5 ml) to $19x$ (25 seeds/95 ml). Replication gave 75–100 seeds per treatment.

The larger 100 × 25-mm Petri dishes were used for the 90-and 95-ml treatments, with the seeds and filter paper placed on a plastic platform inside the dish. The platform's height was adjusted so that seeds were not immersed but were in contact with approximately the same levels of solution as in other treatments. The platform and filter paper were perforated to allow free flow of solution.

Initial experiments were conducted according to the method of Blum et al. (1984), with ferulic acid dissolved in 15 mM MES and the pH adjusted to 5.8 with 1.0 N NaOH. These workers reported osmolalities of up to 25 mosm for 15 mM MES plus 3 mM phenolic acids and saw no inhibition of cucumber radicle growth over this range. In our experiments, however, 15 mM MES inhibited growth of controls compared to distilled deionized water (34.5 vs. 40.9 mm for 25 seeds/5 ml controls, and 37.5 vs. 46.6 mm for 5 seeds/5 ml controls, significantly different at the 0.05 level). Therefore, additional studies were conducted using distilled deionized water as solvent. Sterile techniques (filter sterilization, autoclaving) were not used since preliminary experiments showed no effect on the results (radicle lengths of seedlings in 2 mM ferulic acid, 25 seeds/5 ml, were 23.5 mm in nonsterile solution vs. 21.6 mm in sterile solution, not significantly different at the 0.05 level). Because of concern about oxygen depletion by seed respiration, Petri dishes were opened to ambient air for approximately 5–10 sec every 24 hr. Growth of aerated controls actually was decreased slightly (2–4 mm) compared to nonaerated controls (data not shown).

Seeds were germinated in the dark in a growth chamber at 25–27°C. Radicle lengths of germinated seed were measured in mm after 48 or 72 hr. Seeds were considered to be germinated if the radicle protruded at least 2 mm. The germination rate was approximately 95%.

Chemical Analyses. Chemical analyses were performed on a Bio-analytical Systems Inc. high-pressure liquid chromatograph (HPLC) (West LaFayette, Indiana), using a reverse-phase (C_{18}) column (150 × 4 mm) and detection at 254 nm. A Shimadzu Chromatopac C-R3A (M. B. Nathanson and Associates, Loudon, Tennessee) was used for peak integration and retention time measurements. Compounds were eluted isocratically at 1.5 ml/min with water–methanol–acetic acid (55:45:1, v/v). For analysis, the solution remaining in a Petri dish at 48 or 72 hr was pipetted off, and the dish, filter paper and germinated seeds were rinsed thoroughly with distilled deionized water. The amount of phenolic acid in the resulting solution was then determined.

Data Analysis. All data were subjected to standard analysis of variance procedures. A significance level of $P < 0.05$ was used for all multiple comparison tests.

RESULTS

Radicle growth is a function of both concentration and the amount of phytotoxin available per seed (Figure 1 and 2). Greater inhibition was observed as the amount of ferulic acid available per seed at a given concentration increased. Radicle lengths after 48 hr in buffered 2.0 mM ferulic acid were 71%, 49%, and 47% of controls for the 25 seeds/5 ml, 5 seeds/5 ml, and 25 seeds/95 ml treatments, respectively. The same trends occurred at concentrations of 0.5 and 1.0 mM, with values for the 5 seeds/5 ml treatment intermediate between the 25 seeds/5 ml and 25 seeds/95 ml treatments. No differences in the relative degree of inhibition were noted between 48 and 72 hr (Figure 1). Solution pH remained between 5.6 and 5.8 for all treatments.

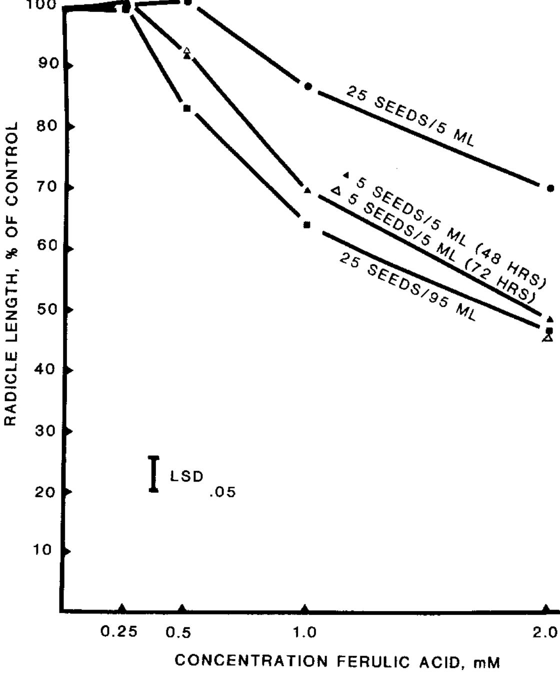

FIG. 1. Effect of seed number and solution volume on inhibition of cucumber radicle growth by ferulic acid in 15 mM MES at pH 5.8.

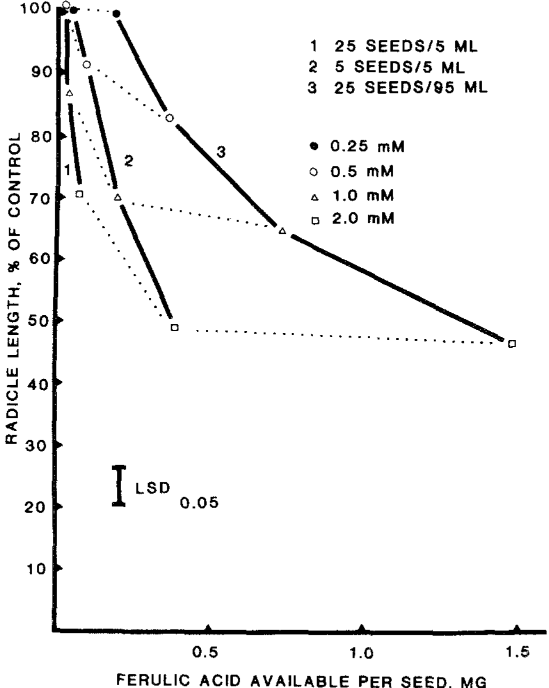

FIG. 2. Effect of the amount of ferulic acid available at pH 5.8 on inhibition of cucumber radicle growth.

In distilled deionized water, inhibition of radicle growth by ferulic acid was greater and differences between the seed number/solution volume treatments more pronounced than in buffered solutions (Figure 3). The initial pH of unbuffered 2.0 mM ferulic acid was 3.0. No inhibition of radicle growth was seen in controls at this pH range (data not presented). The toxicity of phenolic acids, however, is known to decrease as pH increases (Harper and Balke, 1981; Blum et al., 1985), and the final pH values for the 2.0 mM ferulic acid solutions were 5.4, 3.9, and 3.1 for the 25 seeds/5 ml, 5 seeds/5 ml, and 25 seeds/90 ml treatments.

Radicle lengths of 25-seed controls were slightly shorter than the 5-seed treatments in both MES and distilled deionized water. In MES, control radicle lengths at 48 hr were 40.8, 49.1, and 45.4 mm for the 25 seeds/5 ml, 5 seeds/5 ml, and 25 seeds/95 ml treatments. In distilled deionized water, they were 36.8, 43.4, and 40.7 mm for the corresponding treatments.

Ferulic acid concentrations diminished greatly over 48 hr for the 25 seeds/5 ml treatment, with only 9% remaining at 2.0 mM (Table 1). The percentage

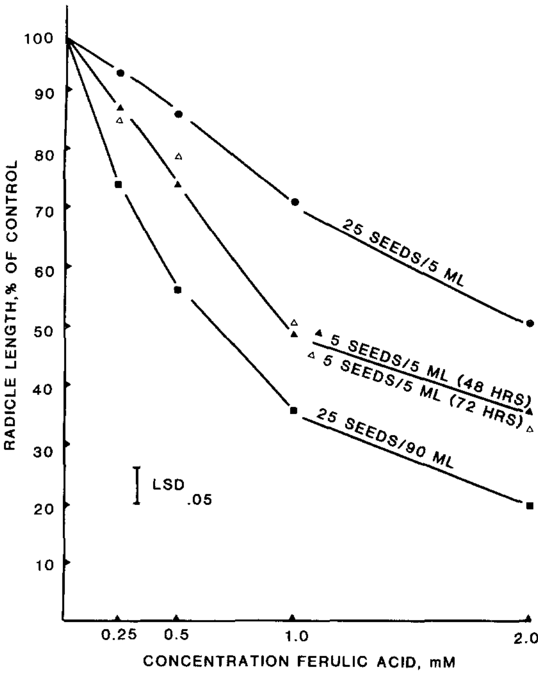

FIG. 3. Effect of seed number and solution volume on inhibition of cucumber radicle growth by ferulic acid in distilled, deionized water.

TABLE 1. PERCENTAGE OF FERULIC ACID REMAINING IN SOLUTION AT 48 AND 72 HOURS FOR TREATMENTS IN 15 mM MES

Initial conc. (mM)	% remaining after 48 (72) hr ± SE[a]		
	25 seeds/5 ml[b]	5 seeds/5 ml[c]	25 seeds/95 ml[b]
0	0	0	0
0.25	0	0	35.1 ± 21.7
0.50	0	1.5 ± 1.0	59.1 ± 14.0
1.0	0	38.9 ± 6.6	81.1 ± 8.1
2.0	8.6 ± 2.5	50.0 ± 2.4 (39.3 ± 8.3)	90.6 ± 1.0

[a] Detection limits were 25 ng/10 μl injection. Values in parentheses are for 72 hr.
[b] $N = 2$.
[c] $N > 5$.

remaining was higher for the 5 seeds/5 ml treatment (50% at 2.0 mM) and highest for the 25 seeds/95 ml treatment (91% at 2.0 mM). As expected, the 95 ml volume stabilized the concentration of ferulic acid relative to the other treatments. After 72 hr, 39% of the ferulic acid remained for 5 seeds/5 ml 2.0 mM treatment. In general, levels of ferulic acid remaining after 48 and 72 hr were lower with distilled deionized water than in buffered solutions (Table 2).

There was significant loss of ferulic acid over 48 hr in water controls with no seeds. This loss increased at 72 hr. Small amounts were lost by physiochemical degradation, and additional amounts could not be recovered due to adsorption on the filter paper. For 2.0 mM ferulic acid, the percentage remaining after 48 hr was 84.4 ± 7.0% (nonsterile) and 85.4 ± 0.5% (sterile) for 0 seeds/5 ml controls. In sterile controls without filter paper, the amount remaining was 92.6 ± 3.1%.

In agreement with the results of Blum et al. (1984), small amounts of metabolic products were detected in Petri dishes of germinating cucumber seedlings containing ferulic acid solutions. These included vanilic acid, protocatechuic acid, and an unknown compound. No differences were apparent in the metabolic products formed in 15 mM MES and distilled deionized water.

In tests with vanillic acid, caffeic acid, and juglone, radicle growth also decreased as the amount of available phytotoxin increased from x (25 seeds/5 ml) to $18x$ (25 seeds/90 ml) (Table 3). Striking results were seen with juglone, a quinone for which pH effects would not be expected to complicate results. With 0.1 mM juglone, radicle lengths were 88% of control with 25 seeds/5 ml, 68% with 5 seeds/5 ml, and 56% with 25 seeds/90 ml.

TABLE 2. PERCENTAGE OF FERULIC ACID REMAINING IN SOLUTION AT 48 AND 72 HOURS IN DISTILLED DEIONIZED WATER

	% remaining after 48 (72) hr ± SE[a]		
Initial conc. (mM)	25 seeds/5 ml[b]	5 seeds/5 ml[c]	25 seeds/90 ml[b]
0	0	0	0
0.25	0	0	10.7 ± 3.4
0.5	0	5.2 ± 2.5 (0)	28.9 ± 25.2
1.0	Trace	10.0 ± 4.7 (0)	76.2 ± 1.7
2.0	4.2 ± 0.1	35.3 ± 5.4 (5.6 ± 2.2)	76.7 ± 2.0

[a]Detection limits were 25 ng/10 μl injection. Values in parentheses are for 72 hr.
[b]$N = 2$.
[c]$N = 5$.

TABLE 3. INHIBITION OF CUCUMBER RADICLE ELONGATION BY VANILLIC ACID, CAFFEIC ACID, AND JUGLONE IN DEIONIZED DISTILLED WATER

	Radicle length, mm (% control)		
Phytotoxin conc. (mM)	25 seeds/5 ml	5 seeds/5 ml	25 seeds/90 ml
Control	40.6 b[a]	46.8 a	38.1 bc
Vanillic acid			
0.5	38.2(94) bc	34.2(73) de	20.5(54) hi
2.0	18.3(45) i	13.8(30) jk	11.6(30) k
Caffeic acid			
0.5	39.9(98) b	49.1(105) a	32.3(85) ef
2.0	30.2(74) f	26.4(56) g	20.2(53) hi
Juglone			
0.1	35.5(88) cd	32.0(68) ef	21.4(56) h
0.5	15.4(38) j	7.9(17) l	5.7(15) l

[a] Means followed by the same letter are not significantly different at the 0.05 level by Duncan's multiple-range test.

DISCUSSION

Effect of Volume and Seed Number in Radicle Elongation Bioassays. The data demonstrate that solution volume and seed number are important factors in radicle elongation bioassays. For any given concentration, larger solution volumes or lower seedling densities increase the amount of phytotoxin available per seed and thereby increase the amount of inhibition observed. Similarly, lower phytotoxin concentrations can produce equivalent or greater inhibitory effects than higher concentrations when the amount available per seed is greater. In the experiments with ferulic acid, radicle lengths 70% of control were observed at 1.0 mM with 25 seeds/95 ml, while with 25 seeds/5 ml, approximately the same level of inhibition was not reached until the greater concentration of 2.0 mM (Figure 1), roughly a twofold difference in initial concentration. Similar doses of phytotoxin, taken up under different conditions of total amount available and concentration, can be expected to result in equivalent levels of inhibition. This is implied by Figure 2.

Our interpretation attributes the disappearance of ferulic acid and the resulting inhibition of growth primarily to uptake of ferulic acid by the germinating seedlings. While there may be some microbial breakdown of ferulic acid to more or less phytotoxic substances (Liebl and Worsham, 1983; Blum et al., 1984), the relative stability of ferulic acid concentrations in our 25 seeds/95 ml treatment argues against this as a complicating factor in these experiments.

Although effects observed in water solution were greater than in buffer, the interpretation of the data is complicated somewhat by pH changes which occurred during the course of the experiment. These results emphasize the importance of buffering pH when conducting bioassays with phenolic acids.

Reasons for differences in control radicle lengths with various seed number–solution volume combinations are not clear. Water did not appear to be limiting in any treatment. At least 0.5 ml of liquid could be removed by pipet at the end of the assay for all treatments. Increasing the solution volume in high-seed-density controls did not increase growth (data not presented). Some cucumber accessions are allelopathic (Putnam and Duke, 1974; Lockerman and Putnam, 1979, 1981a, b). The shorter radicle lengths of high-seed-number controls thus may be due to autotoxic inhibitors produced by cucumbers which accumulate to higher concentrations when there are more seedlings per dish.

In light of our results, most current assays would seem to be conservative in assessing possible allelopathic effects. The 48-hr 25 seeds/5 ml treatment is representative of many current experiments. Greater inhibition, however, was observed with both the 5 seeds/5 ml and 25 seeds/95 ml treatments. Results with 0.5 mM ferulic acid (Figure 1, radicle lengths 83–101% of control) and 0.1 mM juglone (Table 3, radicle lengths 56–88%) suggest that phytotoxic compounds possibly are being overlooked when extracts are screened. While seed number and solution volume have been a concern in designing appropriate growing conditions for assay species, effects of seed number and solution volume on the magnitude of inhibition must be considered in standardizing conditions for bioassays. For cucumber and seeds of similar size, the 5 seeds/5 ml treatment, requiring only 75 ml solution for 15 replicates, may represent a good compromise between conditions of maximum inhibition (25 seeds/90 ml, 270 ml for three replicates) and availability of very small amounts of experimental material.

Possible Implications to Greenhouse and Field Studies. Analogous to any nutrient necessary for plant growth, plants compete for available phytotoxins. The observed effects of volume and seed number in radicle elongation bioassays result from this competition. Numerous herbicide studies have shown similar effects, with greater phytotoxicity in greenhouse and field studies as plant density is decreased (Skipper, 1966; Burrill and Appleby, 1978; Hoffman and Lavy, 1978; Andersen, 1981; Winkle et al., 1981; Khedir and Roeth, 1981; recently reviewed by Appleby, 1985). Hoffman and Lavy (1978), using ^{14}C-labeled atrazine, showed that uptake per plant decreased 50% as soybean density increased, demonstrating competition for this phytotoxin. In one field study, no phytotoxicity was seen at higher densities, while dry-weight reductions of 75% occurred at low density. Effects of similar magnitude were seen when the amount of soil per pot was increased from 225 g (no growth reduction) to 900 g (>90% dry weight reduction) at constant plant density.

Clearly, the phenomenon of competition for phytotoxins should be considered in greenhouse and field studies of allelopathy. High seedling densities and/or small pot volumes may yield results which do not support a hypothesis of allelopathy when, in fact, phytotoxins are present in significant concentrations. In the field, similar effects due to differences in plant density may occur. This suggests that allelopathy may be more important ecologically in situations where overall plant densities are lower because of environmental and other constraints. In these cases, phytotoxin concentrations which cause inhibitory effects in the field may be considerably lower than those shown to be toxic in laboratory assays, especially if soil concentrations are replenished by some mechanism such as daily input of toxins by rains or a continual input from microbial breakdown of plant residues. It has long been supposed that if only small amounts of a phytotoxin are present in the soil, these could be inhibitory if the concentration is high enough in a localized area such as a litter or mulch layer (see Guenzi and McCalla, 1966). This hypothesis is also supported by Figure 2.

Finally, decreased plant growth as plant density decreases is a reversal of effects normally observed when plants compete with one another (see Kira, 1953; Dekker et al., 1983; Harper, 1977). Such results strongly imply the presence of a toxic substance in the soil. Assuming similar effects can be demonstrated for naturally occurring phytotoxins, experiments in which plant density is varied could provide a tool for demonstrating the presence of allelopathic compounds in the soil and separating allelopathic from competitive interference. Further experiments to test these hypotheses are being designed.

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ORIENTATION BEHAVIOR AND EFFECT OF EXPERIENCE AND LABORATORY REARING ON RESPONSES OF *Cotesia melanoscela* (RATZEBURG) (HYMENOPTERA: BRACONIDAE) TO GYPSY MOTH SILK KAIROMONE

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Abstract—*Cotesia melanoscela* responds the same way to the silk of its host, gypsy moth larvae, *Lymantria dispar* L., whether or not it has previously been exposed to hosts. Parasites maintained in the laboratory for six or 25 generations also responded similarly, suggesting that orientation behavior is not modified by laboratory rearing. Details of silk-related orientation behavior were analyzed by tracing paths of female parasites when on or off silk-covered substrates. Females made 31° average turns every 0.5 cm whether or not they were examining silk. They tended to turn back onto a silked area when encountering a border, and their speed of movement when not examining silk was about three times higher than when examining silk.

Key Words—*Cotesia melanoscela*, gypsy moth, *Lymantria dispar*, Lepidoptera, Lymantriidae, orientation behavior, silk kairomone, Hymenoptera, Braconidae.

INTRODUCTION

The gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), parasite *Cotesia* (=*Apanteles*) *melanoscela* (Ratzeburg) is known to respond to a kairomone on gypsy moth silk by intensely examining areas which have silk strands on them (Weseloh, 1976). Kairomone-mediated behavior increases contacts with hosts and oviposition attempts (Weseloh, 1977), and responses to silk decrease over several minutes due to habituation (Weseloh, 1980).

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Other aspects of the interaction remain unexplored, however. For example, while many natural enemies respond to host kairomones (Weseloh, 1981), little is known about their actual orientation mechanisms in most cases. Waage (1978) has suggested that most kairomones perceived on contact cause a decrease in locomotory movement (inverse orthokinetic response) and/or an increase in the rate of turning, either at the edge of the kairomone "patch" or randomly within the patch itself. Strand and Vinson (1982) found that the parasite *Cardiochiles nigriceps* Vierick (Hymenoptera: Braconidae) increased its rate of turning inside a kairomone patch, but also increased rather than decreased its rate of locomotion. Sabelis et al. (1984) showed that starved predatory mites, *Phytoseuilus persimilis* Athias-Henroit (Acarina: Phytoseiidae), walked slower and turned more often than fed mites in a kairomone plume and that both fed and starved mites tended to turn back toward an odor source if they happened to leave it. Another predator, *Coccinella septempunctata* Bruchii (Coleoptera: Coccinellidae), did not respond directly to prey kairomone with such behaviors, but after consuming a prey aphid increased its rate of turning and decreased its locomotory rate (Nakamuta, 1985). Such changes in orientation behavior caused by kairomone perception might also cause specific changes in the orientation movements of *C. melanoscela*.

Another area in which little work on *C. melanoscela* has been done is the effect of previous experience on subsequent parasite behavior. *Pseudeucoila bochei* Weld (Hymenoptera: Cynipidae), for instance, cannot discriminate between parasitized and unparasitized drosophilid hosts until it has had an opportunity to attack unparasitized ones (Van Lenteren, 1976). The already-mentioned *C. nigriceps* recognizes a kairomone patch much more readily after it has parasitized hosts than before so (Strand and Vinson, 1982). Similarly, *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) changes its host preference depending on the particular species of muscoid host puparia that it had previously been exposed to (Cornell and Pimentel, 1978). Even responses to host and microhabitat stimuli change depending on preconditioning, as Vet and Opzeeland (1984) found for two *Asobara* spp. (Hymenoptera: Braconidae: Alysiinae) which attack drosophilids.

A third factor which could affect the behavior of *C. melanoscela* is the number of generations it has been reared in the laboratory. Hoy (1975) showed that reared *C. melanoscela* had slower development, lower progeny production, and lower sex ratios (female–male) than forest-collected parasites. Thus the possibility of behavioral changes is very real.

The present study was undertaken to investigate these areas of research. In particular, differences in host-related behavior of naive and experienced *C. melanoscelus*, responses to silk of parasites reared for different lengths of time in the laboratory, and detailed orientation behavior of *C. melanoscela* when it encounters areas of gypsy moth silk were measured.

METHODS AND MATERIALS

Insects. The *C. melanoscela* adults in these experiments were originally reared from gypsy moth larvae collected in Connecticut forests, and the colonies were maintained on gypsy moths fed an artificial wheat-germ diet (Yamamoto, 1969). One colony was started from gypsy moths collected in spring 1984 (old colony) and one from gypsy moths collected in spring 1985 (new colony).

Naive vs. Experienced Females. This test was run in September 1984 using the old colony when it was ca. five generations old. Female parasites which had emerged and presumably mated the day before were placed in 473-ml, 8-cm-high by 11.5-cm-diam. paper cups with clear plastic lids and honey and water. Parasites were considered experienced after being held for one hr with ca. 20 first and second gypsy moth instars in such containers, while naive insects were held with no hosts. Both groups were then held at least 2 hr without hosts before testing.

Silk substrates were made as follows: a 1.5-cm square of parafilm was wrapped with 1, 5, or 10 strands of silk by dropping a first or second gypsy moth instar and winding the resulting silk strand around the parafilm for the required number of times. The parafilm square was fastened to the bottom of a 9.5-cm glass Petri dish by pressing on the four corners with forceps. The dish was inverted over a mirror so the insect could be easily watched. An experienced or naive *C. melanoscela* female was introduced and observed for 10 min, and the number of contacts with the parafilm, examinations of silk, and total time examining silk were recorded (see Weseloh, 1976, for description of silk-related behavior). Experienced and naive females were alternated in successive tests. Ten replicates were run. The number of contacts and examinations, percentage of contacts leading to examinations, and total time spent examining were each analyzed by two-way analysis of variance and selected treatment comparisons (Ostle, 1963).

Old Colony vs. New Colony. This test was similar to the first one except that all females were naive, the old colony females which were in culture for ca. 25 generations were compared to new colony females in culture for ca. six generations, and only two silk "concentrations" (1 and 10 strands) were used. Ten replicates were run from October 23, to November 21, 1985.

Orientation Behavior. Orientation behavior of *C. melanoscela* females was studied by tracing their paths in the presence or absence of silk. Squares (1.5 cm) of parafilm were wrapped with either 0, 1, 5, or 10 strands of silk and fastened on the bottom of a 95-cm-diam. glass Petri dish, the outside bottom of which was frosted by wet-grinding with No. 600 carborundum grit. The dish was inverted over a glass plate extending over the edge of a counter. A thin 12-cm-diam. plastic disk was fixed to the underside of this glass plate by an arrangement of slides so disks could be quickly exchanged. The whole apparatus

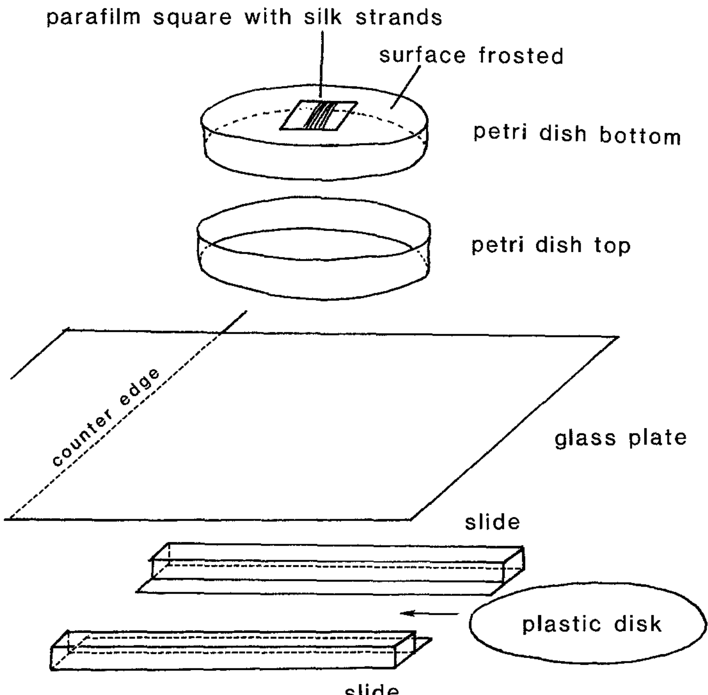

FIG. 1. Exploded diagram of apparatus used for tracing paths of *C. melanoscela* females (drawing by Wilhemina Smith).

(Figure 1) was illuminated from below, and the position of the parafilm and the silk on the parafilm was marked on the plastic disk from below with a felt pen. This was easy to do because the shadow of the pen projected onto the frosted glass bottom of the chamber. A female *C. melanoscela* was introduced, and her path was marked on the plastic disk for 0.5 min, the disk was exchanged for a new one, position of parafilm and silk quickly marked on it, and the female's path was traced for another 0.5 min. This continued for 5 min total. The first group of tests was run in May and June 1985 and used only old colony females (ca. 18 generations in culture). Four replicates for each silk "concentration" were run. Angle changes which occurred when parasites were on or off silk were measured by dividing each traced path into 0.5-cm straight segments (cords), starting from the beginning of the path. The angular changes in direction from one such straight line segment to another were the data recorded (Figure 2).

Angle changes occurring off silk were analyzed by multiple regression analysis (analysis of variance was not used because some females did not move during some time periods, with resulting missing data). The two independent

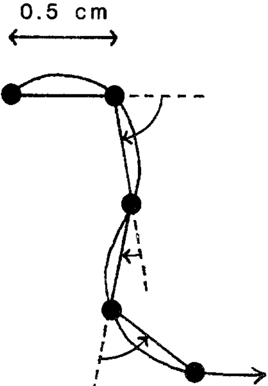

FIG. 2. Diagram of traced path of a *C. melanoscela* female, showing 0.5-cm cords (straight sections) and angular changes in direction as measured by successive cords.

variables were number of silk strands (x_1) and time period (x_2). The dependent variable (y) was the average angle change for one female at a particular time period and number of strands (model: $y = b_0 + b_1x_1 + b_2x_2$). T tests were used to determine differences in angle changes when on or off silk for each time and silk amount combination (46 in all). Jones (1984) pointed out that t tests can be used to make such multiple comparisons if the rejection regions for making a type-I statistical error are modified. This is because when the null hypotheses are true in a many-comparison situation, the probability of rejecting the null hypothesis in one or more of the comparisons can be quite high simply due to chance if the usual probability levels ($P = 0.05$ or 0.01) are used. In order to be 95% confident that the null hypothesis is false in 46 t-test comparisons, one must use a probability level of $1 - (0.95)^{1/46} = 0.001$ for each individual test (Jones, 1984). This was the level used in each comparison.

The above tests were also analyzed to determine the tendency of females to turn back from boundaries associated with silk-covered areas. To generate more replicates, additional tests were run in fall 1985 with new and old colony females. For analysis, results for different colonies and 0 and 1 strands were so similar that these were pooled. The variable analyzed was the proportion of times a female turned back when she went from parafilm to the background Petri dish surface, minus the proportion of times she turned back when going from the background to the parafilm, i.e., the net proportion of turnbacks on parafilm was determined. This was done for each time period for each female. Results for 10 and 20 strands were not pooled, and the proportion of boundary contacts resulting in turnbacks when going from silk to parafilm minus the proportion going from parafilm to silk (net proportion of turnbacks on silk) was also determined for each time period.

To determine trends and significance of data, an iterated, locally weighted regression procedure was used (Cleveland, 1979) to give smoothed scatterplots of net proportion turnbacks vs. time period. Estimates of smoothed value variabilities were made using the bootstrap procedure (Efron and Gong, 1983). In this resampling scheme, random selections with replacement are made from the original data and the weighted regression procedure used on the selections to get a new set of smoothed values at the various time periods. This was done 100 times, giving a range of artificially generated, smoothed net turnback proportions. Using the procedure for finding bias-corrected confidence intervals (Efron and Gong, 1983), 95% confidence intervals could be placed around the original net values. If the confidence interval did not include zero, the true net value could be said to be different from zero with 95% confidence, and thus that the insects tended to turn back most often when approaching the border from a particular side.

To determine how fast females move, the path-tracing apparatus was used with a parafilm square on which 20 silk strands were wrapped. At instances where a female was either examining silk or not, her path was traced as long as possible and the elapsed time measured with a stop watch. The path length was later measured by rolling a plastic, circular protractor marked in degrees over the entire tracing and converting the number of degrees covered to linear distance. Thus speed of movement could be calculated. Thirteen path lengths on silk and 20 off silk from 21 females were so measured (an attempt was made to get both kinds of path lengths from each female, but this was not always possible). The parasites' speeds on and off silk were compared by t test.

RESULTS AND CONCLUSIONS

Naive vs. Experienced Females. There were few differences in parameters whether or not female *C. melanoscela* had been preconditioned by exposure to gypsy moth larvae before tests (Table 1). Experienced females may have been more active, because they contacted the parafilm at a greater frequency than did naive females, but no differences in other parameters occurred. There were some differences in responses to different amounts of silk, but only for the percent of contacts leading to examinations and total examination time. A significant interaction occurred only between silk amount and female type for number of contacts ($F = 3.478$, 2 and 54 df, $P < 0.05$).

Old Colony vs. New Colony. Few differences occurred between old and new colony females in their responses to silk (Table 1). Old colony females spent less time examining than did new colony females, but that was the only difference. No differences occurred between results for different silk amounts, even though trends were what one would expect (i.e., more silk, more activity). All interactions between female type and silk amount were not significant. Thus

TABLE 1. EFFECT OF EXPERIENCE AND LENGTH OF COLONIZATION ON RESPONSE OF *Cotesia melanoscela* TO DIFFERENT QUANTITIES OF SILK, GIVING AVERAGES FOR EACH TREATMENT LEVEL AND VARIABLE AS DETERMINED FOR KIND OF FEMALE AND SEPARATELY FOR SILK CONCENTRATIONS (10 REPLICATES)[a]

Treatment	No. contacts	No. exams.	Contacts leading to examinations (%)	Total time examining (sec)
Experiment 1. Effect of experience				
Female type				
Naive	10.1a	4.0a	37.3a	28.7a
Experienced	15.9b	4.9a	38.4a	42.3a
Silk concentration				
1 silk strand	15.5a	3.1a	20.6a	17.0a
5 silk strands	10.2a	4.0a	43.6b	33.0ab
10 silk strands	13.2a	6.3a	49.3b	56.4b
Experiment 2. Effect of colonization				
Female type				
Old colony (25 gen.)	8.9a	2.3a	22.7a	4.25a
New colony (6 gen.)	12.0a	4.25a	30.1a	15.4b
Silk concentration				
1 silk strand	8.1a	2.2a	23.9a	7.75a
10 silk strands	12.8a	4.35a	28.9a	11.9a

[a] Numbers in each column and each treatment followed by different letters are significantly different at $P < 0.05$ by two-way analysis of variance and selected treatment comparisons.

the old colony females may have been less active than new colony ones, but behavioral differences between the two colonies were small.

Detailed Orientation Behavior. The multiple regression of average angle change away from silk (y) on number of silk strands (x_1) and time period (x_2) was $y = 30.957 + 0.229x_1 - 0.014x_2$, $N = 118$. Using a t test (Ostle, 1963), the coefficients for the x values were not found to be different from 0 (t value for coefficient of $x_1 = 1.707$, 115 *df*, $P < 0.1$, t value for coefficient of $x_2 = -0.018$, 115 *df*, $P > 0.5$). Thus the amount of turning when females were not on silk was not influenced by either the amount of silk present or the passage of time, and averaged $31° \pm 11.7°$ (square root of residual mean square) per turn. When t tests were run to compare rates of turning off silk with rates of turning on silk, only one t value of 46 compared was significant ($t = 3.794$, $P < 0.001$, 35 *df*). Thus with these data there is no reason to expect the parasite to have different turning behaviors when on or off silk.

A different situation exists concerning turnbacks at borders. Figure 3 gives the net proportion turnbacks when on parafilm for all silk concentrations and the net turnbacks when on silk for 10 and 20 strands of silk. For 0 and 1 strands,

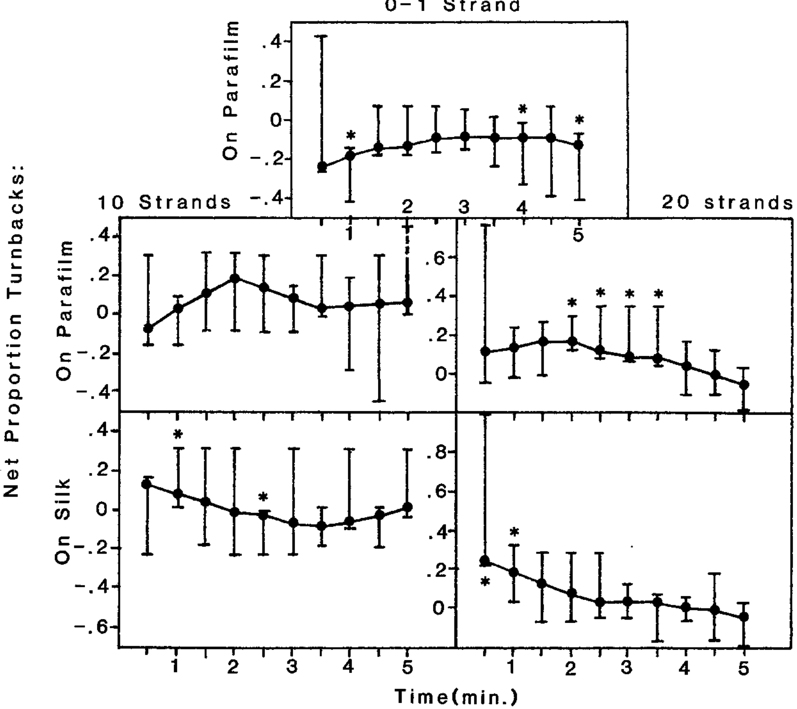

FIG. 3. The relationship between turnbacks at borders for *C. melanoscela* females at different time periods and with different amounts of silk. Data are locally weighted regression values for net proportion turnbacks on parafilm and on silk. Brackets are the 95% bias-corrected bootstrap confidence intervals for each net value. Asterisks highlight values where the confidence interval does not include 0.

all values are below 0, and some are significantly below 0 (i.e., confidence interval does not include 0) with no evident trend over time. This suggests that in the absence or undetectability of silk, females tend not to turn back at borders when they are on parafilm. When silk is detected (10 and 20 strands), many of the net values for on parafilm and silk were positive and, especially for 20 strands, some were significantly so. The net values also tended to decrease with time. Thus females tend to turn back into areas which have silk when they encounter a border, even if that border is somewhat beyond the edge of the substrate which has silk (i.e., at the background-parafilm border). This response decreases with time and could be an important factor in causing the habituation already referred to when *C. melanoscela* females are exposed continuously to silk (Weseloh, 1980).

The speeds of movement of females when on and off silk, respectively,

with 95% confidence intervals, were 0.66 cm/sec ± 0.70 and 2.10 cm/sec ± 1.17. These values were significantly different ($t = 8.392$, 31 *df*, $P < 0.001$). Thus, as with other parasitoids (Waage, 1978), *C. melanoscela* has an inverse orthokinetic response when examining silk.

DISCUSSION

This study suggests that, unlike other parasites mentioned in the introduction, *C. melanoscela* does not learn to recognize gypsy moth silk. The response of *C. melanoscela* to gypsy moth silk is a highly specific one, for the silk of even other lymantriids is examined rather little (Weseloh, 1976). Learning may be more important for parasites which attack a variety of hosts than it is for *C. melanoscela*.

Differences in the behavior of old and new colonies was not evident, but these insects should actually be different in a variety of ways. The ease with which diapause can be altered in laboratory cultures of this species (Hoy, 1975) hints at its genetic plasticity. Possibly the behaviors investigated here are so basic that even under prolonged rearing conditions, they are not greatly changed. The results suggest that the evaluation of these behaviors in the laboratory has some relevence for wild parasites.

Concerning orientation behavior, *C. melanoscela* appears to behave differently from other natural enemies tested (Strand and Vinson, 1982; Sabelis et al., 1984; Nakamuta, 1985) because its rate of turning does not noticably increase when it has detected kairomone. The reason may be related to the natural distribution of kairomone on leaves. When first and third gypsy moth instars which *C. melanoscela* attacks feed on oak leaves, they deposit strands of silk diffusely over the whole surface (personal observation). To find the caterpillar that spun the silk, *C. melanoscela* must thoroughly search all of a rather large leaf. Being a small (ca. 2 mm long) parasite, it can probably do this most successfully if it does not turn so often that it is restricted to one small section of the leaf. By responding to boundaries by turning back, it is able to quickly examine the leaf. By moving slowly, the parasite has time to recognize a host when contacted. Finally, the decrease in the turnback response ensures that the parasite will eventually stop searching if a host is not found.

The orientation behavior of *C. melanoscela* can be broken down into various, simpler behaviors, some of which differ from similar behaviors of other parasites. The differences are related to the host specificity shown by *C. melanoscela* and the distribution of kairomone in relation to the host in nature. When these factors are accounted for, the parasite is seen to be well adapted to find hosts efficiently.

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ATTRACTION TO PHEROMONE SOURCES OF DIFFERENT QUANTITY, QUALITY, AND SPACING: Density-Regulation Mechanisms in Bark Beetle *Ips typographus*[1,2]

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Abstract—The density of bark-beetle colonization of a tree could be regulated by a quantitative effect of the pheromone signal from beetles in the tree (cessation of release of attractive pheromone) or by a qualitative effect (production of pheromone components inhibiting attraction). The quantitative hypothesis was tested on *Ips typographus* by varying the release rate of the two known attractive compounds, 2-methyl-3-buten-2-ol (MB) and (4*S*)-*cis*-verbenol (cV). The highest number of beetles were captured at traps with the highest release rates. The catch was nearly proportional to the release of MB and cV at a distance between traps of 12 m or more. At 6-, 3-, and 1.5-m distances between traps deployed in a triangular arrangement there was still a good discrimination between release rates, but relatively more beetles, especially males, were caught on the blank. The lower release rates caught an equal sex ratio while the highest release rate caught only about 30% males. The qualitative hypothesis was tested by releasing the suspected inhibitors ipsdienol (Id) and ipsenol (Ie), from traps in the same amounts as cV. Only small effects were noted for *I. typographus*. However, the competitor *I. duplicatus* was attracted to Id and inhibited by Ie, while the predator *Thanasimus formicarius* was attracted to both compounds. On the other hand, when the ratio of Id or Ie to cV was 10:1 or 0.1:1 rather than 1:1, they affected the numbers of *I. typographus* attracted. A small amount of Id combined with the attractants increased trap catch, while large amounts of Id or Ie decreased attraction, especially when combined. Attack density regulation is modeled as an effect of both quantitative and qualitative mechanisms acting in sequence.

[1] Coleoptera: Scolytidae.
[2] This study was made within the Swedish project "Odour Signals for Control of Pest Insects."

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Key Words—2-methyl-3-buten-2-ol, *cis*-verbenol, ipsdienol, ipsenol, sex ratio, attraction, inhibition, *Ips duplicatus*, *Thanasimus*, switching, *Ips typographus*, Coleoptera, Scolytidae, Ceeridae.

INTRODUCTION

Regulation of colonization density is a crucial factor in the population biology of the spruce bark beetle, *Ips typographus* (L.). At high population levels the beetle is able to reach attack densities sufficient to overcome the resistance of a healthy tree by a mass attack involving several thousand beetles and governed by aggregation pheromones (Thalenhorst, 1958; Bakke and Riege, 1982). However, the high densities found on trees killed by mass attacks, 3–15 egg galleries/100 cm^2 (Thalenhorst, 1958; Lekander, 1972; Anderbrant, 1986), are often at a level where severe larval competition results in a low number and quality of offspring per female (Botterweg, 1983; Anderbrant et al., 1985). Consequently, the bark bettle needs not only a pheromone mechanism to initiate a mass attack to reach a sufficient attack density, but natural selection would also promote a system telling arriving individuals that a patch is occupied (Alcock, 1982). Arriving beetles may then colonize nearby patches, such as other parts of the tree or adjacent trees. Such "switching," where the attack first focuses on one tree but, in a few days, switches to other trees in the vicinity, has frequently been observed at high "epidemic" population densities both in *Dendroctonus frontalis* (Coulson, 1979), *D. ponderosae* (Geiszler et al., 1980), and in *I. typographus* (J.M. Hoff, unpublished; O. Anderbrant et al., unpublished).

Little is known about variation in pheromone production and response in bark beetles during the colonization phases and how pheromones may be used in regulation of density (Byers et al., 1984). Byers (1983) suggested that colonization density in *Ips paraconfusus* Lanier along the trunk of a felled tree was regulated in part by sex-specific responses to quantitative changes in the rate of pheromone release. Males appeared to avoid higher pheromone concentrations emanating from the densely colonized patches and thus settle in adjacent areas where their pheromone production spreads the area of aggregation and colonization. Schlyter and Löfqvist (1986) showed in the laboratory that walking *I. typographus* females were more attracted to stronger sources of natural pheromone than were walking males. 2-Methyl-3-buten-2-ol (MB) and (4*S*)-*cis*-verbenol (cV) are the essential components of the aggregation pheromone of *I. typographus* (Bakke and Riege, 1982; Schlyter et al., 1987b). The quantity of these two compounds in hindguts is maximal when the unmated male constructs his nuptial chamber under the bark (Birgersson et al., 1984). Later, when the males are mated and females are laying eggs, MB and cV decrease substantially, while ipsdienol (Id) and ipsenol (Ie) reach the same order of magnitude as cV. Id has been indicated as a synergist (Bakke et al., 1977) and Ie as an inhibitor (Bakke, 1981) of the aggregation pheromone.

In *I. typographus*, regulation of density and tree-switching by pheromone could then be hypothesized to take place by one or both of the following mechanisms:

1. *Quantitative Hypothesis.* Initially, males attack in relation to the amount of attractant odor. As the release increases, some of the males attracted to the initial attack site are deflected by high concentrations of pheromone when they reach the source and so attack patches (adjacent bark areas or trees) a short distance away. After mating, the males in the first patch produces less attractant, and the aggregation would switch to the newly attacked areas.

2. *Qualitative Hypothesis.* The higher amounts of inhibitors such as Id and Ie released in the later phases by the earliest arriving males cause an inhibition of attraction, especially in males. Surrounding areas would receive the new attacks and the colonization activity would switch.

From the quantitative hypothesis follows at least two predictions: First, beetles should discriminate between sources of different release rate, and males, compared to females, should be relatively less attracted to the highest rates. Second, discrimination should be significant even when sources are only a few meters apart (as are mature trees in a forest).

From the second, qualitative hypothesis, it follows that the presence of Id and Ie should, especially at the higher release rates or ratios, lower the number of beetles attracted.

In order to test these hypotheses, we have in the field studied beetles landing on sticky traps deployed in triangular arrays at four spacings with three release rates of MB and cV. We have also studied the attraction to pipe traps at constant spacings baited with a concentration range of attractants (MB and cV) with and without Id or Ie, or both, in different proportions.

METHODS AND MATERIALS

Synthetic Substances, Dispensers, and Release Rates. The synthetic substances were released from polyethylene (PE) vials with different sizes of openings (MB, cV, and high rates of Id and Ie) or from glass capillaries (low rates of Id and Ie). Release rates from PE vials were determined from weight loss in a mini wind tunnel, 0.70 m/sec, at 20°C. Weights were taken on a balance to the nearest microgram and corrected for weight changes of empty control vials. The slope with its 95% confidence interval of the regression of weight (y) on time (x) gave the estimated release rate, Table 1. The release rates of Id and Ie from capillaries were measured in a similar wind tunnel. The capillaries were 12 mm long, fused at one end, and filled from the bottom through an inserted thin capillary (Löfqvist, unpublished). The loss of liquid from the capillaries was measured by the change of the position of the meniscus under a stereomicroscope.

MB and cV were released in the field experiments at three release rates

each at a ratio of about 50 : 1, which is close to or larger than that found in male hindguts (Birgersson et al., 1984). The lowest release rate of cV was somewhat higher (about 0.03 mg/day) than intended (0.01) and the medium was lower (0.052 mg/day) than intended (0.10). This altered the target MB–cV ratio somewhat as MB was released at evenly spaced decadic steps. However, the ratio of MB to cV changes the sex ratio very little and it is the MB release that is the most critical in determining the total number caught (Schlyter et al., 1987a). The highest rate, 50 mg MB and 1 mg cV per day, corresponds to the release from 1 m of commercial Borregaard/Hercon Ipslure® dispenser, aged one week (Schlyter et al., 1987b).

Triangle Experiment: Variation of Pheromone Release Rate and Spacing. Three traps arranged in an equilateral triangle constituted a trap group. Each group had a blank trap and various combinations of two of the three possible decadic release rates of MB and cV (Tables 1 and 2). Randomization between replicates took place only within groups, as it was impractical to change the positions of whole trap groups. This means that comparisons of absolute catches between groups are not valid. However, comparisons can be made between groups based on relative catches within a group.

The chemical dispensers were housed in 30-cm sections of commercial drain pipe traps, suspended on 1.5-m-long steel pipes. The black dispenser housing was surrounded by a tubular sticky trap (31 × 19 cm diam.) of No. 4 hardware cloth coated with Stikem Special®.

For the first trapping period (six replicates between the days May 16 and 31), the traps were spaced at 3-, 6-, and 12-m distances in nine trap groups (three baitings × three spacings) (Figure 1). Since the blank caught few beetles even in groups with 3-m spacing, we decided to arrange all nine trap groups in a 1.5-m spacing distance for a second trapping period (eleven replicates between May 31 and June 12, 1982).

The test area was in a clear-cut at 160 m altitude, near Torsby, province of Värmland, Sweden, with a moderately high beetle population (more than 100 trees killed the previous year within 0.7 km). The trap groups were placed in a grid with equal distance between groups (50 m) on a gentle west slope (maximum elevation difference was 20 m).

Quantitatively and Qualitatively Different Baits at Constant Spacings. In the 1982 test, four qualitative combinations of either Id or Ie, both, or neither were released with three rates of MB and cV yielding 12 different baits (Tables 3 and 4). The release rate of Id and Ie was varied to correspond approximately with that of cV.

Black pipe traps with a white funnel at the base (''N 79 with funnel'' in Regnander and Solbreck, 1981) were used at a spacing of about 50 m. Treatment (trap with a bait) positions were randomized after each replicate. The Ie in PE vials was replaced after the first replicate as it began to polymerize.

One set of 12 traps was used in each of two widely separated sites near

TABLE 1. CHEMICALS, RELEASE RATES, AND DISPENSERS FOR 2-METHYL-3-BUTEN-2-OL, *cis*-VERBENOL, IPSDIENOL, AND IPSENOL USED FOR FIELD TRAPPING OF *Ips typographus* IN 1982 AND 1984

Compound	Chemical purity (%)[a]	Source	Nominal release rate (mg/day)	Measured release rate (±95% CI) (mg/day)[b]	Dispensers[c]
1982 experiments (Värmland, Sweden)					
2-Methyl-3-buten-2-ol, (MB)	97	Aldrich	0.5	1.2 ± 0.07	"730" with 2-μl capillary
			5.0	5.8 ± 0.3	"730" with 50-μl capillary
			50.0	57.0 ± 0.8	hard vial, 1-mm hole
cis-Verbenol, (cV)	96	Borregaard	0.01	0.03 ± 0.006	"730" with 5-μl capillary
			0.1	0.05 ± 0.001	"730" with 150-μl capillary
			1.0	1.03 ± 0.05	hard vial, 9-mm hole
Ipsenol, (Ie)	98	Borregaard	0.01	0.004 ± 0.0003	1-μl capillary
			0.1	0.32 ± 0.012	"730" with 20-μl capillary
			1.0	0.54 ± 0.007	"730" with 200-μl capillary
Ipsdienol, (Id)	98	Borregaard	0.01	0.005 ± 0.0005	2-μl capillary
			0.1	0.041 ± 0.003	50-μl capillary
			1.0	0.34 ± 0.02	"730" with 2.9-mm hole
1984 experiment (Grib skov, Denmark)					
MB	97	Aldrich	5.0	5.8 ± 0.3	"730" with 50-μl capillary
cV	99.5	KTH	0.1	0.05 ± 0.001	"730" with 150-μl capillary
Ie	98	Borregaard	0.01	0.004 ± 0.0003	1-μl capillary
			0.1	0.04 ± 0.003	20-μl capillary
			1.0	0.71 ± 0.01	"730," open
Id	98	Borregaard	0.01	0.005 ± 0.0005	2-μl capillary
			0.1	0.041 ± 0.003	50-μl capillary
			1.0	1.16 ± 0.10	two "730," open

[a] Chemical purity estimated by capillary GC. Optical purity for cV > 94% (-)-(4*S*) as estimated by its isopropyl urethane derivate separated on a XE-60-(*S*)-valine-(*S*)-α-phenylethylamide GC column. Ie and Id were racemic.
[b] For measurements of release rates see text.
[c] Dispensers were either PE vials (Kartell, Italy) of two types; "730," a 1-ml vial of soft PE; "hard," a 2-ml vial of hard PE, with capillaries or drilled holes in their lids or a section of glass capillaries fused at one end.

TABLE 2. NUMBER AND SEX RATIO OF SPRUCE BARK BEETLES, *Ips typographus* TRAPPED ON STICKY TRAPS WITH QUANTITATIVELY DIFFERENT BAITS AT FOUR DIFFERENT SPACINGS, VÄRMLAND 1982 (TRAPS WERE PLACED IN EQUILATERAL TRIANGLES)

Pheromone source release rates (mg/day)			Spacing between traps (m)							
			1.5		3		6		12	
MB	cV	Designation	Sum	% males	Sum	% males	Sum	% males	Sum	% males
0	0	Blank								
		with medium and high	198	41.4*[a,b]	86	40.7	16	62.5	6	(33)
		with low and high	86	37.2[a]	13	53.8	7	(43)	0	—
		with low and medium	11	(27)[c]	1	(100)	0	—	1	—
1.2	0.03	Low								
		with high	371	42.0**[a]	109	55.0***	51	54.9*	8	(50)
		with medium	43	44.2	5	(60)	0	—	2	—
5.8	0.05	Medium								
		with high	467	35.1[a]	332	45.8***	153	49.7*	66	31.8[a]
		with low	104	26.0[a]	30	60.0	10	(60)	14	(43)
57	1.0	High								
		with medium	491	30.1[a]	789	33.2[a]	420	39.0[a]	507	32.0[a]
		with low	398	29.2[a]	302	34.8[a]	1359	34.7[a]	246	32.9[a]

[a] Value significantly different from 50% males (95% binomial CI)
[b] Value significantly different from the catch of the high bait (*$P < 5\%$, **$P < 1.0\%$, ***$P < 0.1\%$ by chi-square).
[c] Values in parentheses calculated on less than 15 beetles.

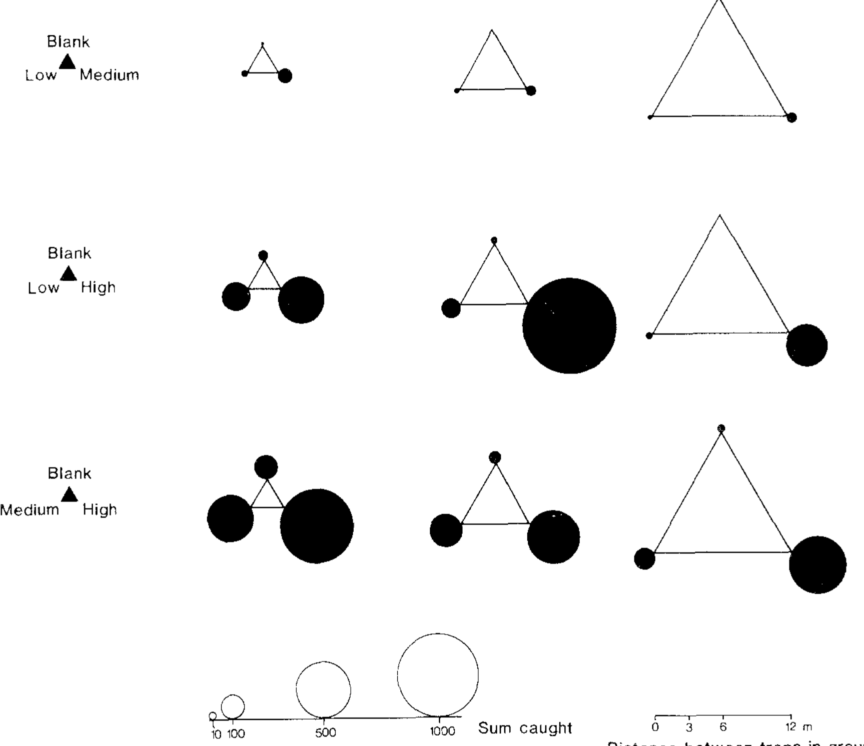


FIG. 1. Total catch on sticky traps with quantitatively different baits at different spacings within triangle trap groups during the first trapping period. Catch data from the 1.5-m spacing during the second period and release rates of baits are shown in Table 2. The different baits are indicated at the black triangles at left. Baits were randomized within trap groups and distance between trap groups was 50 m.

Torsby, province of Värmland, Sweden. Site L, a 3-year-old clear-cut area at 145 m altitude, had a low local beetle population, with no standing trees attacked the previous year within 0.5 km. Site T, a fresh clear-cut at 105 m altitude, had a high local population because many standing trees were killed within the cutting area the previous year, so that a high density of beetles were probably wintering in the duff close to the traps. Trapping was done between May 16 and June 9, 1982.

In the 1984 test, nine combinations of Id/Ie, with MB + cV at a constant medium dose, as well as MB + cV alone as a control (ten different baits) were tested (Table 5).

MB and cV were released with the same type of dispensers as used for the medium release rate in the 1982 test.

TABLE 3. BAITS AND PERCENT MALES IN CATCH OF *Ips typographus* AT THREE GROUPS OF RELEASE RATES AND FOUR QUALITATIVE COMBINATIONS OF ATTRACTANTS AND INHIBITORS, IN PIPE TRAPS, VÄRMLAND, SWEDEN 1982

Bait No.	Release rates (mg/day)				Ratio (Ie + Id)/(MB + cV)	Males in catch (%)		
	MB	cV	Ie	Id		Mean	95% CI	Sample size (*N*)[a]
1	1.2	0.03	0	0	0	52.6	37–68	38
2	1.2	0.03	0.004	0.005	0.007	50.4	42–58	141
3	1.2	0.03	0.004	0	0.003	52.6	37–68	38
4	1.2	0.03	0	0.005	0.004	44.2	37–52	156
1–4	All low baits					48.3	43.2–53.3	373
5	5.8	0.05	0	0	0	46.7	41–52	285
6	5.8	0.05	0.32	0.04	0.062	42.6	38–47	486
7	5.8	0.05	0.32	0	0.054	38.0	32–44	271
8	5.8	0.05	0	0.04	0.007	44.8	40–50	348
5–8	All medium baits					43.1	40.5–45.7	1390
9	57	1.0	0	0	0	33.0	29–37	522
10	57	1.0	0.54	0.34	0.015	26.0	22–30	520
11	57	1.0	0.54	0	0.009	30.2	26–34	519
12	57	1.0	0	0.34	0.006	32.8	29–37	521
9–12	All high baits					30.5	28.6–32.5	2082
1, 5, 9	All baits with only MB + cV					38.5	35.2–41.8	845
2, 6, 10	All baits with MB + cV + Ie + Id					31.8	29.1–34.6	1076
3, 7, 11	All baits with MB + cV + Ie					33.8	30.7–37.1	828
4, 8, 12	All baits with MB + cV + Id					38.6	35.7–41.7	1025
1–12	Site L, all baits					35.4	33.0–37.9	1463
1–12	Site T, all baits					37.6	35.7–39.6	2382

[a] Less than total catch, see Methods and Materials. Samples from sites L and T pooled unless otherwise stated.

Black pipe traps without exterior funnel ("N 79" in Regnander and Solbreck, 1981) were used in two rows with all spacings 10 m and randomized in an incomplete Latin-square design, i.e., a bait could occupy a position only once during a test. Two sets each of 10 traps were placed on two 3- to 4-year-old clear-cuts 1.5 km apart at 50 m altitude, in Esrum forest distinct, Grib skov, northern Sjaelland, Denmark. The beetle population was judged to be fairly high, with about 100 trees killed within 0.5 km the year before but none closer to traps than 100 m at both sites. Trapping was done between May 14 and June 26, 1984.

Statistics. Raw catch data, individual trap catches per replicate (trapping period of one half to several days depending on flight activities), were subjected to a series of transformations, including log, square root, and arcsin $p^{0.5}$ to achieve homogeneous variances before ANOVA (Sokal and Rohlf, 1981). The transformation which yielded homoscedacity for the catches of each treatment in different experiments, as tested by both Cochran's C and Bartlett-Box tests with $P > 5\%$, along with the highest F ratio was chosen and used in ANOVA and MANOVA on each experiment. All beetles from the sticky traps were sex separated. From pipe traps, 260 beetles (or the whole catch if less than 260) from the total catch of each bait on a site, proportionally sampled from the different replicates over the whole trapping period were separated by sex.

RESULTS

Attraction to Sources of Attractive Pheromone of Different Quantity at Different Spacings. The ability of the beetles to discriminate between pheromone sources of different strengths and spaced at various "close" distances was studied in the triangle experiment. The beetles discriminated well between pheromone sources of different strengths (Figure 1), the response being highly dependent on the release rates of MB and cV (Table 2). Even at 3-m spacing, the strongest sources had 65–83% of the beetles caught by a triangle trap group, while the blank caught only a few percent (Figure 2). There was, however, a significant increase in the catch on the blank with decreasing triangle size, and at 1.5 m spacing as much as 17% of the trap group catch was found on the blank trap (Figure 2). Also low and medium release rates had an increased catch at smaller distances when tested in trap groups with a stronger bait present. For the highest release rate, the increase in its proportion of the trap group catch with increased distance was highly significant (Figure 2).

The sex ratios of catch on the blank and low-bait traps were not significantly different from 50% at 3-, 6-, and 12-m distances (Table 2). The percent males caught was significantly less than 50% at the highest release rate at all four distances on all eight traps.

Attraction to Pheromone Sources of Different Quantity and Quality at Con-

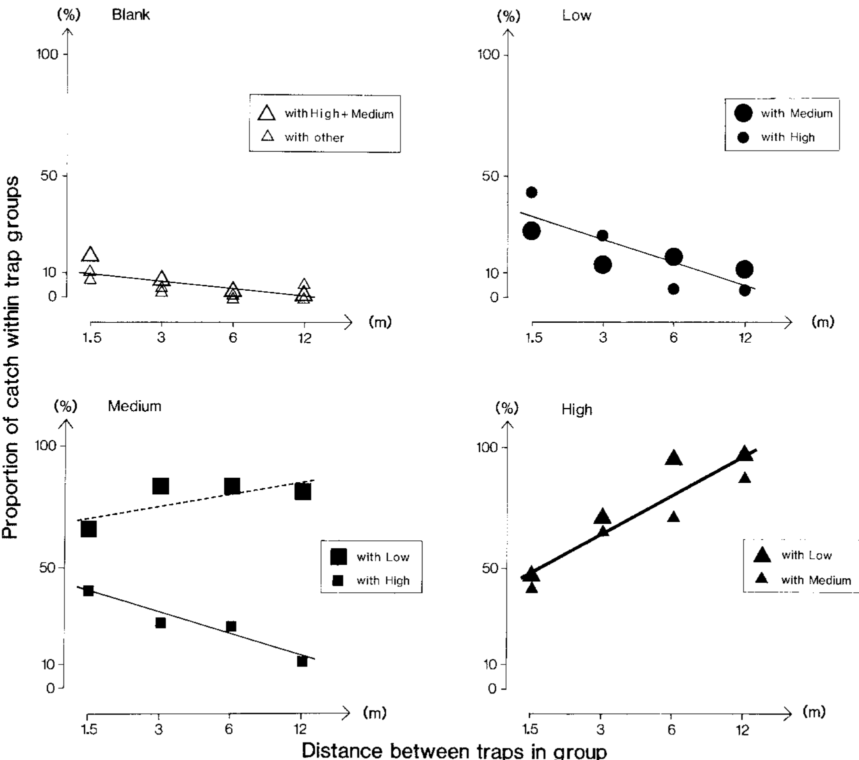


FIG. 2. Relative catches (means) within trap groups as a function of distance between traps. Significance of regression estimated by the slope confidence intervals difference from zero (thick regression line: $P < 1\%$; thin: $P < 5\%$; dotted: $P > 5\%$). Combinations not significantly different when only one line is shown. Release rates and sex ratio statistics shown in Table 2.

stant Spacing. I. typographus catch correlated well to three decadic steps in release rates of MB + cV from pipe traps spaced 50 m apart. At the site with a low local beetle population in the 1982 experiment (site L), the catch was directly proportional (slope close to unity) to the quantity of pheromone released (Figure 3). The addition of Ie, Id, or both in combination to these baits at about the same release rates as cV had very little effect. Multiple ANOVA of the first experiment showed that quantity (amount of MB + cV released) had by far the largest *F* ratio of the main effects. Quality (addition of Ie and/or Id or none) had a smaller but significant effect (Table 4). The interactions between quantity and site could be due to the more direct proportionality between bait and response at site L (Figure 3). One-way analysis of variance showed that baits with the same release of MB + cV usually formed homogeneous subsets (Figure 4).

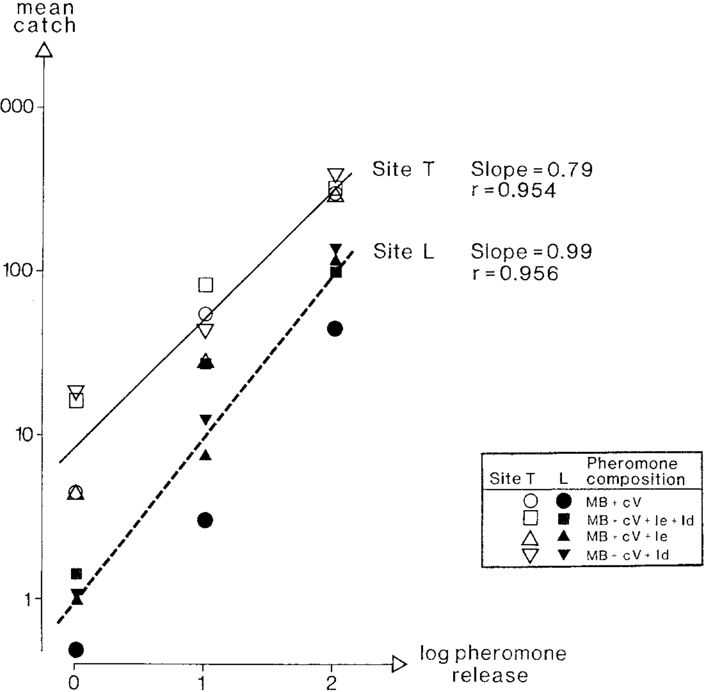


FIG. 3. Dose (log pheromone quantity) and response (untransformed mean trap catches) at sites with a high (T, 8 replicates) and a low (L, 8 replicates) local beetle population. Regression coefficient (slope) different from zero at $P < 1\%$ for both sites. Slope of regression line for site T significantly different from unity at $P < 5\%$. Release rates of the components (MB, cV, Ie, Id) of qualitatively different baits as shown in Table 3.

For the second experiment (1984) multiple ANOVA showed highly significant first-order effects when Ie, Id, or both were tested with cV at either 10 : 1 or 0.1 : 1 ratios (Table 4). The highest ratio of Ie and the combination Ie + Id, decreased the catch significantly as judged by one-way ANOVA (Figure 5). In contrast, the lower amounts of Id, the same as 1 : 1 or a tenth (0.1 : 1) that of cV, increased the catch slightly but significantly compared to the control.

Sex Ratios. An increase in bait strength caused a decrease in the proportion of catch that were males from 50% at the low dose to 30% at the high dose, similar to the ratios caught in the triangle experiment (Table 3). In fact, at the high bait, the pipe traps and the sticky traps caught an almost identical percentage of males, 32.9 and 33.4%, respectively (Tables 2 and 3). The addition of Ie/Id in amounts similar to cV did not give any significant difference between the sex ratio in the catch of the individual baits in the 1982 experiment. However, the three baits with only MB + cV had a higher percentage of males (38.5) than the three baits with both Ie and Id added (31.8%, Table 3). This

TABLE 4. FACTORIAL MANOVA ON EFFECTS ON CATCH IN PIPE TRAPS OF *Ips typographus* OF VARIATION OF MB AND cV AND ADDITION OF Ie AND Id AT CONSTANT RATIO, VÄRMLAND, 1982; AND ADDITION OF Ie AND Id AT DIFFERENT RATIOS TO CONSTANT MB + cV, GRIB SKOV 1984[a]

Effects/factor	Statistics		
	df	*F* ratio	Significance of *F* (%)
Different amount of MB + cV with Ie and Id added at constant ratio[b]			
Within cells	168	—	—
Constant	1	1276.9	<0.1
Main effects			
Quantity (amount of MB + cV)	2	192.0	<0.1
Quality (presence of either Ie, Id, or both or none)	3	5.2	0.2
Site (study area)	1	132.6	<0.1
Interactions			
Quantity × site	2	1.9	15.8
Quantity × quality	6	0.7	67.0
Quantity × quality × site	6	0.7	68.0
Different ratios of Ie and Id added to constant amount of MB + cV[c]			
Within cells	88	—	—
Constant	1	2851.6	<0.1
Main effects			
Ratio (Ie + Id/MB + cV)	2	25.9	<0.1
Quality (presence of either Ie, Id, or both or none)	3	36.2	<0.1
Site (study area)	1	0.3	59.2
Interactions			
Ratio × site	3	0.2	92.0
Ratio × quality	4	2.2	7.4
Ratio × quality × site	6	1.6	14.9

[a] For abbreviation of compounds, see Table 1.
[b] 1982 test, 16 replicates, variable analyzed was log (catch per replicate + 1/2)
[c] 1984 test, 11 replicates, variable analyzed was arcsin (proportion of catch per replicate + 1/100)$^{0.5}$

difference is mainly due to the low male ratio, 26.0%, in the traps with the highest release of MB, cV, Ie, and Id.

Similarily, in the 1984 experiment, the higher amounts of Ie plus Id added together seemed to reduce the percentage of males (Table 5). However, the sex ratios in 1984 were confounded by a constant decrease in the proportion of males caught during the trapping period. During the nine replicates between May 15 and June 8, the percent males (y) in the total catch fell steadily at about 1% per day (x), following the regression equation: $y = 38.2 - 1.20x$ ($r = 0.92$, slope different from zero at $P < 0.1\%$). A similar trend was noted in

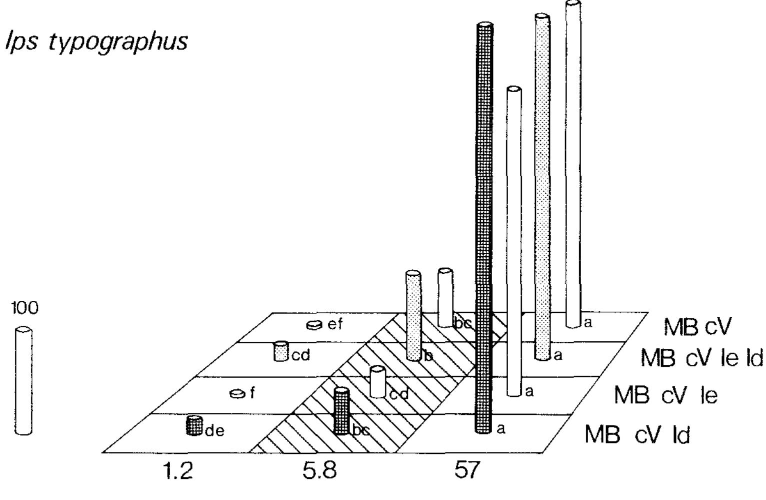


FIG. 4. Response of *Ips typographus* (untransformed mean catches of 8 replicates) towards quantitative variation of MB + cV and addition of Ie and/or Id in well-spaced pipe traps (1982 experiment, site T). Bars with the same letter are not significantly different [$P > 5\%$ by Duncan's multiple range test after ANOVA on catch transformed by log (catch + 1/4)]. Squares with parallel hatching contain baits used in both 1982 and 1984 experiments. Bars with stippled hatching indicate baits with both Ie and Id added, while cross-hatching indicates addition of Id. Sex ratio statistics and release rates are shown in Table 3.

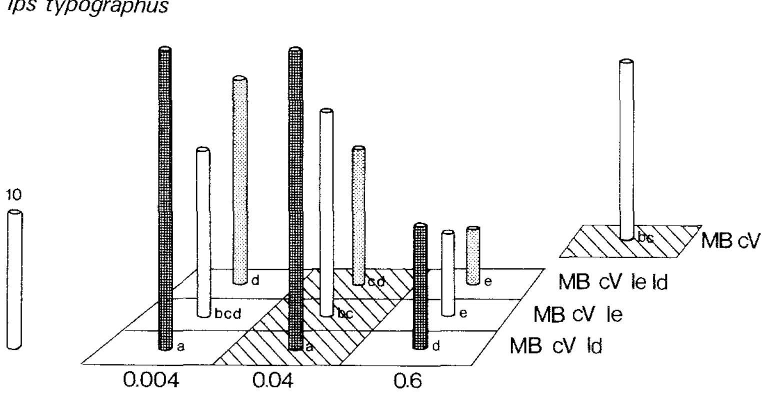
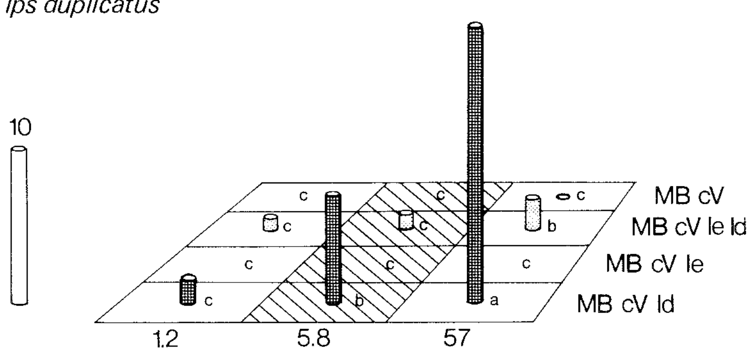


FIG. 5. Response of *Ips typographus* (untransformed means) to addition of Ie and/or Id (1984 experiment, 11 replicates, two sites pooled) to MB + cV at 5.8 + 0.05 mg/day. Statistics and hatchings as in Figure 4, except that arcsin (proportion of catch per replicate + $1/100)^{0.5}$ was used in ANOVA.

TABLE 5. BAITS AND PERCENT MALES IN CATCH OF *Ips typographus* AT CONSTANT RELEASE OF ATTRACTANTS WITH IPSENOL AND IPSDIENOL ADDED AT DIFFERENT RATIOS IN PIPE TRAPS, GRIB SKOV, DENMARK, 1984

Bait No.	Release rates (mg/day)				Ratio (Ie + Id)/(MB + cV)	Males in catch (%)		
	MB	cV	Ie	Id		Mean	95% CI	Sample size (*N*)[a]
1	5.8	0.05	0.004	0.005	0.0015	25.4	19–32	169
2	5.8	0.05	0.04	0.04	0.14	19.3	19–28	109
3	5.8	0.05	0.71	0.58	0.22	15.4	7–28	39
1–3	All baits with Ie and Id added					22.1	17.9–26.7	317
4	5.8	0.05	0.004	0	0.0007	26.3	19–35	118
5	5.8	0.05	0.04	0	0.007	27.7	21–35	155
6	5.8	0.05	0.71	0	0.12	27.0	18–39	63
4–6	All baits with only Ie added					27.1	22.6–32.1	336
7	5.8	0.05	0	0.005	0.0009	27.9	23–34	244
8	5.8	0.05	0	0.04	0.007	31.3	26–37	243
9	5.8	0.05	0	0.58	0.10	28.7	21–38	101
7–9	All baits with only Id added					29.4	25.9–33.2	588
10	5.8	0.05	0	0	0	30.3	23–38	145

[a] Equal to total catch, see Methods and Materials. Samples from the two test sites pooled as sex ratio of total catch on each site showed no significant difference.



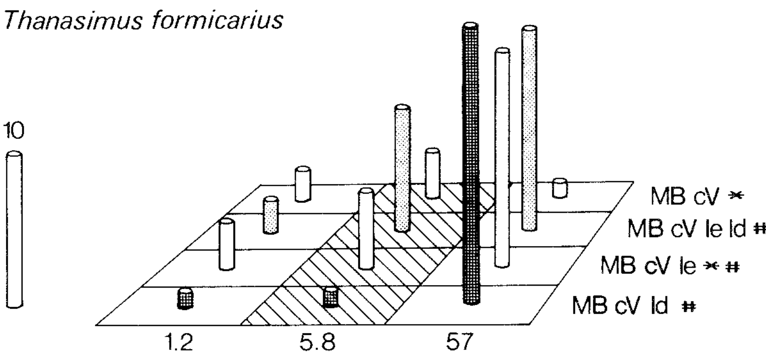


FIG. 6. Response of *I. duplicatus* (untransformed mean catches) in 1982 experiment, sites pooled. Statistics and hatchings as in Figure 4. Response of *Thanasimus formicarius* (total sums caught, sites pooled) in 1982 experiment. Hatching as in Figure 4. Qualitative bait combinations followed by the same symbol (*, #) are not significantly different ($P > 5\%$, Wilcoxon matched-pairs signed-ranks test).

1982 in Värmland, but the smaller number of replicates during this period makes a statistical analysis less valid.

Other Species. In contrast to *I. typographus*, *I. duplicatus* (Sahlb.) did not react to a change in MB + cV release, but responded only to addition of Id and/or Ie and was attracted strongly only to baits containing high levels of Id (Figure 6). Inclusion of Ie reduced the catch clearly and significantly at all release rates. The predator *Thanasimus formicarius* (L.) (Coleoptera: Cleridae) was attracted predominantly towards baits with high amounts of Ie and/or Id (Figure 6). The smaller predator *T. femoralis* (Zett.) was attracted in a similar pattern but in very small numbers.

DISCUSSION

Methodology and its Relationship to Natural Behavior. In order to arrive at meaningful biological interpretations of results from trapping with synthetic compounds, three elements of the methods must be scrutinized in particular: trap design, purity of chemicals, and release rates of chemicals.

The traps used in this study were either a sticky trap with silhouette, which require beetles to land in order to be trapped, or pipe traps with holes which require a beetle both to land and enter a hole to be trapped. Thus, both trap types reflect the landing rate, which is one of the most important parameters in the host colonization process (Byers et al., 1984). The two trap types also had very similar sex ratios in their catches, both here and in a parallel study (Schlyter et al., 1987a).

The chemicals used in this study were all of fairly high chemical purity (Table 1), and the cV was of correct ($>94\%$ *S*) enantiomeric composition while Ie and Id were racemic (50/50 *R/S*). The natural composition of Ie and Id in *I. typographus* is probably not racemic (Francke et al., 1980). Attractive synergistic properties of these compounds could thus have been concealed by the presence of an unnatural enantiomer (Wood, 1982; Vité et al., 1985). Possible inhibitory properties of a compound might also be hidden by an incorrect enantiomeric composition.

The release rates of the different pheromone components are very critical in a study of this kind, because they must ultimately be compared to natural pheromone sources. It is difficult to determine the actual release rate during attraction in the field and so we must rely on laboratory estimates until further studies are done (see Table 1). *I. typographus* males produce high amounts of MB when initiating the nuptial chamber, while production of cV is dependent on the amount and enantiomeric composition (optical purity) of α-pinene in the bark (Birgersson et al., 1984; unpublished). For males attacking a resin-rich standing tree, the release of MB and cV per male has been estimated by air entrainments to be on the average about 100 μg/day and 1–2 μg/day, respectively, per male (Schlyter et al., 1987b; Birgersson et al., unpublished). Typically, as more than 1000 males might attack a standing tree, this would yield a release of at least 100 mg/day of MB and 1–2 mg/day of cV per tree, which corresponds to the highest dose used per trap in the present study.

Quantitative Hypothesis. There was a dramatic effect on the response of *I. typographus* with a change in dose of attractive pheromone components. In two of three sites the catch was directly proportional to the release rate. This finding is in contrast to the generally held concept that in pheromone studies one usually gets only a doubled response with a 10-fold increase in dose. The nearly proportional relationship could be related to the MB component of the pheromone, which has an unusually steep dose–response curve both in EAGs and in field tests (Dickens, 1981; Schlyter et al., 1987a). The beetles were able to differ-

entiate between pheromone sources of different strengths only 1.5 m apart. This indicates they can sense which area is likely to provide the better habitat in terms of avoiding competition. However, "low" attractant releasing areas could not be identified as either a beginning or terminating colonization site based on the quantitative hypothesis alone.

The almost equal sex ratio obtained both on sticky traps and in pipe traps with the low releasing baits as compared to female biased sex ratios on higher releasing baits indicates that an area with low numbers of males in the earliest attack phases, producing a weak pheromone signal (Figure 7A, B), would then attract relatively more males and result in a mass attack (Figure 7C).

The nearly equal sex ratio obtained on weak baits in pipe traps with funnel in our study indicates that the low proportion of males always found in pipe traps in mass trapping (Bakke et al., 1983) is not only due to an intrinsic property of the pipe traps, but is mainly due to the high pheromone doses used. Males are proportionally less attracted to strong pheromone sources than females. They may well be deflected from a strong pheromone source, such as a tree under mass attack (Figure 7C, D) and start colonization in a patch with lower amounts of pheromone (Byers, 1983). In a Y-tube walking bioassay, Schlyter and Löfqvist (1986) showed that females chose stronger sources of natural male pheromone more often than males did. At a strong pheromone source, flying males may reach a threshold concentration releasing landing or avoidance further away than at a weaker source. The reason for the sexual differences could then be that, although both sexes are equally attracted from a distance (Schlyter et al., 1987a), males are physiologically more sensitive to the pheromone (Dickens, 1981). Males might respond further away than females by entering the "landing step" in the attraction sequence and so land in less populated areas and expand the colonization area (Schlyter et al., 1987a).

The quantitative mechanism, if invoked alone, would predict that males be attracted to completely colonized trees (where all males are mated, Figure 7E) with a low release of attractive pheromone, something which would be nonadaptive.

Qualitative Hypothesis. Males could separate a new and weak pheromone source from an old and weak source of MB and cV by the release of Id and Ie from the older source. More specifically, it could be of interest for a male to assess the ratio of Ie and Id to MB and cV. Addition of Ie and Id in the same quantity as cV (Ie + Id : cV = 0.9 : 1) had no effect in the 1982 test, even at the high release rate (0.9 mg/day). When these compounds were added in the 1984 test at approximately the same release rate (1.3 mg/day), but at a much higher ratio to cV (25 : 1), a clear reduction in catch resulted.

As reported by Bakke (1975), the competitor *I. duplicatus* is strongly attracted to baits containing Id, and this was also shown in our test in Värmland (species not present in Denmark). *I. duplicatus* was also inhibited by Ie at much lower doses than *I. typographus*, an effect also found in Norway (Bakke, per-

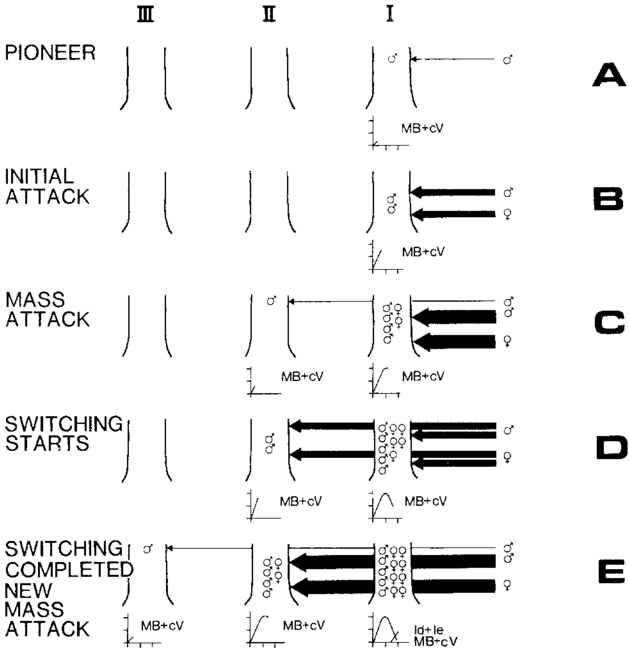


FIG. 7. (A) At the low densities in the *pioneer stage*, density is increased by recruiting more beetles by the increased release of aggregation pheromone (MB + cV). (B) If the first pioneering males are not killed by the resin flow in a short time (hours–1 day), they will produce large amounts of pheromone, and if the beetle population is high, the attracted beetles will start an *initial attack* to exhaust the defensive resin flow. (C) In the *mass attack* phase the tree (I) is taken over, and large numbers of females and males are attracted. A small proportion of males are deflected and land on an adjacent tree (II). (D) While attraction declines towards the first tree as a result of the decline in production of attractive pheromone components, attraction increases towards the second tree, which starts a *switch* in attraction. (E) The first "*switching*" is now *completed* as very few beetles are attracted to the first tree, which now produces only small amounts of attractants (MB + cV) but increasing amounts of inhibitory compounds (Ie + Id and others). The second tree (II) has now become the focus of attraction, where a *new mass attack* starts, while a small fraction of males again is deflected and lands on a third tree (III). At high population levels, this kind of density regulation behavior could mean the death of hundreds or thousands of standing trees, while at lower normal population levels, the colonization may proceed only along patches on a single fallen tree. The small graphs under tree trunks represent log pheromone release and are a simplification and condensation of Figures 3 and 4 in Birgersson et al. (1984). The marks on the x axis represent the attack phases 1, 3, and 6, corresponding to males boring in cortex bark (1), having completed nuptial chamber (3), and being joined by egg-laying females (6).

sonal communication). As *I. duplicatus* is the weaker competitor for breeding substrate when attacking at the same time as *I. typographus* (Anderbrant and Schlyter, unpublished), it is surprising that *I. duplicatus* is attracted to a blend of chemicals (MB, cV, and Id) produced by *I. typographus* in an already colonized patch. This question can probably not be resolved before we gain a better understanding of the quantity and chirality of Id and Ie produced by these two competing species in different phases.

The two *Thanasimus* species were little attracted to baits with MB and cV, but baits with addition of Ie and Id were attractive kairomone sources, which corresponds well to the results of Bakke and Kvamme (1981).

Addition of Id alone showed an interesting effect on *I. typographus*: at low release rates and proportions (0.1 : 1 and 0.8 : 1 of Id to cV) it increased the catch somewhat, while at the higher rate (12 : 1. Id : cV) it decreased the catch (Figure 5). This multifunctional (Rudinsky, 1973) effect of Id is not shared by Ie, which seems to decrease attraction in all baits where it is added alone (Figures 4 and 5). The reason for this difference between the compounds might be that Id is produced somewhat earlier than Ie (Birgersson et al., 1984; unpublished). Verbenone, reported by Bakke (1981) as an inhibitor of trap catches and often produced in considerable quantities in galleries in later phases (Leufvén and Birgersson, 1987) could also contribute to the message of an "old patch."

Other means of communication than pheromonal, such as acoustic, could also affect the colonization density by producing spacing patterns (Byers, 1984). However, the stridulation in *I. typographus* is of low amplitude (Rudinsky, 1979) and would result in a short communication distance (millimeters–centimeters) and could contribute little directly to the switching between patches of the scale discussed here.

Conclusion. Using the chemical data from Birgersson et al. (1984), biological data from Schlyter and Löfqvist (1986), and this study, we propose a combined hypothesis in a graphic model of how attack density could be regulated by the aggregation switching to other trees (areas) at a high population level, as shown in Figure 7. The switching is effected by both the quantitative and qualitative mechanisms acting in sequence. A high quantity of MB and cV is the mass attack signal and also enlarges the area of attack as many of the males land on adjacent trees. The aggregation switching to new areas or trees is completed by a decrease of MB and cV in combination with an increase of the proportion of Id and Ie and possibly other later-occurring substances at the initial colonization area.

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INFLUENCE OF PHEROMONE CHIRALITY ON RESPONSE BY *Oryzaephilus surinamensis* AND *Oryzaephilus mercator* (COLEOPTERA: CUCUJIDAE)[1]

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Abstract—The response of the sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.), and the merchant grain beetle, *O. mercator* (Fauvel), to synthetic racemic and chiral macrolide aggregation pheromones was assessed in pitfall olfactometers. *O. mercator* utilizes the *R* enantiomers of (*Z*)-3-dodecen-11-olide and (*Z,Z*)-3,6-dodecadien-11-olide. *O. surinamensis* utilizes the *R* enantiomers of (*Z,Z*)-3,6-dodecadien-11-olide and the synergist (*Z,Z*)-5,8-tetradecadien-13-olide in combination with achiral (*Z,Z*)-3,6-dodecadienolide. For both species, the racemates of the respective chiral pheromones were effective attractants. The respective *S* enantiomers were inactive for both species and had no effect on the biological activity of the active antipodes. No diel periodicity in responsiveness to pheromones was detected in *Oryzaephilus* spp. reared either on a 12:12 light–dark photoperiod or in darkness. Nonpheromone macrolides, naturally released in trace amounts by *Oryzaephilus* spp., did not affect the aggregation response of either species to its pheromones when these additional macrolides were combined with the pheromone mixtures.

Key Words—*Oryzaephilus surinamensis*, *Oryzaephilus mercator*, aggregation pheromone, macrolide lactone, (Z)-3-dodecen-11-olide, (*Z,Z*)-3,6-dodecadien-11-olide, (*Z,Z*)-5,8-tetradecadien-13-olide, (*Z,Z*)-3,6-dodecadienolide, (*Z*)-5-tetradecen-13-olide, enantiomers, racemate, chirality, aggregation behavior, Coleoptera, Cucujidae.

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INTRODUCTION

The sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.), and the merchant grain beetle, *O. mercator* (Fauvel), are long-lived pests of stored food products throughout the world. In the United States, *O. surinamensis* was ranked first in importance as a pest of stored products and processed food and second as a problem on raw grain, whereas *O. mercator* was listed as an established pest of processed food in general (Mueller, 1982). Utilization of pheromones for suppression and monitoring of insect populations could contribute to the development of integrated control programs (Burkholder, 1981, 1982) for these economically important species.

Seven male-produced, macrolide lactone aggregation pheromones have been identified for grain beetles in the genera *Oryzaephilus* and *Cryptolestes* in the family Cucujidae (Oehlschlager et al., 1987, and references therein). In the genus *Oryzaephilus* (Figure 1), the pheromones have been identified as (Z)-3-dodecen-11-olide (I) and (Z,Z)-3,6-dodecadien-11-olide (II) for *O. mercator*, and II, (Z,Z)-3,6-dodecadienolide (III), and (Z,Z)-5,8-tetradecadien-13-olide (IV) for *O. surinamensis* (Pierce et al., 1985). An additional compound, (Z)-5-tetradecen-13-olide (V), a pheromone synergist in *C. turcicus* (Millar et al., 1985a) and *C. pusillus* (Millar et al., 1985b) is produced in trace amounts by *O. surinamensis* and *O. mercator* (Oehlschlager et al., 1987), but has not been tested in *Oryzaephilus* spp. In laboratory bioassays utilizing the natural products, *O. mercator* responded best to a mixture of I and II, while IV synergized the response to a mixture of II and III for *O. surinamensis* (Pierce et al., 1985). Macrolides I, II, and IV each possess one chiral center and thus could exhibit biological activity as the *R* or *S* enantiomers or some combination thereof.

Our objectives were: (1) to determine the biological activities of the *R*, *S*, and racemic forms of the macrolide aggregation pheromones for both *Oryzaephilus* spp.; (2) to determine the biological activities of additional macrolide lactones released in trace amounts by males of both species (Oehlschlager et al., 1987); and (3) to assess whether or not either or both species show any diel periodicity in pheromone response.

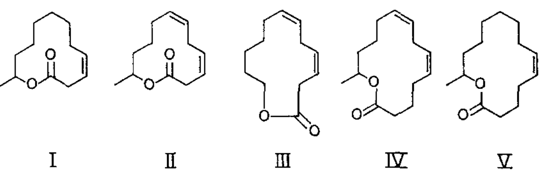

FIG. 1. Structures of macrolides I, II, III, IV, and V.

METHODS AND MATERIALS

Experimental Insects. O. mercator and *O. surinamensis* were reared on large-flake rolled oats and brewer's yeast (95 : 5, w/w) at 28–30°C and 40–60% relative humidity in darkness (Pierce et al., 1981). Additionally, beetles used for some bioassays were reared as above but on a 12 : 12 hr light–dark cycle (lights on from 0600 to 1800 hr).

Macrolide Lactones. Macrolide lactones used for bioassays were as follows: (*R*,*S*)-I, (*R*)-I, and (*S*)-I were synthesized by Oehlschlager et al. (1983). Racemic II was synthesized by Millar and Oehlschlager (1984). (*R*)-II was isolated from volatiles of feeding *O. mercator* (see below), enantiomeric excess >99.5% (Oehlschlager et al., 1987). (*S*)-II was synthesized by the method of Millar and Oehlschlager (1984). III was synthesized by Millar and Oehlschlager (1984). (*R*,*S*)-IV, (*R*)-IV, and (*S*)-IV were synthesized by Millar and Oehlschlager (1984). (*R*,*S*)-V, (*R*)-V, and (*S*)-V were synthesized by Millar et al. (1983).

Collection of Natural II. Beetle-produced volatiles for preparative separation of (*R*)-II were obtained by aerating *O. mercator* adults of mixed sex and age maintained on rolled oats in all-glass systems for periods of seven days (Pierce et al., 1981, 1985). Charcoal-filtered, humidified air was drawn by aspiration through the culture contained in a vertically oriented chamber and then through a trap filled with Porapak Q® (50–80 mesh, Applied Sciences Division, Milton Roy Laboratory Group, State College, Pennsylvania). Volatiles were recovered by extraction of the Porapak Q with purified pentane in a Soxhlet extractor for 24 hr and concentrated by distilling off the pentane through a Dufton column.

Instrumental Methods. A Hewlett-Packard 5830A gas chromatograph equipped with a 18835B capillary inlet system and a flame-ionization detector (FID) was employed for analyses by gas chromatography (GC). Samples were analyzed on a 50-m × 0.5-mm-ID open-tubular glass column coated with Superox 4 (Alltech Associates, Deerfield, Illinois). The temperature program for analytical GC was 70°C for 2 min, then 4°C/min to 180°C, holding for 30 min or less. A Varian 1200 gas chromatograph equipped with a 10 : 1 effluent splitter, FID, and thermal gradient collector (Brownlee and Silverstein, 1968) was used for isothermal (175°C) micropreparative separation of II from Porapak Q-trapped volatiles of feeding *O. mercator*. The column was a 3-m × 3.17-mm-OD stainless-steel tube packed with 5% OV-101 on Chromosorb G (80–100 mesh). Before micropreparative separation, an aliquot of the pentane extract was concentrated to 30–50 μl by evaporation under a gentle stream of nitrogen at −10°C. For both chromatographs, helium was the carrier gas, and the injection port and detector temperatures were 260°C and 270°C, respectively.

Quantification of macrolides in purified pentane for bioassay was done using methyl myristate as an internal standard.

Mass spectra were determined on a Hewlett-Packard 5895A GC/MS/DS fitted with a 30-m × 0.25-mm-ID fused silica column coated with Carbowax 20 M (J & W Scientific, Rancho Cordova, California) with helium as the carrier gas.

Bioassay Procedures. A two-choice, pitfall olfactometer (Pierce et al., 1981) was utilized to test macrolide attractiveness. Filter paper disks treated with a 10-μl aliquot of either an experimental stimulus in purified pentane or purified pentane as a control were put singly into the bottoms of two glass vials suspended from holes in the bottom of a plastic Petri dish arena. Twelve beetles of mixed sex were released into the dish, and the lid was replaced. Bioassays for each test solution were replicated 12 times simultaneously (i.e., 12 olfactometers). After 2 hr in darkness, the numbers of beetles in experimental and control vials were recorded. The raw data were analyzed with a t test for correlated, paired data. Results were expressed as the mean percent response of the total number of beetles per treatment.

Bioassays were conducted at 23°C and 60% relative humidity using test beetles 5–12 weeks posteclosion. Except for bioassays examining the effect of photoperiod on pheromone response, test insects had been reared in darkness. To ensure a uniform state of preconditioning for *O. mercator*, each replicate of 12 beetles was held in a 60-ml glass vial without food for 20 hr at 23°C in darkness prior to a bioassay. To obtain maximum responsiveness from *O. surinamensis*, up to 1500 beetles were preconditioned without food for 48 hr at 23°C in darkness in a 6-liter Erlenmeyer flask, through which charcoal-filtered, humidifed air was drawn at 1.5 liters/min. Since response of *O. surinamensis* in the two-choice olfactometer was extremely sensitive to population density (Pierce et al., 1983), test beetles were maintained at a reduced population density of 1000 beetles/kg medium for at least one week before bioassays commenced.

RESULTS AND DISCUSSION

O. mercator. Olfactory response by mixed-sex *O. mercator* to racemic I and II and the *R* and *S* enantiomers thereof over a 10,000-fold dose range is presented in Figures 2–4. It was previously shown (Pierce et al., 1985) that response by *O. mercator* to a 1:1 mixture of natural I and II was numerically superior to that to either compound alone, although the beetles responded well to either pheromone. The 1:1 mixture of I and II approximated the natural ratio produced by the bioassay beetles. Good aggregation response was shown to the *R* enantiomers of macrolides I (Figure 2C), II (Figure 3C), and the 1:1 mixtures

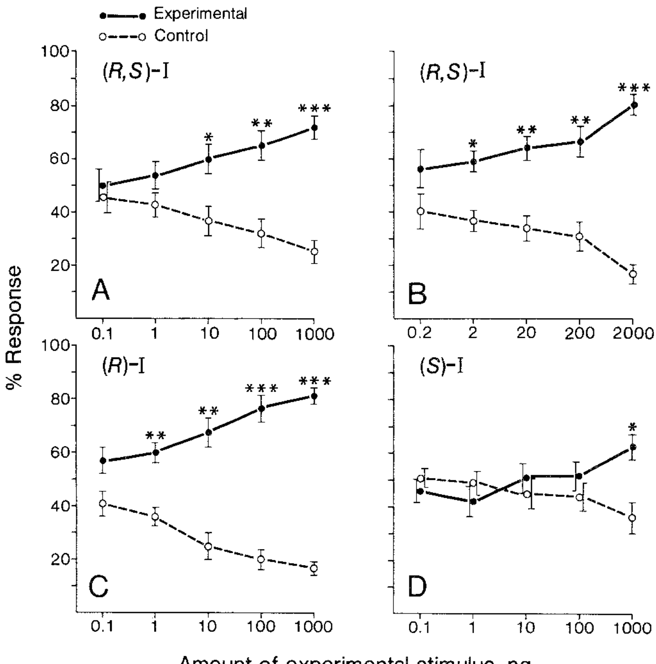

FIG. 2. Response ($\bar{X} \pm$ SE) by mixed-sex *O. mercator* in two-choice, pitfall bioassay to macrolides (R,S)-I, (R)-I, and (S)-I. Significant response (t test) to experimental stimulus indicated by the following: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $N = 12$ replicates.

of (R)-I and (R)-II (Figure 4C), with the thresholds for significant positive response at 1–2 ng of experimental stimulus. A similar response occurred to equivalent amounts each of (R)-I (Figure 2B) and (R)-II (Figure 3B) presented as the racemates and to the 1 : 1 mixture of racemic I and II (Figure 4B). These response patterns were equivalent to those shown towards natural I and II by mixed-sex *O. mercator* (Pierce et al., 1985). Based on the same weights of macrolides bioassayed, however, lower aggregation responses were demonstrated towards racemic I (Figure 2A), II (Figure 3A), and the 1 : 1 racemic mixture (Figure 4A) compared to the responses shown to the *R* enantiomers thereof (Figures 2C–4C).

No significant positive response was shown to the *S* enantiomers of I and II alone (Figures 2D, 3D) or combined (Figure 4D) except at high doses. These moderately positive responses can be explained by small optical impurities in (S)-I and (S)-II (enantiomeric excess >99%) (Oehlschlager et al., 1987).

Recently, *O. mercator* was found to produce chirally pure I and II in the

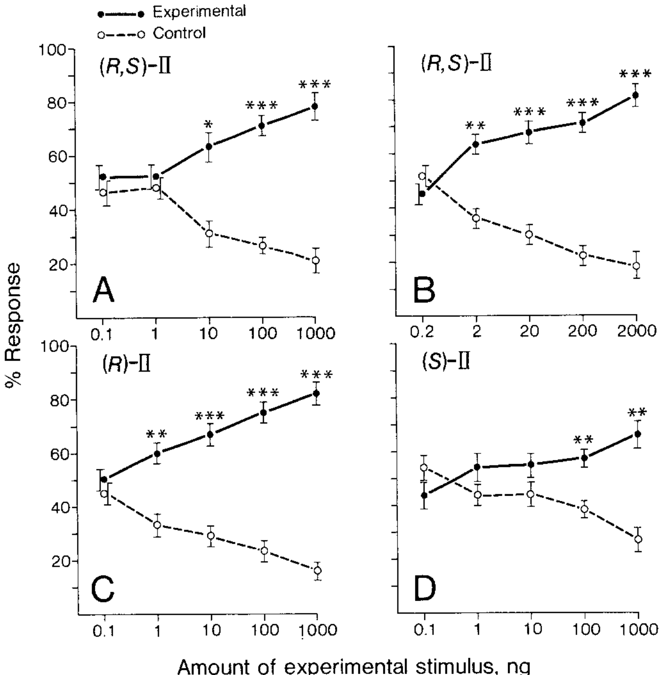

FIG. 3. Response ($\bar{X}$ ± SE) by mixed-sex *O. mercator* in two-choice, pitfall bioassay to macrolides (*R*,*S*)-II, (*R*)-II, and (*S*)-II. Significant response (*t* test) to experimental stimulus indicated by the following: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. $N = 12$ replicates.

R configuration (Oehlschlager et al., 1987). Figures 2–4 indicate that the inactive *S* enantiomers of I and II had no effect other than dilution on the biological activity of (*R*)-I and (*R*)-II.

O. surinamensis. Moderate amounts of (*R*,*S*)-II (Figure 5A) or achiral III (Figure 5B) elicited a low aggregation response from mixed-sex *O. surinamensis*; the positive response was enhanced when (*R*,*S*)-II and III were combined in a 2:1 mixture (Figure 5C). The racemate of the synergist IV elicited no significant response when bioassayed alone over a 6-ng to 6-μg dose range (data not shown). When combined with either (*R*,*S*)-II (Figure 5D) or III (Figure 5E), (*R*,*S*)-IV enhanced the positive response to either of the former two macrolides and extended the ranges of positive response at the highest experimental doses. The 2:1:6 mixture of (*R*,*S*)-II, III, and (*R*,*S*)-IV (Figure 5F) evoked the strongest aggregation response from mixed-sex *O. surinamensis* over the 10,000-fold dose range. The natural pheromone ratio of II, III, and IV produced by the bioassay beetles was approximately 1:1:3 (Pierce et al., 1985).

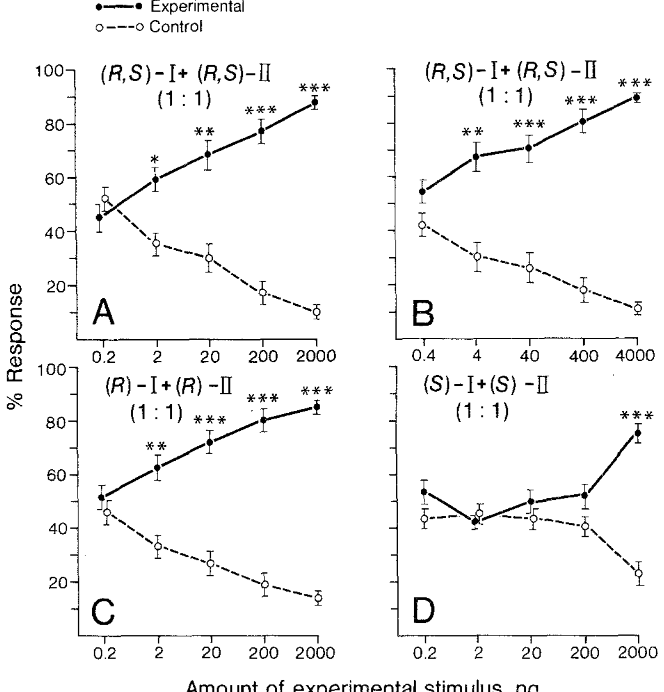

FIG. 4. Response ($\bar{X}$ ± SE) by mixed-sex *O. mercator* in two-choice, pitfall bioassay to 1 : 1 mixtures of macrolides I and II in the racemic, *R*, or *S* forms. Significant response (*t* test) to experimental stimulus indicated by the following: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $N = 12$ replicates.

When bioassayed individually over a 3-ng to 3-μg dose range, the *R* or *S* enantiomers of IV elicited no significant response (data not shown), as was found for racemic IV. The addition of (*R*)-IV to the 2 : 1 mixture of (*R*, *S*)-II and III to give a 2 : 1 : 3 mixture (Figure 6A) elicited a strong aggregation response, similar to that elicited by the 2 : 1 : 6 mixture of (*R*, *S*)-II, III, and (*R*, *S*)-IV (Figure 5F). The substitution of (*S*)-IV for (*R*)-IV in the 2 : 1 : 3 mixture resulted in a low response (Figure 6B) like that shown towards the 2 : 1 mixture of (*R*, *S*)-II and III (Figure 5C). Therefore, the *R* enantiomer of IV appears to be the active synergist. No inhibition of response by the *S* enantiomer of IV is indicated.

Figure 7 illustrates the response by *O. surinamensis* to either (*R*)-II and (*R*)-IV or (*S*)-II and (*S*)-IV in combination with achiral III. The response patterns to (*R*)-II, (*R*)-II + III, and (*R*)-II + III + (*R*)-IV over a 10,000-fold dose range (Figures 7A–C) were similar to those when equivalent amounts of (*R*)-II and (*R*)-IV as racemates were tested (Figures 5A, C, F). Likewise, these aggre-

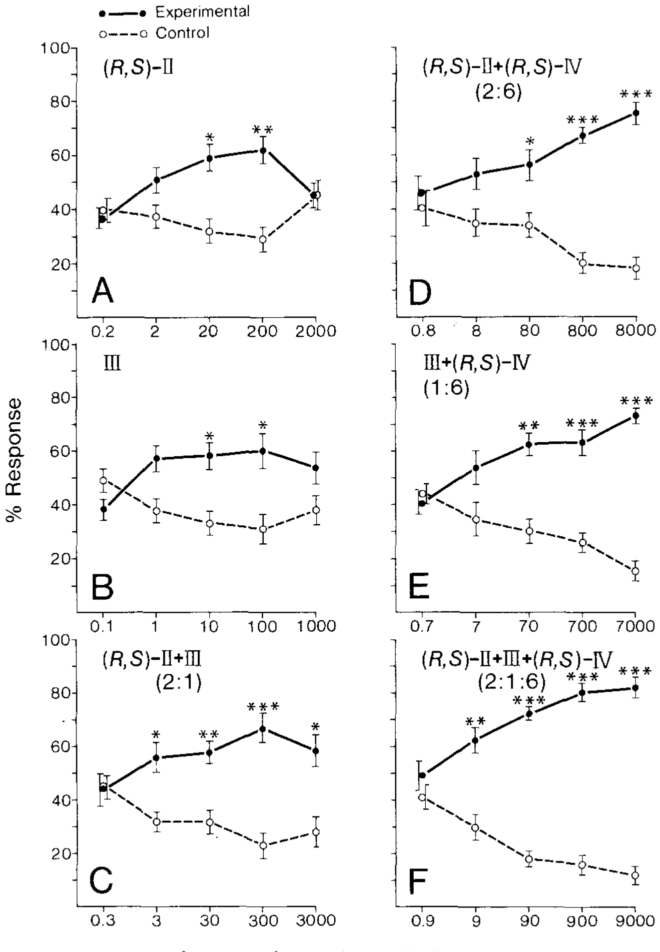

FIG. 5. Response ($\bar{X} \pm$ SE) by mixed-sex *O. surinamensis* in two-choice, pitfall bioassay to macrolides (*R*,*S*)-II, III, and (*R*,*S*)-IV. Significant response (*t* test) to experimental stimulus indicated by the following: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $N = 12$ replicates.

gation responses also agreed with those exhibited earlier by *O. surinamensis* to the natural pheromones (Pierce et al., 1985). Solitary (*S*)-II was inactive except at the 1-μg dose (Figure 7D), which can be explained by a small optical impurity (enantiometic excess >99%) (Oehlschlager et al., 1987). The addition of III (Figure 7E), or of III and (*S*)-IV to (*S*)-II (Figure 7F) elicited response patterns resembling that to solitary III (Figure 5B).

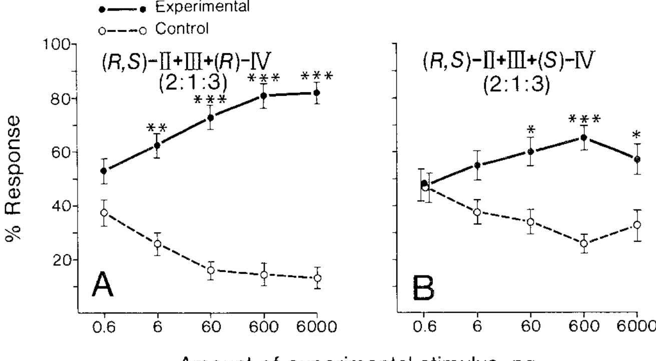

FIG. 6. Response ($\bar{X} \pm$ SE) by mixed-sex *O. surinamensis* in two-choice, pitfall bioassay to macrolides (*R*, *S*)-II, and III combined with (*R*)-IV or (*S*)-IV. Significant response (*t* test) to experimental stimulus indicated by the following: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. $N = 12$ replicates.

O. surinamensis produces chirally pure II and IV in the *R* configuration (Oehlschlager et al., 1987). Taken together, Figures 5, 6, and 7 indicate that *O. surinamensis* utilizes the *R* enantiomers of II and IV. The *S* enantiomers appear to be inactive alone and do not inhibit or affect response to the *R* enantiomers.

Additional Macrolides. Feeding males of both *Oryzaephilus* spp. produced trace amounts of additional macrolide lactones as detected by GC-MS (Oehlschlager et al., 1987). For *O. mercator* these trace macrolides were III, IV, and V, while *O. surinamensis* produced trace amounts of V. When these compounds were bioassayed individually over a 0.1- or 0.2-ng to 1- or 2-μg dose range, III and the *R*, *S*, and racemic forms of IV and V elicited no response from mixed-sex *O. mercator*, and (*R*)-, (*S*)-, and (*R*, *S*)-V elicited no response from mixed-sex *O. surinamensis* (data not shown).

The five macrolides (I–V) were bioassayed in five-part mixtures (Table 1). For either *Oryzaephilus* spp. the presence of additional macrolides in the respective pheromone mixtures did not affect response to the aggregation pheromones over a 10,000-fold dose range (compare Table 1 with Figures 4B and 5F).

Interspecific Communication. Since both *Oryzaephilus* spp. utilize (*R*)-II as a common pheromone, solitary (*R*)-II and the pheromone mixture of the other species were tested to further investigate possible interspecific communication between *O. mercator* and *O. surinamensis* (Table 2). Solitary (*R*)-II elicited strong aggregation response from *O. mercator* and comparatively lower response from *O. surinamensis*. For either species, response to (*R*)-II was not affected by the pheromone(s) of the other species.

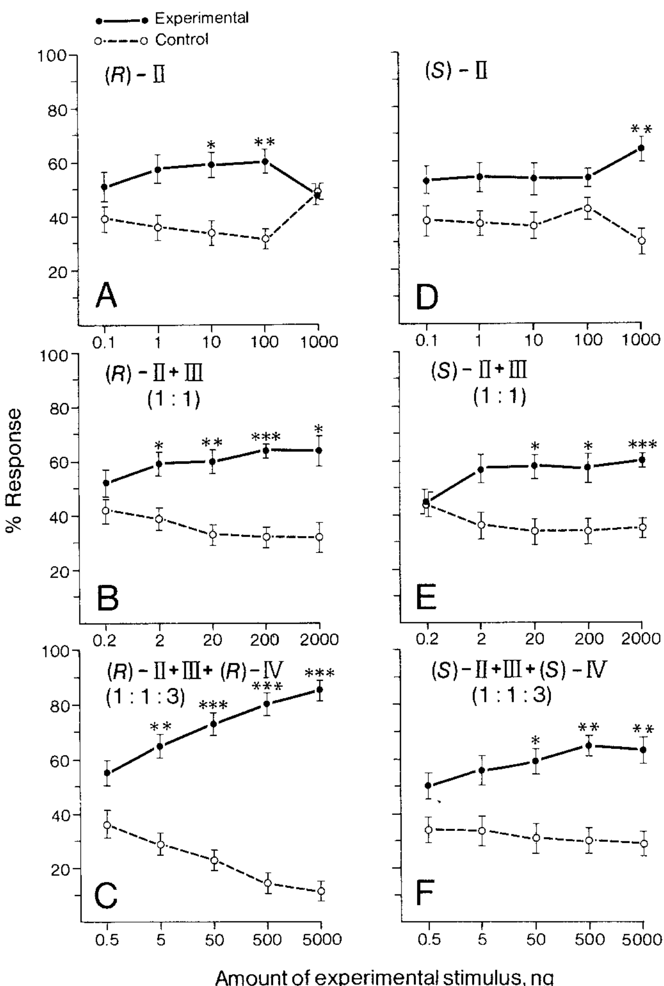

FIG. 7. Response ($\bar{X} \pm$ SE) by mixed-sex *O. surinamensis* in two-choice, pitfall bioassay to macrolides: chiral II, III, and chiral IV. Significant response (*t* test) to experimental stimulus indicated by the following: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $N = 12$ replicates.

Diel Response Patterns. For both *Oryzaephilus* spp., there was no apparent diel rhythm in pheromone response to moderate amounts of the respective pheromone mixtures tested in mid-photophase and mid-scotophase (Table 3). Beetles reared in darkness showed no difference in pheromone response when compared to beetles reared on a 12 : 12 hr light–dark photoperiod. *Oryzaephilus* spp. and the maize weevil, *Sitophilus zeamais* Motsch. (Walgenbach et al., 1983),

TABLE 1. RESPONSE BY MIXED-SEX *O. mercator* AND *O. surinamensis* IN TWO-CHOICE, PITFALL BIOASSAY TO MIXTURES OF MACROLIDES I, II, III, IV, AND V

Amount (ng) of I + II + III + IV + V in experimental stimulus[a]	Response, % ($\overline{X}$ ± SE)[b]			
	O. mercator		*O. surinamensis*	
	Experimental stimulus	Pentane control	Experimental stimulus	Pentane control
0.2 + 0.2 + 0.1 + 0.6 + 0.2	50.3 ± 5.0	46.9 ± 4.2 NS	47.5 ± 6.7	46.8 ± 6.2 NS
2 + 2 + 1 + 6 + 2	62.9 ± 4.8	33.6 ± 4.6 **	61.2 ± 4.6	31.3 ± 4.2 **
20 + 20 + 10 + 60 + 20	69.0 ± 4.5	28.9 ± 4.7 ***	69.0 ± 4.6	26.8 ± 3.7 ***
200 + 200 + 100 + 600 + 200	73.8 ± 3.6	22.0 ± 3.2 ***	76.1 ± 4.2	17.9 ± 3.4 ***
2000 + 2000 + 1000 + 6000 + 2000	85.9 ± 2.7	12.0 ± 3.2 ***	84.2 ± 4.2	9.0 ± 3.1 ***

[a] Macrolides I, II, IV, and V were the racemates.

[b] Significant response (t test) to experimental stimulus indicated by the following: $***P < 0.001$; $**P < 0.01$; NS, not significant. $N = 12$ replicates per treatment, 12 adults per replicate.

TABLE 2. RESPONSE BY *O. mercator* AND *O. surinamensis* TO COMMON MACROLIDE AGGREGATION PHEROMONE ALONE AND IN COMBINATION WITH PHEROMONES FROM OPPOSITE SPECIES

Test species[a]	Experimental stimulus	Amount of experimental stimulus (ng)	Response, % ($\bar{X} \pm$ SE)[b]	
			Experimental stimulus	Pentane control
O. mercator	(*R*)-II	0.1	52.1 ± 4.7	45.3 ± 4.8 NS
		1	60.9 ± 4.1	33.1 ± 3.8 **
		10	67.8 ± 4.0	30.8 ± 3.9 ***
		100	71.5 ± 3.7	23.6 ± 3.8 ***
	(*R*)-II + III + (*R*)-IV	0.1 + 0.1 + 0.3	55.4 ± 5.1	40.8 ± 4.3 NS
		1 + 1 + 3	61.8 ± 4.5	31.9 ± 4.6 **
		10 + 10 + 30	66.7 ± 4.1	30.0 ± 4.0 ***
		100 + 100 + 300	74.1 ± 3.6	25.9 ± 3.2 ***
O. surinamensis	(*R*)-II	1	55.0 ± 5.9	39.1 ± 5.7 NS
		10	60.2 ± 4.2	36.5 ± 3.5 *
		100	63.4 ± 4.1	33.8 ± 4.0 **
	(*R*)-I + (*R*)-II	1 + 1	50.9 ± 5.6	41.1 ± 5.1 NS
		10 + 10	59.4 ± 4.4	34.1 ± 3.9 *
		100 + 100	61.9 ± 4.1	34.3 ± 3.0 **

[a] All bioassays for each species (mixed sex) were completed in a separate 2-hr session.
[b] Significant response (*t* test) to experimental stimulus indicated by the following: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS, not significant. $N = 12$ replicates per treatment, 12 adults per replicate.

are similar in their lack of diel periodicity in responsiveness to pheromones. However, another long-lived, stored-product beetle, the granary weevil, *Sitophilus granarius* (L.), definitely exhibits such periodicity (Faustini et al., 1982).

Practical Implications. Response to attractive volatiles in *O. mercator* and *O. surinamensis* appears soon after eclosion and apparently extends throughout the long, adult lives of these beetles (Pierce et al., 1983). Moreover, neither species showed any diel periodicity in responsiveness, despite the photic conditions under which they were reared. Therefore, macrolide lures for *Oryzaephilus* in field traps might be effective under variable conditions of photoperiod throughout adult life.

Our results also indicate that the cost-effective racemates of the chiral macrolide pheromones could be used in biomonitoring programs for *Oryzaephilus* spp. Similarly in cucujid beetles of the genus *Cryptolestes*, the inactive antipodes of the chiral macrolide pheromones were not inhibitory (Wong et al., 1983; Millar et al., 1985a,b). Nonpheromone macrolides (Tables 1 and 2) naturally produced in trace amounts had no effect on response by *Oryzaephilus* spp. to their respective pheromones. Since these trace macrolides as well as the *S* enantiomers of I and IV are aggregation pheromones of other cucujid beetles (Oehlschlager et al., 1987), lures might be formulated to trap *Cryptolestes* spp. together with *Oryzaephilus* spp. In this regard, it was possible to monitor simultaneously for both a cucujid and tenebrionid grain beetle using pheromones for both species together in the same field trap (Lindgren et al., 1985). Such combinations of lures for several species would greatly reduce the cost of pheromone-based monitoring programs.

Taxonomic and Ecological Implications. The fact that *O. mercator* and *O. surinamensis* utilize the common pheromone (*R*)-II reflects the close taxonomic relationship of these two species (Slow, 1958). Although they are distinct species that do not interbreed (Slow, 1958), they are morphologically very similar (Loschiavo and Sabourin, 1982). On the other hand, chromosomal studies revealed that the genera *Oryzaephilus* and *Cryptolestes* are not very closely related (Robertson, 1959). Differences in pheromone complexes provide chemotaxonomic evidence in support of this distant relationship. *Cryptolestes* spp. use as aggregation pheromones either macrolides that are unique to the genus, macrolides released in trace amounts by *Oryzaephilus* spp. and not used as pheromones by the latter spp., or macrolides of the *S* configuration (Oehlschlager et al., 1987). The chiralities of the nonpheromone, trace macrolides in *Oryzaephilus* spp. are unknown, however. Enzymatic specificities for macrolide biosynthesis most likely differ in the two genera.

Since our bioassay data indicated that *O. mercator* could be significantly attracted to *O. surinamensis* aggregation pheromone (Table 2), other semiochemicals such as sex pheromones (Burkholder, 1982), so far unknown for these two species, ultimately might be involved in species recognition. How-

TABLE 3. EFFECT OF TIME OF DAY AND REARING PHOTOPERIOD ON RESPONSE BY *O. mercator* AND *O. surinamensis* TO MACROLIDE AGGREGATION PHEROMONE

Test species[a]	Photoperiod[b]	Test time (hr)	Amount of experimental stimulus (ng)[c]	Response, % ($\bar{X} \pm$ SE)[d]	
				Experimental stimulus	Pentane control
O. mercator	D	1200	20	61.1 ± 4.4	33.3 ± 4.8 **
	D	1200	200	75.0 ± 2.5	21.5 ± 2.2 ***
	D	2400	20	62.0 ± 4.9	33.8 ± 4.7 **
	D	2400	200	81.1 ± 3.3	17.5 ± 3.0 ***
	LD	1200	20	63.3 ± 3.8	31.7 ± 4.5 **
	LD	1200	200	80.6 ± 2.6	19.4 ± 2.6 ***
	LD	2400	20	66.0 ± 5.2	31.9 ± 5.7 **
	LD	2400	200	77.6 ± 3.6	18.9 ± 3.2 ***

O. surinamensis	D	1200	50	70.3 ± 4.0	19.6 ± 2.9 ***
	D	1200	500	87.5 ± 3.2	9.0 ± 3.0 ***
	D	2400	50	71.3 ± 4.4	22.4 ± 4.2 ***
	D	2400	500	88.0 ± 2.8	7.0 ± 2.8 ***
	LD	1200	50	74.6 ± 4.2	23.9 ± 4.4 ***
	LD	1200	500	86.2 ± 4.4	9.4 ± 2.8 ***
	LD	2400	50	71.9 ± 4.5	20.1 ± 2.7 ***
	LD	2400	500	83.2 ± 1.9	9.1 ± 1.9 ***

[a] Mixed sex.
[b] D = beetles reared in darkness; LD = beetles reared on a 12:12 light–dark photoperiod.
[c] Experimental stimulus = (*R*)-I + (*R*)-II (1:1) for *O. mercator*, and (*R*)-II + III + (*R*)-IV (1:1:3) for *O. surinamensis*.
[d] Significant response (*t* test) to experimental stimulus indicated by the following: $^{***}P < 0.001$; $^{**}P < 0.01$; NS, not significant. $N = 12$ replicates per treatment, 12 adults per replicate.

ever, in a large, three-dimensional habitat such as stored grain, cross-attraction to (Table 2) or tolerance of (Table 1) the pheromones of a coinhabiting species might be expected. Similar cross-attraction and tolerance occurs among ambrosia beetles that inhabit another spacious host resource, the sapwood of coniferous trees (Borden et al., 1981). In contrast, mutual inhibition of response occurs between two species of bark beetles, *Ips pini* (Say) and *I. paraconfusus* Lanier, that compete for an essentially two-dimensional resource, the inner bark of trees (Birch and Wood, 1975).

Aggregation pheromones in Coleoptera are thought to have arisen initially as mechanisms to cause aggregation at a suitable food source (Shorey, 1973). In many bark beetles (Borden, 1985) and grain beetles (Burkholder, 1982), aggregation pheromone production is dependent on food and is thought to signal the presence of a food-rich habitat. Although pheromone production was enhanced in the presence of food in all *Cryptolestes* spp. investigated to date (H.D. Pierce, Jr. et al., 1984; Millar et al., 1985a,b), macrolide aggregation pheromones may serve as especially critical signals of food reserves for *Oryzaephilus* spp. since only trace amounts of pheromones were released by either *O. mercator* or *O. surinamensis* deprived of food (unpublished observations). Furthermore, *Oryzaephilus* spp. prefer food of high oil content, and fatty acids are hypothesized to be precursors of the macrolide pheromones (H.D. Pierce, Jr. et al., 1984).

One mechanism contributing to the maintenance of species specificity is differential resource utilization. In this regard, a model for the colonization of new habitats by *Tribolium* spp. (Tenebrionidae) (Ghent, 1963; Ziegler, 1976) has also been applied to *Oryzaephilus* spp. (Pierce et al., 1983). The more dispersive *O. surinamensis* may be a primary colonist suited to exploit widely distributed, sparsely populated, and very fresh habitats, whereas *O. mercator* may be a secondary colonist better able to invade and persist in habitats already occupied by populations of the same or different species (Pierce et al., 1983). In support of this model, young, reproductivity vigorous *O. surinamensis* (but not *O. mercator*) were extremely sensitive to overcrowding by conspecifics, as evidenced both by decreased pheromone production (A.M. Pierce et al., 1984) and perception (Pierce et al., 1983). The strong attractiveness of (*R*)-II to *O. mercator* which allows it to be attracted to habitats already colonized by *O. surinamensis* is consistent with the proposed roles for *O. surinamensis* and *O. mercator* as primary and secondary colonists, respectively, of temporary habitats.

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CHIRALITY OF MACROLIDE PHEROMONES OF GRAIN BEETLES IN THE GENERA *Oryzaephilus* AND *Cryptolestes* AND ITS IMPLICATIONS FOR SPECIES SPECIFICITY[1]

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Abstract—The chiralities of macrolide lactone aggregation pheromones of five species of economically important grain beetles have been determined by capillary gas chromatographic separation of the diastereomeric (*S*)-*O*-acetyllactate derivatives of the hydroxy methyl esters derived from boron trifluoride-catalyzed cleavage of the macrolides in methanol. Chirally pure (*Z*)-3-dodecen-11-olide (I) is produced in the *S* configuration by *Cryptolestes ferrugineus* (Stephens) and in the *R* configuration by *Oryzaephilus mercator* (Fauvel). (*Z,Z*)-3,6-Dodecadien-11-olide (II) is produced in the *R* configuration by both *O. mercator* and *O. surinamensis* (L.). (*Z,Z*)-5,8-Tetradecadien-13-olide (IV) is produced in the *R* configuration by *O. surinamensis* and as a 85 : 15 mixture of *R* and *S* isomers by *C. turcicus*. (*Z*)-5-Tetradecen-13-olide (V) is produced in the *S* configuration by *C. pusillus* (Schönherr) and as a 33 : 67 mixture of the *R* and *S* isomers by *C. turcicus* (Grouvelle). The results indicate that in these cucujids, species specificity in pheromone response is maintained at least in part by pheromone chirality.

Key Words—Chiral semiochemicals, pheromones, enantiomeric composition, enantiomeric synergism, *Cryptolestes ferrugineus, Cryptolestes pusillus, Cryptolestes turcicus, Oryzaephilus mercator, Oryzaephilus surinamensis,* Coleoptera, Cucujidae, (Z)-3-dodecen-11-olide, (Z,Z)-3,6-dodecadien-11-olide, (Z)-5-tetradecen-13-olide, (Z,Z)-5,8-tetradecadien-13-olide.

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INTRODUCTION

Since the first discovery of an aggregation pheromone response in cucujid grain beetles (Borden et al., 1979), our investigations in the genera *Cryptolestes* and *Oryzaephilus* have yielded a new class of aggregation pheromones, the macrolide lactones. In the five economically important species studied, we found seven macrolide pheromones (Table 1) (H.D. Pierce, Jr. et al., 1984). Laboratory bioassays revealed that for each species, insects of mixed sex are attracted best to those mixtures of macrolides produced by feeding males (H.D. Pierce, Jr. et al., 1984). In four species, superior attraction was achieved with binary mixtures (Wong et al., 1983; Millar et al., 1985a, b; A.M. Pierce, et al., 1984; Pierce et al., 1985), whereas for *Oryzaephilus surinamensis* (L.), a ternary mixture of macrolides was maximally attractive (A.M. Pierce et al., 1984; Pierce et al., 1985). The males of some species produce macrolides that are pheromones for other species (Table 1). In some instances, different species share common pheromones. Maintenance of species recognition in this sympatric group must therefore involve several mechanisms.

We report the determination of the chiralities of the male-produced macrolides in five species of cucujids. This information, in combination with previously reported bioassay data, allows delineation of the role of chirality in maintenance of species recognition.

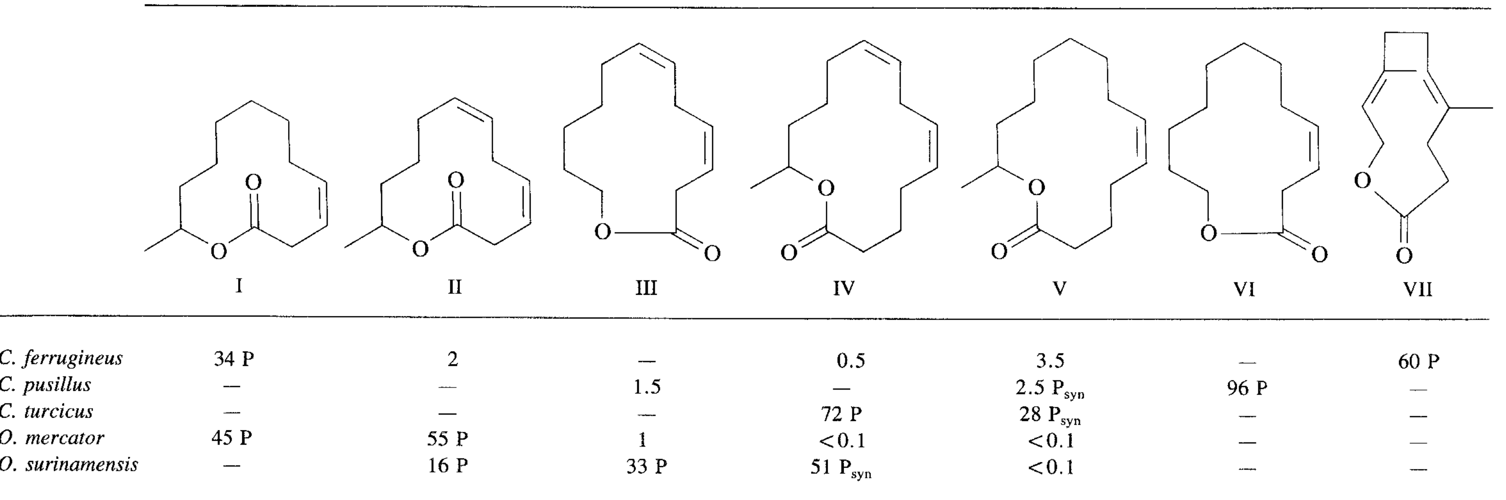
METHODS AND MATERIALS

Synthetic Macrolides. Synthetic I in both its racemic and chiral forms was available from previous studies (Oehlschlager et al., 1983). Synthetic racemic II and IV and synthetic chiral IV were prepared as described by Millar and Oehlschlager (1984). Racemic and chiral V were prepared as described by Millar et al. (1983). The remaining macrolides (III, VI and VIII) in Table 1 are achiral and were previously prepared in the references mentioned above.

Insect-Derived Macrolides. Pentane extracts of Porapak Q collected volatiles from feeding adults of each species were prepared as previously described (H.D. Pierce, Jr. et al., 1984). The macrolide composition as determined by gas chromatography (GC) of each extract is given in Table 1.

Derivation of Macrolides and Analysis. Synthetic macrolides or Porapak Q extracts of beetle-produced volatiles (0.5–5.0 μg) were dissolved in methanol (50–100 μl) containing 5% boron trifluoride etherate at −20°C and sealed in glass ampoules. The reactions were maintained at 70°C overnight. This process yielded the corresponding hydroxyl methyl esters, which were isolated by dilution of each reaction with pentane (100 μl) and washing with water (3 × 50 μl). The pentane solution was dried over finely powdered sodium sulfate and filtered through glass wool. A portion (30 μl) of the filtrate was derivatized by treatment in a clean ampoule with pyridine (15 μl of a 50 mg/ml solution in

TABLE 1. DISTRIBUTION OF MACROLIDES IN *Cryptolestes* AND *Oryzaephilus* SPECIES[a]



	Distribution (%)						
	I	II	III	IV	V	VI	VII
C. ferrugineus	34 P	2	—	0.5	3.5	—	60 P
C. pusillus	—	—	1.5	—	2.5 P_{syn}	96 P	—
C. turcicus	—	—	—	72 P	28 P_{syn}	—	—
O. mercator	45 P	55 P	1	<0.1	<0.1	—	—
O. surinamensis	—	16 P	33 P	51 P_{syn}	<0.1	—	—

[a] Composition of macrolide fraction determined by gas chromatographic analysis of captured volatiles from insects of mixed sex (only males of each species produce pheromones) feeding on rolled oats (A.M. Pierce, et al., 1984; H.D. Pierce, Jr. et al., 1984). P = pheromone; P_{syn} = pheromones inactive alone but which act as synergists. Pheromones active in bioassays conducted on insects of mixed age and sex. — = Not detected in species by single ion monitoring of the most prominent fragment ion in the mass spectrum of the macrolide when the macrolide fraction was subjected to GC-MS. Probable detection limits 0.01%. Chemical names of I–VII are as follows: I: (Z)-3-dodecen-11-olide; II: (*Z*, *Z*)-3,6-dodecadien-11-olide; III: (*Z*, *Z*)-3,6-dodecadienolide; IV: (*Z*, *Z*)-5,8-tetradecadien-13-olide; V: (Z)-5-tetradecadien-13-olide; VI: (Z)-3-dodecenolide; VII: 4,8-dimethyl-(*E*,*E*)-4,8-decadien-10-olide.

TABLE 2. RETENTION TIMES OF MACROLIDES, DERIVED HYDROXY METHYL ESTERS, AND THEIR (*S*)-*O*-ACETYLLACTATE DIASTEREOMERS[a]

		Column and temp. program[b]		Retention time (min)[b]			
						(*S*)-*O*-Acetyllactates	
Macrolide	Source	DB-1	SPB5	Macrolide	Hydroxy methyl ester	*R* macrolide	*S* macrolide
1 (*R*, *S*)-I	Synthetic	1		15.16	19.50	29.99	30.42
2 (*S*)-I	Synthetic	1		15.16	19.52	30.05 (<1%)	30.49 (>99%)
3 (*S*)-I	*C. ferrugineus*	1		15.20	19.48	ND[c]	30.45
4 (*R*, *S*)-I	Synthetic		1	9.81	13.63	21.93	22.31
5 (*R*)-I	*O. mercator*		1			21.86	ND
6 (*R*, *S*)-II	Synthetic		1	9.81	13.42	21.47	21.80
7 (*R*)-II	*O. mercator*		1			21.38	ND
8 (*R*)-Dihydro-I	*O. mercator*		1			22.13	ND
9 (*R*)-Tetrahydro-II	*O. mercator*		1			22.12	ND
10 (*S*)-Dihydro-I	Synthetic		1			22.15(1%)	22.56(99%)
11(*R*,*S*)-Tetrahydro-II	Synthetic		1			22.15	22.55

12 (*R*)-Tetrahydro-II	*O. surinamensis*		1			22.14(99%)	22.51(1%)
13 (*R*, *S*)-IV	Synthetic		1	12.63	16.15	27.91	28.42
14 (*R*)-IV	Synthetic		1	12.62	16.24	27.89(96%)	28.36(4%)
15 (*R*,*S*)-IV	*C. turcicus*		1	12.61	16.18	27.85(85%)[d]	28.35(15%)[d]
16(*R*,*S*)-Tetrahydro-IV	Synthetic		1			30.12	30.80
17 (*R*)-Tetrahydro-IV	*O. surinamensis*		1			30.08	ND
18 (*R*)-Tetrahydro-IV	*O. surinamensis*	2				30.07	ND
19 (*R*,*S*)-V	Synthetic		1	12.63	16.31	28.75	29.30
20 (*R*)-V	Synthetic		1		16.47	28.68(94%)	29.26(6%)
21(*R*,*S*)-V	*C. turcicus*		1	12.61	16.35	28.69(34%)[d]	29.30(66%)[d]
22 (*R*,*S*)-V	Synthetic	2				28.92	29.35
23 (*R*)-V	Synthetic	2				28.92 (96%)	29.39 (4%)
24 (*S*)-V	*C. pusillus*	2				ND	29.35
25 (*R*,*S*)-Dihydro-V	Synthetic	2				29.97	30.41
26 (*S*)-Dihydro-V	*C. pusillus*	2				ND	30.40

[a] Analysis by coupled gas chromatography–mass spectrometry as previously described (Slessor et al., 1985) confirmed the identity of the observed peaks as the desired *O*-acetyllactate diastereomers (major fragment ions at m/z 115, 87, 43).
[b] Gas chromatographic conditions: Column DB-1: 30 m × 0.25 mm ID fused silica column programmed as follows: (1) 60°C for 2 min, 7°C/min to 180°C, 2°C/min to 240°C; (2) 80°C for 2 min, 10°C/min to 180°C, 2°C/min to 220°C. Column SPB5: 30 m × 0.25 mm ID fused silica column programmed as follows: (1) 100°C for 2 min, 10°C/min to 180°C, isothermal for 2 min, 10°C/min to 215°C.
[c] ND = not detected
[d] Average of three determinations.

ether) and (*S*)-*O*-acetyllactyl chloride (30 μl of a 25 mg/ml solution in methylene chloride), as described by Slessor et al. (1985).

The *O*-acetyllactyl methyl esters derived from synthetic and naturally occurring macrolides I, II, IV, and V were analyzed by capillary gas chromatography on Hewlett-Packard 5890 and 5880A instruments fitted with flame-ionization detectors. The injector and detector temperatures were 250°C. Helium was the carrier gas. Columns and oven temperature programs are listed in Table 2.

Microhydrogenations were performed by placing 30–50 μl of the hexane solution of the macrolides under one atmosphere of hydrogen in the presence of a few milligrams of 5% Pd on $BaSO_4$. The solution was separated from catalyst by syringe prior to treatment with $BF_3 \cdot CH_3OH$.

The chirality of I in *C. ferrugineus* (Stephens) was determined by comparison of the retention times (Table 2) for the *O*-acetyllactate derivatives (IX) derived from synthetic (*R*,*S*)-I (entry 1) and synthetic (*S*)-I (entry 2) with the *O*-acetyllactate derived from natural I produced by *C. ferrugineus* (entry 3). The same procedure was used for the determination of the chirality of IV in *C. turcicus* (Grouvelle) (entries 13–15), and of V in *C. turcicus* (entries 19–21) and *C. pusillus* (Schönherr) (entries 22–24).

Both I and II reduce to the same saturated macrolide. The determination of the chirality of I in *O. mercator* (Fauvel) involved reduction of natural I, isolated by micropreparative GC, to its dihydro derivative followed by the usual derivatization. Comparison of the retention times of the *O*-acetyllactate derivatives of synthetic racemic tetrahydro II (entry 11), synthetic dihydro (*S*)-I (entry 10) and dihydro (entry 8) derived from *O. mercator* yielded the configuration of the natural material. The retention values obtained above (Table 2) for the *O*-acetyllactate derivative of tetrahydro II (entry 11) enabled us to determine the chirality of tetrahydro II derived from reduction of II produced by *O. mercator* (entry 9) and *O. surinamensis* (entry 12). Likewise, both IV and V reduce to the same saturated macrolide. Knowledge of the chirality of V in *C. pusillus* (entry 24) from the experiments described above allowed assignment of the chirality of the corresponding *O*-acetyllactate of the dihydro derivative (entries 25, 26). This in turn can be used to assign chirality in the *O*-acetyllactate of tetrahydro IV derived from *O. surinamensis* (entry 18).

RESULTS AND DISCUSSION

Chirality of Macrolides I, II, IV, and V. Conversion of macrolides I, II, IV, and V to the corresponding hydroxyl methyl esters (VIII) and thence to *O*-acetyllactate derivatives (IX) is not accompanied by any apparent racemization of the chiral center or double bond isomerization as evidenced by the conversion of synthetic chiral macrolides to single diastereomers (Figure 1). Diastereomers

I,II,IV,V VIII IX

FIG. 1. Conversion of macrolide pheromones to *O*-acetyllactate methyl esters.

(IX) obtained from each racemic macrolide were baseline-separated by the chromatographic systems used. In all cases, the diastereomers (IX) derived from macrolides possessing the *R* configuration eluted prior to those derived from the corresponding macrolides possessing the *S* configuration. From previous work on this method, it is known that errors due to kinetic resolution during formation of the *O*-acetyllactates (IX) and to differential weight response of the diastereomers are less than 3% (Slessor et al., 1985). The enantiomeric compositions calculated for each macrolide in Table 3 are therefore not corrected for either of these variables.

Cross-Attractancy in Cucujids. The aggregation pheromone of the rusty grain beetle, *C. ferrugineus*, consists of I and VII (Wong et al., 1983). These two macrolides, each attractive alone, also act synergistically. One would not expect the rusty grain beetle to be cross-attracted (Table 4) to other cucujids in Table 1 for two reasons. First, *C. ferrugineus* is the only species that produces VII. Second, although both *C. ferrugineus* and *O. mercator* share I as a pheromone, the former produces (Table 3) and responds (Wong et al., 1983) only to (*S*)-I, whereas the latter produces (Table 3) and responds (Pierce et al., 1987) only to (*R*)-I.

The macrolide pheromones produced by *C. pusillus* are V and VI (Table 1) (Millar et al., 1985a). Macrolide V acts to synergize response to the second component, VI, which is active alone. Although *C. pusillus* produces (*S*)-V of high chiral purity (Table 3), no preference for one enantiomer of V acting as a synergist for VI was detected by laboratory bioassays (Millar et al., 1985a). Cross-attraction (Table 4) of *C. pusillus* to other cucujids in Table 1 is not expected since VI, a singly active pheromone component for this species, is not produced by any other species. Although V is common to all cucujids investigated, it is not attractive alone to *C. pusillus*.

The flour mill beetle, *C. turcicus*, utilizes IV and V as its aggregation pheromone (Table 1). As with *C. pusillus*, V is inactive alone but synergizes response to IV which is attractive alone (Millar et al., 1985b). Both IV and V are produced by *C. turcicus* as mixtures of *R* and *S* enantiomers and with opposite chiral enrichments. Macrolide IV is rich in *R* enantiomer (85 : 15, *R*:*S*)

TABLE 3. CHIRALITIES OF MACROLIDES OF *Cryptolestes* AND *Oryzaephilus* SPECIES[a]

	I	II	IV	V
C. ferrugineus	*S*			
C. pusillus				*S*
C. turcicus			*R*:*S*(85:15)	*R*:*S*(33:67)
O. mercator	*R*	*R*		
O. surinamensis		*R*	*R*	

[a] Single *S* or *R* indicates >99% one enantiomer.

and V is rich in *S* enantiomer (33:67, *R*:*S*). Careful analysis of the responses of *C turcicus* to the chiral isomers of the singly active macrolide, IV (Millar et al., 1985b), reveals that mixtures of IV containing both *R* and *S* isomers are more attractive than either chiral isomer alone. This is the only known case of enantiomeric synergism in the cucujids. Synergism between enantiomers also occurs in the scolytids, *Gnathotricus sulcatus* (Borden et al., 1976) and *Ips pini* (Lanier et al., 1980).

It is probable that *C. turcicus* would not be cross-attracted to *C. ferrugineus*, even though both IV and V are produced by the latter. In *C. turcicus*, the V:IV ratio is 0.38, whereas, in *C. ferrugineus*, it is 13.0. Furthermore, IV and V are only minor components of *C. ferrugineus* volatiles. Attraction of *C. turcicus* to *C. pusillus* would not be expected since both share only V which is inactive alone in both species. Attraction of *C. turcicus* to *O. mercator* is unlikely because both IV and V are produced by the latter only as minor components (Table 1). Attraction of *C. turcicus* to *O. surinamensis* would not be expected for two reasons. The ratio of IV to V produced by *O. surinamensis* (>510) is drastically different from that emitted by *C. turcicus*. Secondly, IV produced by and attractive to *C. turcicus* is a mixture of *R* and *S* enantiomers, whereas *O. surinamensis* produces (*R*)-IV of high chiral purity (Table 3).

The merchant grain beetle, *O. mercator*, utilizes macrolides I and II as its aggregation pheromone (Table 1; A.M. Pierce, et al., 1984; Pierce et al., 1985). Both I and II are active alone and additively (A.M. Pierce et al., 1984; Pierce et al., 1985). *O. mercator* produces (*R*)-I and (*R*)-II in high chiral purity (Table 3), and this species responds only to the *R* enantiomer of each component of its aggregation pheromone (Pierce et al., 1987). This species would not be expected to be attracted (Table 4) to *C. ferrugineus* because the latter produces only *S*-I (Table 3) (Wong et al., 1983). Cross-attraction of *O. mercator* to *O. surinamensis* is possible since both species produce (*R*)-II (Table 3) which is attractive alone to *O. mercator* (Pierce et al., 1987).

The sawtoothed grain beetle *O. surinamensis* utilizes only three macrolides (II, III, and IV) as pheromones (A.M. Pierce, et al., 1984; Pierce et al., 1985). Macrolides II and III are attractive alone and additively in 1:1 mixture, whereas IV is not attractive but synergizes response to II and III (H.D. Pierce, Jr. et al., 1984; Pierce et al., 1985).

O. surinamensis produces (Table 3) and responds (Pierce et al., 1987) to the *R* isomers of II and IV. Cross-attraction (Table 4) of *O. surinamensis* to *C. ferrugineus* due to the production of minor amounts of II and IV by the latter is not likely since the II:IV ratio in *C. ferrugineus* does not approximate that found in *O. surinamensis* (Table 1). Cross-attraction of *O. surinamensis* to *C. pusillus* is not likely since the only *O. surinamensis* pheromone produced by *C. pusillus* is III and this is a minor component in the volatiles of the latter. Attraction of *O. surinamensis* to *C. turcicus* would not be expected to occur

TABLE 4. CROSS ATTRACTION OF *Cryptolestes* AND *Oryzaephilus* SPECIES[a]

Responding species	Emitting species				
	C. ferrugineus	*C. pusillus*	*C. turcicus*	*O. mercator*	*O. surinamensis*
C. ferrugineus	+	– [1]Emits no VII	– [1]Emits no VII	– [1]Emits no VII [3]Emits *R*-I	– [1]Emits no VII
C. pusillus	– [1]Emits no VI	+	– [1,2]Emits no VI	– [1,2]Emits no VI	– [1,2]Emits no VI
C. turcicus	– [2]Emits insufficient IV relative to V Insufficient amounts of IV and V	– [1,2]Emits no IV	+	– Emits insufficient IV and V	– [2,4]Emits only *R*-IV
O. mercator	– [3]Emits only *S*-I	– [1]Emits no I or II	– [1]Emits no I or II	+	+ (moderate-good) Emits no I
O. surinamensis	– [1]Emits insufficient II	– [1]Emits no II or IV Insufficient III	– [2]Emits no II or III	+ (low) Emits insufficient IV, III relative to II	+

[a] 1 to 4 refer to corresponding mechanisms of species recognition as given in Conclusion. Macrolides listed refer to pheromone components identified as being required for response by responding species that are missing in emitting species. + = responding species is expected to be attracted to emitting species; – = responding species not expected to be attracted to emitting species.

even though these two species produce major amounts of IV. This component of the *O. surinamensis* pheromone blend is not attractive unless II and III are present (H.D. Pierce, Jr. et al., 1984; Pierce et al., 1985). Both of the latter components are absent in *C. turcicus* volatiles. Attraction of *O. mercator* to *O. surinamensis* is likely since each species uses (*R*)-II as an active pheromone component. In addition, *O. mercator* produces a minor amount of III which is singly a pheromone of low activity for *O. surinamensis*.

CONCLUSION

The only cucujid investigated to date likely to exhibit significant cross-attraction is *O. mercator* to *O. surinamensis* (Table 4). The group of insects investigated utilize several mechanisms for species recognition. The major mechanisms identified are: (1) presence of a unique pheromone (*C. ferrugineus*, VII; *C. pusillus*, VI); (2) use of synergistic pheromones that are inactive alone but synergize response to other pheromones (*O. surinamensis*, IV; *C. pusillus* and *C. turcicus*, V); (3) response to only one chiral isomer of a pheromone (*C. ferrugineus* and *O. mercator*, I); and (4) synergism between enantiomers of a pheromone (*C. turcicus*, IV).

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METHYLBUTYNOL EFFECTIVELY REPLACES METHYLBUTENOL, A PHEROMONE COMPONENT OF *Ips typographus* (L.) (COLEOPTERA: SCOLYTIDAE)

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Abstract—In field experiments in Sweden, the constituent 2-methyl-3-buten-2-ol of the aggregation pheromone of the spruce bark bettle *Ips typographus* (L.) was effectively replaced by 2-methyl-3-butyn-2-ol.

Key Words—*Ips typographus*, spruce bark beetle, Coleoptera, Scolytidae, pheromones, aggregation pheromone, methylbutenol, methylbutynol, (*S*)-*cis*-verbenol, ipsdienol, traps.

I. INTRODUCTION

Bark beetle pheromones may contain several components. One or several constituents are necessary and considered to be irreplaceable; others may contribute more or less to the overall attractive effect and are not essential. Little is known about the specific behavioral functions of the individual constituents of bark beetle pheromones.

The spruce bark beetle *Ips typographus* (L.) produces a number of substances in varying proportions (Birgersson et al., 1984). (*S*)-*cis*-Verbenol and

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2-methyl-3-buten-2-ol are claimed to be essential for eliciting aggregation response (Bakke, 1976; Bakke et al., 1977). Varying results have been obtained by the addition of ipsdienol to the essential compounds to increase attraction (Bakke et al., 1983; Schlyter, 1985; Regnander, personal communication).

Recent studies in Czechoslovakia have indicated that it is possible to replace 2-methyl-3-buten-2-ol with another substance, and patent applications for effective blends have been filed (Konečný et al., 1984, 1985). It has been claimed (Ozols et al., 1982) that methylbutenol as well as ipsdienol can be replaced by other substances, but the identity of those substances has not been revealed. To our knowledge, until now there are no documented cases in which essential bark beetle-produced pheromone components have been effectively replaced by a compound of different structure.

In this paper we present evidence from field experiments conducted in Sweden in 1985 that 2-methyl-3-butyn-2-ol can effectively replace 2-methyl-3-buten-2-ol in baits for trapping *Ips typographus*.

METHODS AND MATERIALS

Chemicals. As standards for the field experiments, commercially available Celamerck plastic bag dispensers containing 1500 mg 2-methyl-3-buten-2-ol, 70 mg (S)-*cis*-verbenol, and 15 mg ipsdienol were used. The release rate of the blend was expected to be about 50 mg/day, but it was ca. 30 mg/day in the experiments.

The experimental blends of the following substances were prepared in the laboratory: 2-methyl-3-buten-2-ol (ME), 2-methyl-3-butyn-2-ol (MY), (*S*)-*cis*-verbenol (CV), and ipsdienol (ID).

ME and MY were purchased from Fluka A.G. The identity of the compounds was checked by NMR and the purity by GC. None of these alcohols contained any detectable trace of the other compound (secured detection level 1/1000), either at the start or at the end of the experiments. MY gave only one peak in the GC trace, while ME contained 22% of an impurity most probably consisting of 2-methyl-3-butan-2-ol, which appears to be a by-product formed in the manufacturing process. Racemic ID was synthesized according to Baeckström et al. (1983). The CV was a gift from Borregaard (95% ee) and was purified by liquid chromatography to >99% chemical purity. The main impurity removed was verbenone.

Immediately before the field tests, special glass dispensers of our own design were prepared with the experimental blends. All blends contained CV combined with either ME or MY, in some blends ID was also added. The substances were used in the following proportions by weight: ME and MY 100, CV 5, and ID 1.

Sufficient chemical (5 ml or more) was added to each dispenser to last the

duration of the experiments. Release rates were determined gravimetrically. In three- to five-week experiments release rates varied between 34 and 79 mg/day (two faulty dispensers released 115 and 202 mg/day). The mean release rate was 56 mg/day ($N = 18$). In the long-term experiment (see below) the mean release rate was 35 mg/day, and the dispensers were still effective after more than three months.

Field Experiments. The attractiveness of the chemicals was tested in two experiments of similar design, one 18-day experiment (replacement) in which ME was replaced by MY and another three-month experiment (long-term) in which the long-term attractiveness of MY + CV was studied. In the replacement experiment, 1979 model Swedish traps with collecting funnel were used; in the long-term experiment, 1979 model Norwegian traps without funnel were used (cf Regnander and Solbreck, 1981).

The traps were placed in fresh clear-cuts 35 km north of Uppsala which contained slash and stumps of predominantly Norway spruce. The traps were set up in groups of three with distances of 6 m between traps and 50 m between groups. Each trap in a group was baited with one of three treatments (see below). All traps were placed in a single row strung parallel to the southern forest edge of the clear-cuts at a distance of 50–75 m from the forest.

The replacement experiment was conducted for 18 days. The treatments (baits) were: Commercial lure, MY + CV, and ME + CV. Five groups with three traps in each were used. The catches were collected when a nearby monitoring trap indicated that there had been sufficient flight activity. After each collection, the positions of the traps within each group were interchanged randomly. The catches were collected five times. The five groups and the five trapping periods yielded a total of 25 replicates.

The long-term experiment, at a distance of 5 km from the replacement experiment, was conducted for 102 days. The treatments were: Commercial lure, MY + CV, and ME + CV + ID. A randomized block design with 10 replicates (groups) was used. The treatments within each block remained in the same position during the entire experimental period. The catches were collected at various intervals.

RESULTS

Replacement of Methylbutenol with Methylbutynol. A total of 40,630 *Ips typographus* was caught in this experiment. Table 1 summarizes the results of the comparison between the methylbutynol blend and blends containing methylbutenol. A two-way analysis of variance (3 treatments, 25 blocks) confirmed that there were no significant differences in catch efficiency between the three pheromone blends ($F = 0.98$).

The results show that methylbutynol can effectively replace methylbutenol

TABLE 1. COMPARISON OF ATTRACTIVENESS OF THREE PHEROMONE BLENDS: MEAN CATCHES OF *Ips typographus* PER REPLICATE FOR SUCCESSIVE TRAPPING PERIODS (N = 5) AND ENTIRE EXPERIMENTAL PERIOD (N = 25).

Trapping period 1985	No. of beetles		
	Commercial lure (ME + CV + ID)	MY + CV	ME + CV
May 15–19	538.4	439.0	400.8
May 20–27	519.2	441.0	661.8
May 28	631.4	604.6	597.4
May 29	799.0	747.0	721.0
May 30–June 2	376.6	303.4	336.4
Mean catch ± SD (25 replicates)	572.9 ± 224.4	507.0 ± 234.1	543.5 ± 275.6

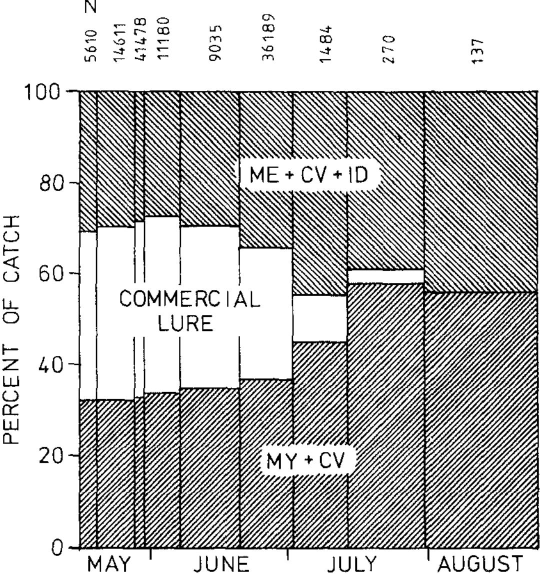

FIG. 1. Relative trap catches of *Ips typographus* in three treatments with different pheromone blends during successive trapping periods. The catches are given as each treatment's percentage of the total catch during each period (N on top of figure = total number of beetles in period = 100%).

in the attraction of *I. typographus*. The commercial lure was not significantly more attractive than the other blends, despite the addition of ipsdienol.

Long-Term Performance of Methylbutynol. A total of 119,994 *Ips typographus* was trapped between mid-May and late August, giving a mean of 4000 beetles per trap. After one and a half months of the experimental period, the trap catches decreased sharply (Figure 1, N).

The attractiveness of the three treatments to *I. typographus* differed towards the end of the experimental period (Figure 1). Higher catches for the traps baited with commercial lure were recorded during the initial three to four weeks. However, two-way analysis of variance did not reveal any significant differences between the three treatments for trap catches up to 35 days. Later, attractiveness of the commercial dispensers decreased strongly, while the experimental glass dispensers remained attractive until the end of the experiment. MY with CV was at least as attractive as the blend containing ME, CV, and ID over a 102-day period.

The results indicate that 2-methyl-3-butyn-2-ol can effectively replace 2-methyl-3-buten-2-ol in blends for trapping *Ips typographus* over long time periods. Ipsdienol was obviously not required for effective trapping in late season.

DISCUSSION

The experiments show that 2-methyl-3-buten-2-ol can be replaced by 2-methyl-3-butyn-2-ol. We are, however, aware of the impurity of the methylbutenol used (see Methods and Materials).

Several thorough studies have been made on substances produced by *Ips typographus*. It is unlikely that 2-methyl-3-butyn-2-ol would have been overlooked. In any case, it can hardly be expected to be released in nature by the beetles in such amounts that it may act as an effective component in combination with (*S*)-*cis*-verbenol to attract the beetles. A possible release of 2-methyl-3-butyn-2-ol by the host plant has not been studied, but such release is improbable.

In the replacement experiment, no significant differences in catches were found between the three mixtures containing methylbutynol or methylbutenol. In the long-term experiment, MY plus CV was not less attractive than the blend ME + CV + ID. Thus, the usefulness of adding ipsdienol to baits for *I. typographus* can be questioned, particularly since it is so attractive for clerid beetles which prey upon *I. typographus* (e.g., Bakke and Kvamme, 1981; Schlyter, 1985).

The release rate of our glass dispensers was adjusted to the expected release rate of the commercial lure used as the standard. In the experiments the actual release rates of the standards were, on average, lower than those of our glass dispensers. According to studies by Sauerwein (1981), Bakke et al. (1983),

and Regnander (personal communication), this difference in release rates is not expected to significantly affect trap catches. This appears confirmed by the first 35 days of the long-term experiment, during which similar blends in the two types of dispenser gave similar catches, while the drop in catch of the commercial dispensers after these 35 days seems to signify a drastic drop in release and attraction.

Interaction between neighboring treatments could be a source of experimental error in trapping experiments, in our case an overestimation of the least attractive treatment. However, the results of Schlyter (1985) indicate low interaction at a 6-m trap distance.

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SEX PHEROMONES OF RICE MOTH, *Corcyra cephalonica* STAINTON
I. Identification of Male Pheromone

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Abstract—Behavioral observations of the rice moth (*Corcyra cephalonica*, Pyralidae, Galleriinae) in the laboratory have shown that a male wing-gland pheromone induces attraction of female moths. This pheromone was identified as a blend of (*E,E*)- and (*Z,E*)-farnesal. Wing-gland extracts or synthetic compounds were shown to be attractive to females by inducing walking.

Key Words—Male pheromone, olfactometer, identification, synthesis, farnesal, Lepidoptera, Pyralidae, Galleriinae, *Corcyra cephalonica*, rice moth, 3,7,11-trimethyl-2,6,10-dodecatrienal.

INTRODUCTION

The rice moth, *Corcyra cephalonica* Stainton (Pyralidae, Galleriinae), is a major pest of stored products in subtropical countries (Balachowsky, 1972). In India, the biology of this insect has been extensively studied and different aspects of the sex pheromone-mediated behavior were reported by Singh and Sidhu (1976). They concluded that: (1) the female emits a sex attractant; (2) "calling" females or abdominal-tip extracts were able to stimulate both males and females; and (3) the male produces an aphrodisiac pheromone.

These results suggest that the pairing system in the rice moth is little different from that found in more widely studied genera such as the tortricids and noctuids. However, although female-emitted pheromones are well-known in

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pyralid subfamilies like the Phycitinae, Pyraustinae, and Crambinae, they have not previously been found in Galleriinae. In this subfamily, male pheromones emitted from wing glands (Vöhringer, 1934; Barth, 1937; Atkinson, 1982; Farine, 1983) evoke a searching behavior in females (Zagatti, 1981; Flint and Merkle, 1983; Greenfield and Coffelt, 1983). It seems that the female responds to male pheromone by walking rather than by flying toward the male, as was originally proposed (Röller et al., 1968; Dahm et al., 1971; Finn and Payne, 1977).

The reported occurrence of female-emitted pheromones in the rice moth led us to reconsider its communication system, since it could represent a step in the evolution of mating behavior in this subfamily.

METHODS AND MATERIALS

Insects. In France, a laboratory-reared strain, fed on *Sorghum*, was obtained from India (G.B. Pant University of Agriculture and Technology), and this strain was compared with "wild" moths found on stored cacao pods in southwestern France. Larvae were fed on rice and vegetables (complementary diet for dogs: MacAni KC 62) and kept in a warm room (27°C, 70% relative humidity) under a 16:8-hr light–dark cycle. Adults were sexed as soon as they emerged (generally half an hour prior to scotophase), and maintained separately thereafter.

Behavioral observations were conducted in a dark room at 25–27°C, with only illumination from a red lamp (Osram 5463; 40 W). Calling and courtship behavior were analyzed using a video-tape recorder (Sanyo VTR 7100) and video camera (Sanyo VC 510). All observations and tests were done between the second and third hour of the scotophase. Moths were 0 (3 hr), 1, or 2 days old when tested.

In England, moths from a laboratory culture originating in Malawi were used. Larvae were fed on a medium of wheat feed (10 parts), dried yeast (1 part), and glycerol (2 parts). Cultures were maintained at 25°C under constant light, and moths were sexed and separated each day.

Olfactometric Tests. Our previous work on galleriine moths (Zagatti, 1981), led us to reconsider the bioassay of pheromones, especially as sex attraction was never quantified for such walking insects. Bioassays were performed in a glass-tube olfactometer (Sower et al., 1973). The tube (70 cm long, 3.5 cm in diameter with an airflow of 1.5 liters/min) was divided into seven sections, each 10 cm in length (Figure 1). Section 0 was at the downwind end and section 6 at the upwind end of the tube.

From 6 to 11 moths were released into section 0 of the tube 30 min before the test, and clean air was passed over them continuously.

Either living insects (A) or hexane extracts (B) were used as pheromone

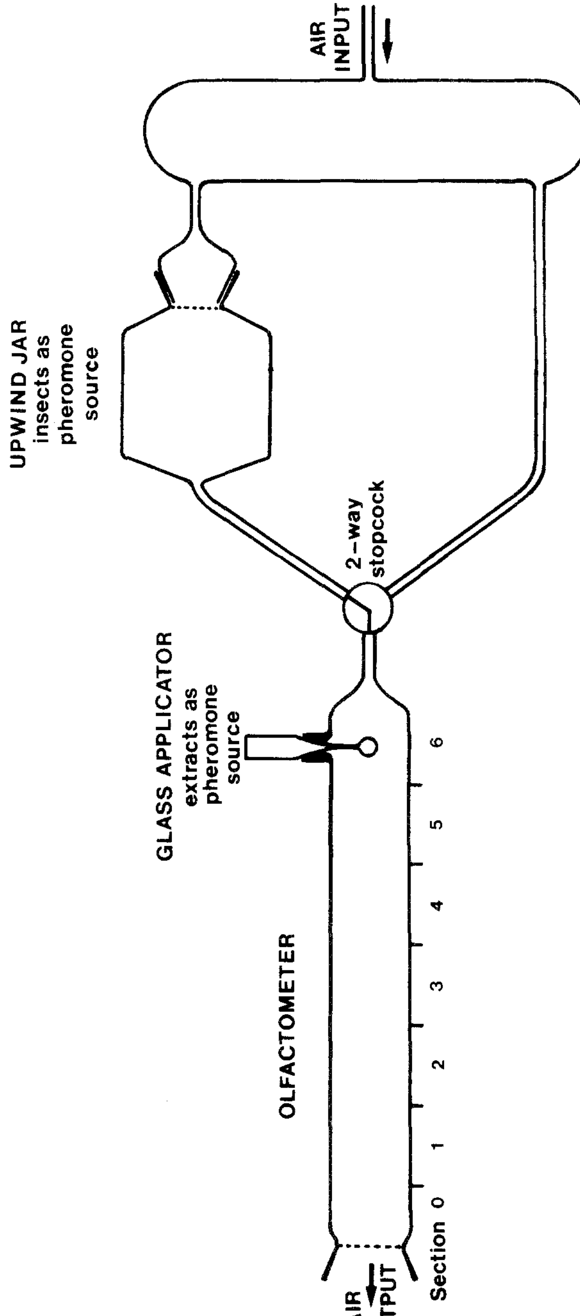

FIG. 1. Diagram of the olfactometer developed for testing the attractiveness of living insects or hexane extracts.

source. (A) The airflow passed through a glass jar containing five males or six females, upwind from the olfactometer tube (Figure 1). (B) The extracts were placed onto an applicator, which consisted of a ground glass rod ending in a small ball (6 mm in diameter). When in place, the ball was situated in the center of the tube, in section 6.

Computation of Results. During a test period of 5 min, the number of moths in each section is recorded every minute, and the position of the moths is given by the formula:

$$X_t = \sum_{i=0}^{6} i\,(n_i)$$

where n_i is the number of moths in section i at the time t. The movement of the moths at each minute is:

$$S_t = X_t - X_0$$

where X_t is the position of the moths at the time t and X_0 the position of the moths at the beginning of the test.

Since the value of S_t depends on the initial distribution of the moths in the tube (X_0), it has to be balanced by the maximum attraction: S_{max} (i.e., the value of S_t if all moths are attracted toward section 6).

$$S_{max} = \sum_{i=0}^{6} (6 - i)n_i$$

where n_i is the number of moths in section i at $t = 0$.

Finally, we define D_t as the index of relative attraction at the time t:

$$D_t = \frac{S_t}{S_{max}} \times 100$$

The tests were not considered when S_{max} was lower than 20.

Statistical analyses were performed using nonparametric tests (Mann-Whitney test, Siegel, 1956); each treatment was repeated eight times.

Pheromone Collection. In France, forewings of 53 males from the Indian strain were clipped and soaked in 1 ml hexane for 2 hr at room temperature. The crude extract was then filtered through glass wool and reduced to ca. 100 μl under N_2 flow. This extract was kept at −30°C until use. An extract of 21 males from the French strain was used for comparison.

For the olfactometric tests, 5 μl of the extract, which correspond to 2.5 male equivalents (ME) were applied to the applicator.

In England, volatiles emitted by male moths were collected on activated charcoal or Porapak Q resin. From one to four virgin male moths were placed in a silanized glass vessel (15 × 4.5 cm). Laboratory air was drawn into the vessel through a filter containing activated charcoal (12 × 2.5 cm; 10–18 mesh)

at 2 liters/min, and out through a collection filter for up to 8 hr. Activated charcoal collection filters contained 5 mg of activated charcoal (0.05–0.1 mm) held between stainless-steel screens (300 mesh) fused into Pyrex glass tubing (6 mm OD, 4 mm ID). Adsorbed volatiles were removed by eluting with dichloromethane (20 μl and then two 10-μl portions) as described by Grob and Zürcher (1976). Porapak collection filters contained 100 mg Porapak Q (50–80 mesh) held between glass wool plugs in a Pasteur pipet (5 mm ID). Adsorbed volatiles were eluted with dichloromethane (200 μl and then two 100-μl portions). Both types of filter were washed well with dichloromethane before use and dried with a nitrogen stream. Dodecyl acetate (1 μg) was added to the solutions of volatiles as internal standard for GC analysis.

Pheromone Identification. In France, male wing-gland extracts were analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS). GC analyses were performed on a Carlo Erba Fractovap 2900 with flame ionization detector (FID), equipped with a nonpolar column (50 m WCOT SE-52, 0.2 mm ID; Girdel, Suresnes, France) heated from 45 to 160°C (25°/min) and from 160 to 210°C (5°/min) and with a Girdel 300 chromatograph (FID) equipped with a polar column (34 m WCOT Carbowax 20 M, 0.3 mm ID; Girdel), operated isothermally at 180°C. GC-MS was performed on a Girdel 32 gas chromatograph coupled to a Nermag R 10-10C quadrupole mass spectrometer (electron impact at 70 eV). The chromatograph was fitted with a fused silica capillary column (25 m WCOT CPSil 5 CB, 0.22 mm ID; Chrompack, Middelburg, The Netherlands) and operated isothermally at 180°C. Chemical ionization–mass spectrometric data were obtained using ammonia as reactant gas at 92.5 eV.

In England, the original work was carried out in 1977 on a Varian 7200 gas chromatograph (FID) using packed GC columns (1.8 m × 2 mm ID glass columns packed with 2.5% SE-30 + 0.25% Carbowax 20 M on 100–120 mesh Chromosorb G AW DMCS, or 1.5% Carbowax 20 M on 100–120 mesh Chromosorb G AW DMCS; temperature programmed from 120° to 220°C at 4°C/min; nitrogen carrier gas at 25 ml/min). GC-MS was carried out with a Pye 104 gas chromatograph coupled to a Varian CH5 DF mass spectrometer via a double-stage Watson-Biemann separator (5-ft × $\frac{1}{4}$-in. glass column packed with OV-225 nonextractable polymer-coated column operated isothermally at 130°C with helium gas at 40 ml/min as the carrier). The results were later checked on fused silica capillary columns coated with CPSil 5 CB (chemically bonded methyl silicone; Chrompack) and CP Wax 57CB (chemically bonded Carbowax 20 M; Chrompack) (25 m × 0.32 mm ID columns; splitless injection; oven temperature 70°C for 2 min, then programmed at 20°C/min to 100°C and then at 2°C/min to 200°C; helium carrier gas at 0.4 kg/cm^2).

Synthesis. For synthesis of (*E,E*)- and (*Z,E*)-farnesyldiphenylurethanes (Bates et al., 1963), a mixture of commercial (Janssen; Beerse, Belgium) far-

nesol (9 ml) [containing (*E*,*E*)-, (*Z*,*E*)-farnesol, and nerolidol], diphenyl-carbamyl chloride (10 g), and anhydrous pyridine was heated at 60°C under Ar for 4 hr. After cooling, the resulting reaction mixture was dissolved in ether and the organic phase was extracted with 2 N HCl, $NaHCO_3$, and brine and dried over Na_2SO_4. The brown oil obtained after evaporation of the solvent was treated with MeOH and left in the cold. White crystals formed which were recrystallized several times from MeOH to provide pure (*E*,*E*)-farnesyldiphenylurethane ($C_{28}H_{35}O_2N$, F = 60–61°C, M^+ at *m/z* 417, base peak at *m/z* 169).

The combined mother liquors were purified by column chromatography (Silicagel 60 Merck). Pure (*Z*,*E*)-farnesyldiphenylurethane was isolated as a colorless liquid by repeated preparative HPLC (Jobin-Yvon Miniprep, hexane–ethyl acetate 9:1). $C_{28}H_{35}ON$: M^+ at *m/z* 417, base peak at *m/z* 169.

For synthesis of (*E*,*E*)- and (*Z*,*E*)-farnesol (Bates et al., 1963), in a typical experiment, 680 mg of farnesyldiphenylurethane are dissolved in a mixture of 3.5 ml EtOH, 0.1 ml H_2O, and 0.4 g KOH, and heated at 60°C for 5 hr. After cooling, the mixture was extracted with Et_2O, and the organic phase was washed with H_2O and dried over Na_2SO_4. The pure alcohols were obtained after column chromatography over Silicagel (Merck) with hexane containing increasing amounts of ethyl acetate. Yield: 310 mg (85%). $C_{15}H_{26}O$: M^+ at *m/z* 222, base peak at *m/z* 69.

(*E*,*E*)-Farnesol was further identified by GC-MS comparison with an authentic sample provided by Dr. W. Pickenhagen, Firmenich S.A., Geneva, Switzerland.

For synthesis of (*E*,*E*)- and (*Z*,*E*)-farnesal, pure (*E*,*E*)-farnesal was obtained from the corresponding alcohol by oxidation with a 100-fold excess of freshly prepared MnO_2 in hexane solution according to Corey and Achiwa (1969), followed by Kugelrohr distillation (3 mm Hg/120°C bath temperature).

When (*Z*,*E*)-farnesol was treated in the same manner, (*Z*,*E*)-farnesal containing slowly increasing amounts of (*E*,*E*)-farnesal was obtained. Therefore, it is recommended to prepare farnesal immediately before use.

RESULTS

Observation of Mating Behavior. The mating behavior of *C. cephalonica* occurs a few hours after the emergence of adults. When kept separately, both male and female moths have a similar behavior; they display rhythmic wing-fanning although it seems more constant and frantic with males. This behavior is observed at the beginning of the scotophase and lasts 3 or 4 hr until copulation.

When male and female moths are kept together in small cages, sexual encounters seem to occur at random. Moths are very agile and continuous walk-

ing or jumping results in frequent contacts; therefore it is difficult to evaluate the significance of such movements for sexual behavior.

Before mating occurs, the wing-fanning male tries to mount the female from behind, then to grasp her with his labial palpi. After copulation, the male releases his grip and rests motionless in a tail-to-tail posture.

Cross Attraction. The results of cross-attraction tests are summarized in Figure 2; five males were used to attract females and six females to attract males (eight replicates each).

Females were obviously attracted by males (Figure 2a), but males also were attracted by females (Figure 2b) although the observed indexes D_t were

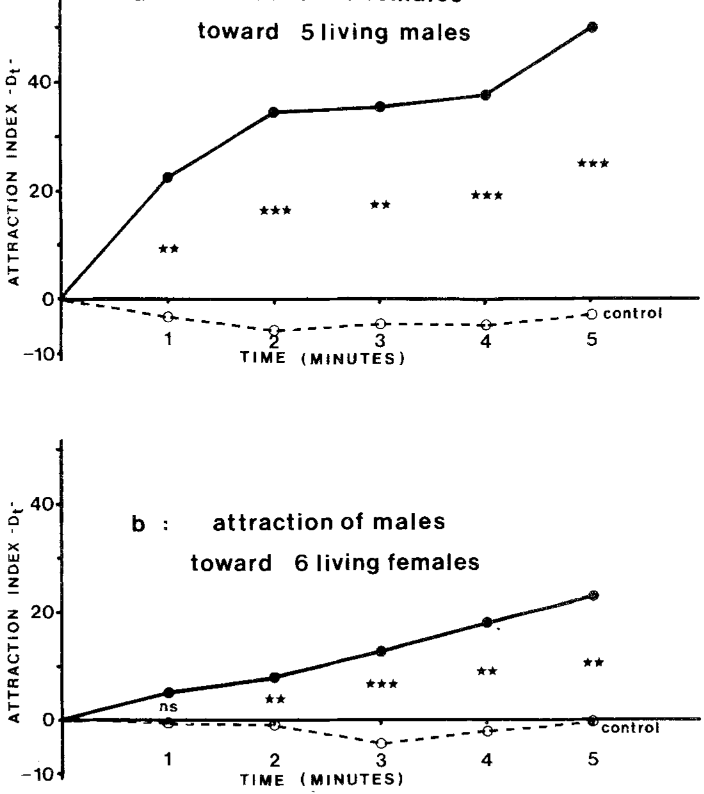

FIG. 2. (a) Relative attraction of 58 females by five males; (b) relative attraction of 69 males by six females. Statistical analysis (Mann-Whitney test): difference with respect to the control (test carried out with 76 females and 68 males, the chamber being empty). ns: not significant; $**P < 0.01$; $***P < 0.001$ for eight replicates.

lower. When males are placed upwind, the females exhibit: (1) raising of the antennae, (2) increase in wing-fanning, and (3) upwind orientation by walking. Attraction is easy to observe with females, which begin to walk only after the stimulation, but is more difficult with males, which are constantly moving.

Attraction of Females toward Male Wing-Gland Extract. The results (Figure 3) are in agreement with cross-attraction tests: virgin females are attracted by male wing extract when tested with 2.5 ME. The attraction of males toward female extract is discussed in a subsequent paper (Hall et al., 1987).

These results clearly show that, in this species, sexual attraction is mediated at least in part by olfaction. As in other Galleriinae, a male pheromone attracts females.

Chemical Identification. GC analyses of extracts from the forewings of the Indian and French strains showed two peaks in a 79:21 ratio. GC-MS data in electron impact mode revealed two sesquiterpenic isomers, with characteristic ions at *m/z* 69 ($C_5H_9^+$) and *m/z* 84 ($C_5H_8O^+$). Chemical ionization mass spectrometry with ammonia indicated a molecular weight of 220 (*m/z* 238).

The computerized spectra library (Sidar data acquisition system) strongly suggested farnesal [(*E*,*E*)-3,7,11-trimethyl-2,6,10-dodecatrienal] as the main compound.

This structure was confirmed by GC comparison of synthetic (*E*,*E*)-farnesal with the natural extract. The minor compound was identified as (*Z*,*E*)-farnesal, which can also be produced by spontaneous isomerization of (*E*,*E*)-farnesal.

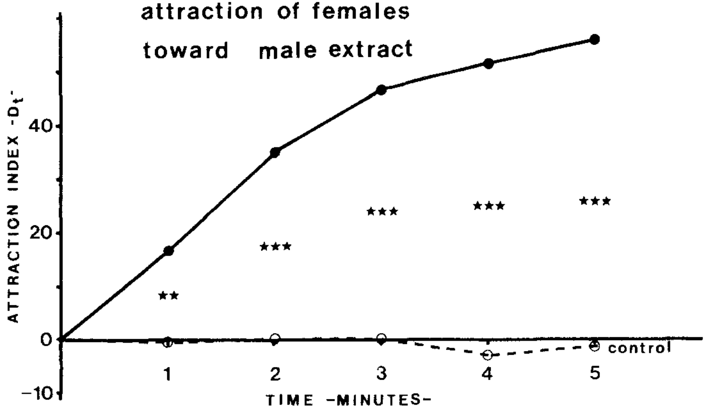

FIG. 3. Relative attraction of 67 females by the male forewing extract (2.5 ME). Statistical analysis (Mann-Whitney test): difference with respect to the control (test carried out with 61 females using 5 μl hexane). $**P < 0.01$; $***P < 0.001$ for eight replicates.

TABLE 1. RETENTION DATA FOR FARNESAL ISOMERS ON FUSED SILICA CAPILLARY COLUMNS

	ECL^{a}	
Isomer	CP Sil 5 CB	CP Wax 57CB
Z,Z	12.60	14.91
Z,E	12.87	15.35
E,Z	12.90	15.49
E,E	13.13	15.81

[a] Equivalent chain lengths relative to retention times for straight-chain acetates.

Analyses of volatiles collected from male moths in England showed two components with no other component more than 1% of the major peak. These components were identified as (*E,E*)- and (*Z,E*)-farnesal by GC-MS and co-chromatography of the natural and synthetic materials on packed and capillary GC columns. All four isomers of farnesal could be distinguished even on packed GC columns, and they were completely resolved on the CP Wax 57CB capillary column. Retention data for the isomers are shown in Table 1.

The *EE/ZE* ratio varied between 95:5 and 89:11, and extraction of the forewings of the moths after entrainment, according to the French procedure, gave ratios identical to those obtained by entrainment of the same moths. The ratio did not seem to vary consistently with age of the moths or time of collection. Addition of a catalytic amount of iodine to the natural extract or entrainment and exposure of the solution to sunlight for 10 min changed the ratio to 77:23.

When male pheromone was collected in the dark, approximately 5 μg of (*E,E*)-farnesal was obtained from four moths over 6 hr. When collections were carried out under low light intensity (approx. 20 lux), the male moths exhibited much more vigorous wing-fanning and 25 μg of (*E,E*)-farnesal could be collected from four moths over 6 hr. Extraction gave approx. 0.5 μg (*E,E*)-farnesal per male moth.

A blend of synthetic farnesal isomers (70% *EE* to 30% *ZE*) evoked a strong attraction of virgin females in olfactometric tests (Figure 4a). The responses obtained with "pure" isomers (Figure 4b) were rather inconclusive. Both (*E,E*)- and (*Z,E*)-farnesal attracted females, but a GC quantitation of the test compounds showed a rapid increase in the relative amount of the minor compound after three or four replicates (10 days), soon reaching an equilibrium.

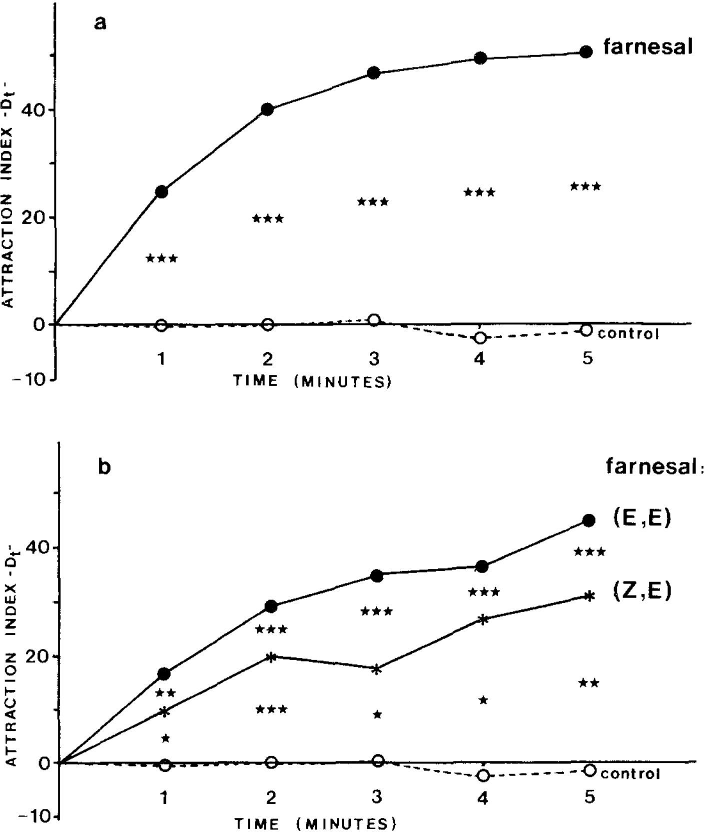

FIG. 4. Relative attraction of females by synthetic farnesal. (a) 50 μg of a 70/30 blend of (*E,E*)- and (*Z,E*)-farnesal (N = 69, eight replicates). (b) (*E,E*)- and (*Z,E*)-farnesal tested separately (25 ng) (N = 42 and 45 respectively, five replicates). Statistical analysis (Mann-Whitney test): difference with the control (61 females, 5 μl hexane, eight replicates): $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The differences between (*E,E*)- and (*Z,E*)-farnesal are not significant.

DISCUSSION

As in other Galleriinae, males of *C. cephalonica* emit a pheromone in order to attract females. Their calling behavior consists of wing-fanning which can be observed from the beginning of the scotophase. By gently abrading the male forewings, scales are easily removed to reveal a pouch located along the costal vein very close to the wing articulation and filled with a yellowish liquid. This pouch is similar to the wing gland of *Galleria mellonella* (Barth, 1937) or *El-*

dana saccharina (Atkinson, 1982; Farine, 1983), and is likely to be the site of male pheromone production.

The first study dealing with *C. cephalonica* (Singh and Sidhu, 1976) implied that males were attracted by females as in the Phycitinae, which represent a pyralid subfamily closely related to Galleriinae (Minet, 1981). The results of our cross-attraction tests show that males actually attract females, and although a pheromone is also produced by females, it is not the primary attractant pheromone mediating the sexual communication of this species (see following paper in this issue).

All our observations suggest that female *C. cephalonica* moths walk rather than fly to calling males, and this would seem to be a general characteristic of Galleriinae. Observations have shown the occurrence of walking behavior in *Eldana saccharina* (Zagatti, 1981) and *Achroia grisella* (Greeenfield and Coffelt, 1983). In trapping experiments with *Galleria mellonella* (Finn and Payne, 1977) and with *Achroia grisella* (Dahm et al., 1971; Spangler, 1984), it was necessary to position sticky traps baited with male moths or synthetic pheromone on the ground in order to catch female moths. It is thus likely that in the Galleriinae the females mate close to the emergence site.

To our knowledge, farnesal has only been reported once before in an insect secretion: in the mandibular glands of the ant *Lasius fuliginosus* (Bernardi et al., 1967). Terpenoid compounds of any sort have only rarely been found to be produced by moths. 6-Methyl-5-hepten-2-one and the corresponding alcohol were found in the abdominal brushes of the noctuid, *Phlogophora meticulosa* (Aplin and Birch, 1970), and pinocarvone in the brushes of another noctuid, *Apamea monoglypha* (Aplin and Birch, 1970). More notably, the monoterpene lactone (3*S*,4*R*)-*trans*-3,7-dimethyl-6-octen-4-olide, is produced in the wing glands of the male galleriid, *Eldana saccharina* (Kunesch et al., 1981; Vigneron et al., 1982), and the diterpene, (*E*)-3,7,11,15-tetramethyl-2-hexadecen-1-ol [(*E*)-phytol], is produced in the wing glands of the male phycitid, *Ephestia elutella* (Phelan et al., 1986).

The biosynthetic origin of the farnesal produced by male *C. cephalonica* moths is unknown. It could possibly be derived from farnesol or farnesyl pyrophosphate in the diet, although it is more likely to have been biosynthesized from mevalonate in the insect tissue. The juvenile hormone JH3, the methyl ester of 10,11-epoxyfarnesoic acid, has been shown to be biosynthesized from mevalonate (Schooley et al., 1973), and both farnesal and farnesol were found in cultures of corpora allata from the tobacco hornworm, *Manduca sexta*, where they are probably precursors of JH3 (Baker et al., 1983).

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SEX PHEROMONES OF RICE MOTH, *Corcyra cephalonica* STAINTON
II. Identification and Role of Female Pheromone

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Abstract—Laboratory investigations of mating behavior in the rice moth, *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae; Galleriinae) showed that male moths are attracted at short range to live, virgin female moths and to female abdominal-tip extract. Volatiles collected from virgin female moths contained one component eliciting an electroantennographic (EAG) response from the male moth, and the chemical, spectroscopic, and chromatographic data on this component were consistent with that of synthetic 6,10,14-trimethyl-2-pentadecanol. This compound caused an EAG response from the male moth and attracted male moths in the bioassay. The pheromone is thought to play a role in courtship, and the synthetic material was shown to cause the male moths to search for a mate and attempt copulation.

Key Words—Rice moth, *Corcyra cephalonica*, Lepidoptera, Pyralidae, Galleriinae, female pheromone, courtship pheromone, olfactometer, electroantenogram, 6,10,14-trimethyl-2-pentadecanol.

INTRODUCTION

Studies of mating behavior in the rice moth, *Corcyra cephalonica* (Lepidoptera: Pyralidae; Galleriinae) have shown that female moths are attracted to male moths, and male moths are attracted to female moths (Zagatti et al., 1987). The male moths were found to produce (*E*,*E*)- and (*Z*,*E*)-farnesal (3,7,11-trimethyl-2,6,10-dodecatrienal) from glands in the forewings, and the synthetic

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compounds were shown to attract walking female moths (Zagatti et al., 1987). This paper describes identification of the female-produced pheromone and studies to establish its role in the mating behavior of this species.

METHODS AND MATERIALS

Insect Material. Laboratory cultures of *C. cephalonica* were maintained as described by Zagatti et al. (1987). The identification work in London was carried out with moths from cultures of Malawi origin. Moths from cultures originating in India were used for the bioassay work in France.

Pheromone Collection and Purification. For bioassay work, the abdominal tips (three posterior segments including ovipositor) of 38 virgin female moths were excised 2 hr into the scotophase. The tips were extracted in 1 ml hexane for 2 hr and the extract filtered through glass wool and reduced to 100 μ under nitrogen.

For the identification work, ovipositor or whole-body washes from virgin female moths were made under a variety of conditions: age 0–7days; at various times throughout a 12 hr–12 hr light–dark cycle or under constant low light; in heptane, ether, or dichloromethane.

To collect pheromone by entrainment, 20–30 virgin female moths were placed in a silanized, 3-liter, bolt-head flask with two paper tissues. Laboratory air was drawn at 1 liter/min into the bottom of the flask through a charcoal filter (12 $\times$ 2 cm; 10–18 mesh) and out through two glass filters (1.5 cm ID) packed with 2.5 g Porapak Q (50–80 mesh) purified by washing well with dichloromethane. Pheromone was collected continuously under natural lighting conditions at 20–25°C. Twice per week, the Porapak filters were extracted with a total of 25 ml dichloromethane, and dead moths were replaced with freshly emerged ones.

After seven weeks of entrainment, the Porapak filter extracts were combined and evaporated to 5 ml on a rotary evaporator at or below room temperature. Florisil (0.5 g, 100–200 mesh) was added and the remaining solvent removed on the rotary evaporator. The coated Florisil was deposited on the top of a column of 9.5 g Florisil (30 $\times$ 1 cm) made up in pentane. This was then eluted with 50-ml aliquots of diethyl ether–pentane mixtures containing 0, 2, 5, 10, 20, 30, 50, 75, and 100% ether. Fractions of 10 ml were collected.

Gas Chromatography (GC). Columns packed with SE-30 or Carbowax 20 M and fused silica capillary columns coated with CPSil 5 CB or CP Wax 57CB were used as described in Zagatti et al. (1987). Retention data are given as the equivalent chain length (ECL) relative to the retention times for straight-chain saturated acetates.

Electroantennography (EAG) and Linked Gas Chromatography-Electroantennography (GC-EAG). Male *C. cephalonica* moths were prepared for

EAG recording as described by Beevor et al. (1986), except that the glass microelectrodes were inserted into the interstitial membrane between annuli of the intact antennal flagellum. The recording electrode was inserted into the distal end of one antenna, and the reference electrode into the proximal end of the other. The microelectrodes were filled with saline (Roelofs and Comeau, 1971) with 0.2% w/v agar added in order to reduce evaporation and avoid crystallization of dissolved salts in the microelectrodes.

Test samples were syringed onto the inner walls of a disposable Pasteur pipet and blown over the antennal preparation with a pulse of nitrogen, as described by Beevor et al. (1986). Typically, an interval of 5 min was allowed between successive stimuli for the preparation to recover fully.

Linked GC-EAG analyses with packed and fused silica capillary GC columns were carried out as described by Moorhouse et al. (1969) and Beevor et al. (1986). The GC column effluent was split 1 : 1 between the EAG preparation and the flame ionization detector. The GC columns used were as described above.

Microchemical Reactions. For bromination, a dilute solution of bromine in carbon tetrachloride (5 μl) was added to the sample dissolved in carbon tetrachloride (5 μl), and the mixture analyzed after 20 min at room temperature. The carbon tetrachloride was purified by passage through neutral alumina.

Reductions were carried out by adding a few grains of lithium aluminium hydride to the sample dissolved in dichloromethane (15 μl).

Oxidations were carried out with a saturated solution of pyridinium chlorochromate in dichloromethane for 20 min at room temperature. The pyridinium chlorochromate was washed with ether before use, and the dichloromethane was passed through neutral alumina.

In all microchemical reactions, blanks for assay by GC or EAG were prepared under identical conditions using pure solvent instead of the test solution.

Mass Spectrometry (MS). Linked GC-MS analyses of natural and synthetic materials were carried out in EI mode at 70 eV with a VG Micromass 7070F instrument and VG 2000 data system. This was coupled via an all-glass jet separator to a Pye 104 gas chromatograph fitted with a glass column (2.5 m × 2 mm ID) packed with 15% Carbowax 20 M on Chromosorb W and operated isothermally at 200°C.

Synthesis. 5,9,13-Trimethyl-2-pentadecanol (compound A) was prepared by a sequence starting with three successive Julia reactions (Julia et al., 1960) as shown in Figure 1. The "one-pot" modification (Biernacki and Gdula, 1979) was used in these reactions. The yields became progressively poorer, but intermediates were available from a preparation of 4,8-dimethyldecanal, the aggregation pheromone of *Tribolium* spp. (Breuer et al., 1982). The yields are quoted for products purified by chromatography if necessary and distillation.

6,10,14-Trimethyl-2-pentadecanol (compound B) was prepared from far-

FIG. 1. Synthesis of 5,9,13-trimethyl-2-pentadecanol (A). Reagents: (i) Mg/Et_2O; cyclopropylmethyl ketone; H_2SO_4; (ii) $KOAc/CH_3CN$/Adogen 464; (iii) $K_2CO_3/MeOH$; (iv) $H_2/PtO_2/EtOH$; (v) pyridinium chlorochromate/CH_2Cl_2; (vi) CH_3MgI/Et_2O. Yields are for chromatographically homogeneous products after distillation.

nesol by standard reactions involving two-carbon homologation with ethyl acetoacetate, as shown in Figure 2.

Bioassays. The olfactometric bioassay used was that described by Zagatti et al. (1987) and consisted of a glass tube olfactometer divided into seven sections. The positions of moths released into the olfactometer were recorded every minute for 5 min, and the attraction index (D_t) calculated as described by Zagatti et al. (1987).

In order to determine the range of action of the female pheromone, the responses of individual male moths to female extract (1 female equivalent, FE) were recorded. In all, 99 male moths were observed in the olfactometer for 3-min periods, and the time and direction of their first shift were recorded along with their initial position. As controls, 28 males were observed under the same

FIG. 2. Synthesis of 6,10,14-trimethyl-2-pentadecanol (B). Reagents: (i) $H_2/PtO_2/EtOH$; (ii) $CBr_4/PPh_3/CH_2Cl_2$; (iii) ethyl acetoacetate/Na/EtOH; (iv) 150°C/DMSO/H_2O; (v) $NaBH_4/EtOH$. Yields are for chromatographically homogeneous products after distillation.

conditions with hexane (5 μl) on the applicator, and there was no significant difference between the numbers of moths moving upwind and downwind after 3 min ($\chi^2 = 3.7$ with 4 *df*). The percentages of moths making an initial upwind shift after periods of 30 sec, 1 min 30 sec, and 3 min were calculated for different initial positions, and these were compared with the corresponding results in the control tests using a 2×2 χ^2 analysis.

The precise role of the female-emitted pheromone in mating behavior was investigated using the synthetic pheromone. A solution of the pheromone (100 ng) in hexane (1 μl) was deposited onto the inner walls of a disposable Pasteur pipet, and air was blown at 200 ml/min through the pipet and over a pair of male moths caged in a small cylindrical box (5×8 cm diameter). A total of 15 pairs of males was tested with the pheromone and 15 pairs with hexane (1 μl) as controls. The results were recorded as the percentages of moths showing the following types of behavior within a 2-min observation period (Table 3): (1) calling posture (stationary, fanning wings); (2) search for females (walking, fanning wings); and (3) copulation attempts (moths circling and trying to mount one another).

The test and control results were totally different, obviating the need for statistical analysis.

RESULTS

Evidence for Female-Emitted Sex Pheromone. During initial studies of pheromone-mediated behavior in *C. cephalonica*, it was shown that live virgin female moths can attract male moths (Zagatti et al., 1987). A crude hexane extract of female abdominal tips (2.5 FE) was also shown to be attractive (Figure 3), demonstrating the olfactory nature of this phenomenon.

In these tests, it seemed that male moths close to the pheromone source were markedly more stimulated than those further downwind. By recording the responses of individual male moths at different distances from the pheromone source it was shown that the percentage of moths responding to female extract (1 FE) by an upwind shift decreased sharply at distances greater than 13 cm from the source (Figure 4). At distances greater than 33 cm from the source, there was no significant difference between the responses in the presence or absence of the pheromone.

Pheromone Identification. Whole-body washes or ovipositor washings from virgin female *C. cephalonica* moths caused EAG responses from the male moth, and linked GC-EAG analyses suggested that a single EAG-active component was present. Amounts of this component obtained under a variety of conditions were much less than 1 ng/female, and purification of these solvent washes by GC or liquid chromatography did not remove large amounts of impurities which prevented further analytical work.

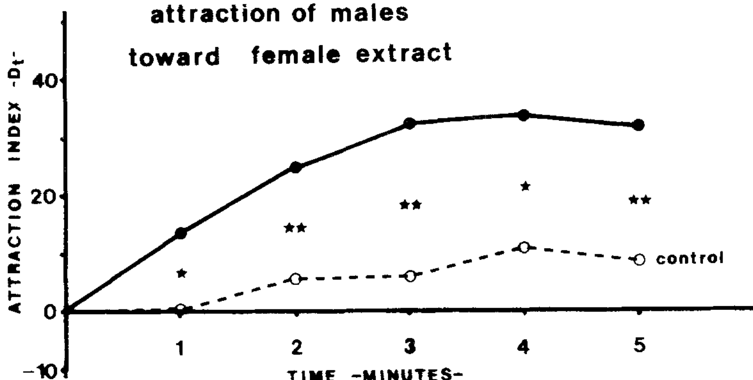

FIG. 3. Attraction indices for male *C. cephalonica* moths to female abdominal tip extract (2.5 FE; 68 males) and hexane (5 μl; 64 males) as control (eight replicates). Statistical analysis by Mann-Whitney test: difference between treatment and control significant at $^{*}P = 0.05$; $^{**}P = 0.01$.

Aeration of virgin female moths and collection of the volatiles emitted on Porapak Q gave significant amounts of pheromone that could be purified by liquid chromatography on Florisil. Assay of the fractions by EAG showed that essentially all the activity was eluted with 20% ether in pentane. Thus the crude Porapak extract (0.5 μl from 5 ml) gave an EAG response greater than 0.8 mV; the two active fractions (0.5 μl from 10 ml) each also gave an EAG response greater than 0.8 mV with no other fractions giving responses greater than 0.29 mV.

Linked GC-EAG analysis of the crude Porapak extract on a GC column packed with Carbowax 20 M showed only one EAG-active component at ECL 15.36 (total response 1.0 mV; no other response greater than 0.15 mV). Analysis of the active fractions from liquid chromatography similarly showed one active component at the same retention time (total response 1.9 mV; background response 0.20 mV), and these fractions were sufficiently pure for carrying out microchemical reactions and GC-MS analysis on the active component. Approximately 5 μg of this purified material was obtained after seven weeks of entrainment (1000–1500 moth days).

When microgram quantities of synthetic long-chain hydrocarbons, aldehydes, acetates, and alcohols were chromatographed on Florisil under the same conditions, hydrocarbons were eluted with pentane, aldehydes with 2–5% ether in pentane, acetates with 5% ether in pentane, secondary alcohols with 10–20% ether in pentane, and primary alcohols with 50% ether in pentane. The elution behavior of the active component was thus consistent with that of a long-chain, secondary alcohol.

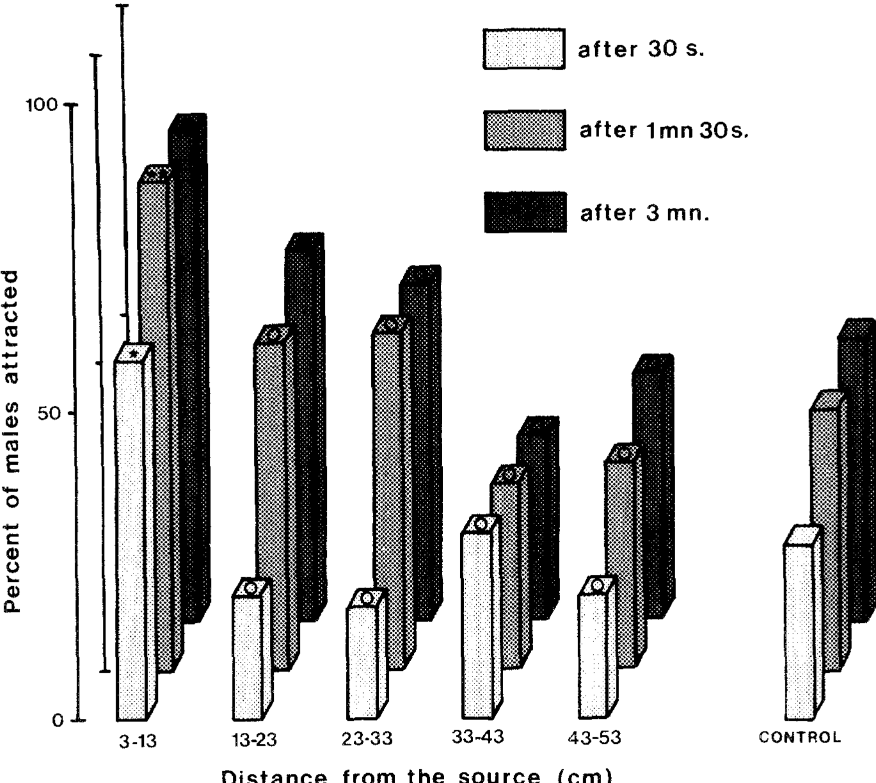

FIG. 4. Responses of male *C. cephalonica* moths to female abdominal tip extract (1 FE; 99 males) and hexane (1 μl; 28 males) as control, relative to their initial distance from the source and the time after the start of the stimulation. The vertical axis represents the percentage of males which moved at least one section upwind in the olfactometer. Statistical analysis by χ^2 test: difference between treatment and control not significant, o; significant at $*P = 0.05$; $**P = 0.01$.

Bromination of the crude Porapak extract caused no loss in EAG activity. GC analyses on a GC column packed with Carbowax 20 M showed that the purified active component was unaffected by bromination. Bromination of standard synthetic compounds showed that unsaturated compounds and aldehydes reacted but saturated acetates, alcohols, and ketones were unchanged.

On treatment of the crude Porapak extract with lithium aluminium hydride in dichloromethane, EAG activity was lost, but the activity was completely restored when the reaction mixture was hydrolyzed with water. These results were consistent with the active component being a saturated alcohol.

Reaction of the purified active component with pyridinium chlorochromate

was monitored by GC analysis on columns packed with SE-30 and Carbowax 20 M. The active component at ECL 14.44 and 15.36 on the two columns reacted to give a product at ECL 14.30 and 14.33. These shifts in ECL on the two GC phases of −0.14 and −1.03, respectively, were consistent with a methyl carbinol-to-methyl ketone conversion (Table 1).

The mass spectrum of the active component (Figure 5a) showed ions of highest mass number at *m/z* 255 and 252, interpreted as loss of CH_3 and H_2O from a saturated, 18-carbon alcohol of molecular weight 270. The remainder of the spectrum suggested an aliphatic rather than alicyclic structure. The GC retention times for the active component on fused silica capillary columns (Table 2) indicated that, if the component was a saturated 18-carbon alcohol with the alcohol in the 2 position, it must have several branches in the aliphatic chain. These branches would be favored fragmentation points in the mass spectrum (e.g., Carlson et al., 1978), and 5,9,13-trimethyl-2-pentadecanol (A) was prepared as a model compound. This compound had a mass spectrum (Figure 5c) significantly different from that of the active component and slightly longer GC retention times (Table 2). In view of the known shorter retention times of

TABLE 1. RETENTION DATA FOR NATURAL AND SYNTHETIC ALCOHOLS AND CORRESPONDING CARBONYL COMPOUNDS ON PACKED GC COLUMNS[a]

	ECL	
Compound	SE-30	Carbowax 20 M
Natural pheromone component	14.44	15.36
Natural pheromone component + pyridinium chlorochromate	14.30	14.33
(difference)	(−0.14)	(−1.03)
1-Heptadecanol	15.75	17.70
Heptadecanal	15.05	15.30
(difference)	(−0.70)	(−2.40)
2-Heptadecanol	15.04	16.31
2-Heptadecanone	14.88	15.31
(difference)	(−0.16)	(−1.00)
3-Heptadecanol	14.95	16.00
3-Heptadecanone	15.00	15.00
(difference)	(+0.05)	(−1.00)
4-Heptadecanol	15.00	15.86
4-Heptadecanone	14.70	14.43
(difference)	(−0.30)	(−1.43)

[a]Glass columns (1.8 m × 2 mm ID) packed with 2.5% SE-30 + 0.25% Carbowax 20 M or 1.5% Carbowax 20 M on 100–120 mesh Chromosorb G AW DMCS; temperature programmed from 120°C to 220°C at 4°C/min; nitrogen carrier gas at 25 ml/min. ECL = equivalent chain length.

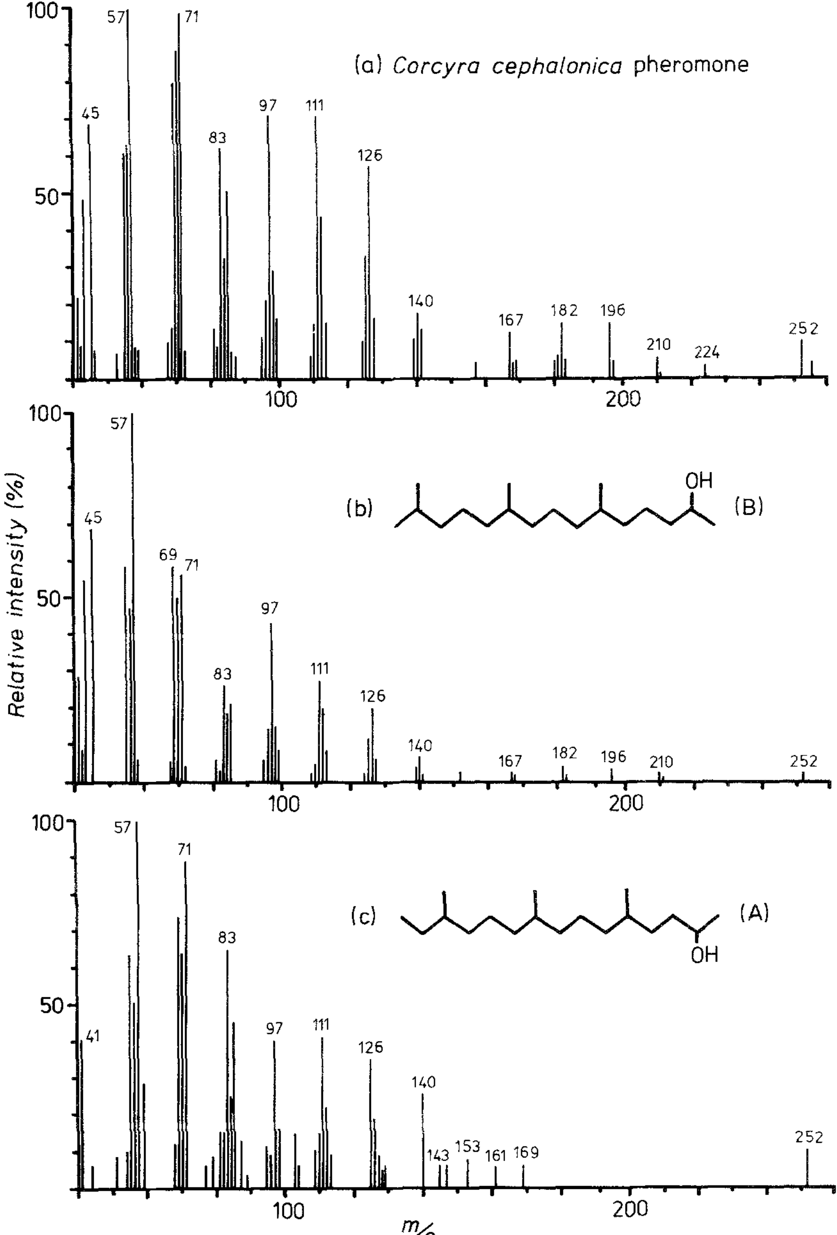

FIG. 5. Electron impact mass spectra of (a) EAG-active component in volatiles collected from virgin female *C. cephalonica* moths; (b) synthetic 6,10,14-trimethyl-2-pentadecanol (B); (c) synthetic 5,9,13-trimethyl-2-pentadecanol (A).

TABLE 2. GC DATA FOR NATURAL AND SYNTHETIC COMPOUNDS ON FUSED SILICA CAPILLARY COLUMNS[a]

	CP Sil 5 CB	CP Wax 57CB
	ECL	
Natural pheromone component and		
6,10,14-Trimethyl-2-pentadecanol (B)	14.42	15.50
5,9,13-Trimethyl-2-pentadecanol (A)	14.52	15.57
2-Octadecanol	16.02	17.45
	Peak width at half height (sec)	
National pheromone component	3.4	8.0
6,10,14-Trimethyl-2-pentadecanol	9.0	11.4

[a]Columns (25 m × 0.32 mm ID) coated with CP Sil 5 CB (chemically bonded methylsilicone) or CP Wax 57CB (chemically bonded Carbowax 20 M); oven temperature 70°C for 2 min, then programmed at 20°C/min to 100°C, then at 2°C/min to 200°C; helium carrier gas at 0.4 kg/cm^2

iso-branched fatty acid methyl esters relative to the *anteiso*- isomers (e.g., Body, 1984), 6,10,14-trimethyl-2-pentadecanol (B) was then prepared. This compound had a mass spectrum (Figure 5b) that was essentially identical with that of the active component, and identical retention times on fused silica capillary GC columns (Table 2). The latter were confirmed by cochromatography of the natural and synthetic materials. The GC peak for the synthetic mixture of four diastereoisomers was broadened relative to that for the natural component on both columns (Table 2), suggesting that the natural component consisted of only one or a few of the possible diasteroisomers.

Compound B elicited a significant EAG response from male *C. cephalonica* moths, while compound A showed no activity. Responses from the same moth to 3.5 ng of material delivered through the GC-EAG link were 2.31, 1.23, and 0.0 mV for the natural pheromone component, compound B, and compound A, respectively. The lower EAG response to the synthetic mixture of diastereoisomers and enantiomers also suggests that the natural material consists of only one or a few of the possible isomers.

Bioassays of Synthetic Pheromone. Both candidate compounds A and B were tested as attractants in the olfactometer. At the 200 ng level, compound B was highly attractive to the male moths whereas A was inactive even at the 400 ng level (Figure 6a). The attraction indices for 200 ng of B were considerably higher than those evoked by 2.5 FE of female abdominal tip extract (Figure 3), and the tests were repeated with lower amounts of the synthetic material (Figure 6b). Male moths were significantly attracted to 2 ng of compound B.

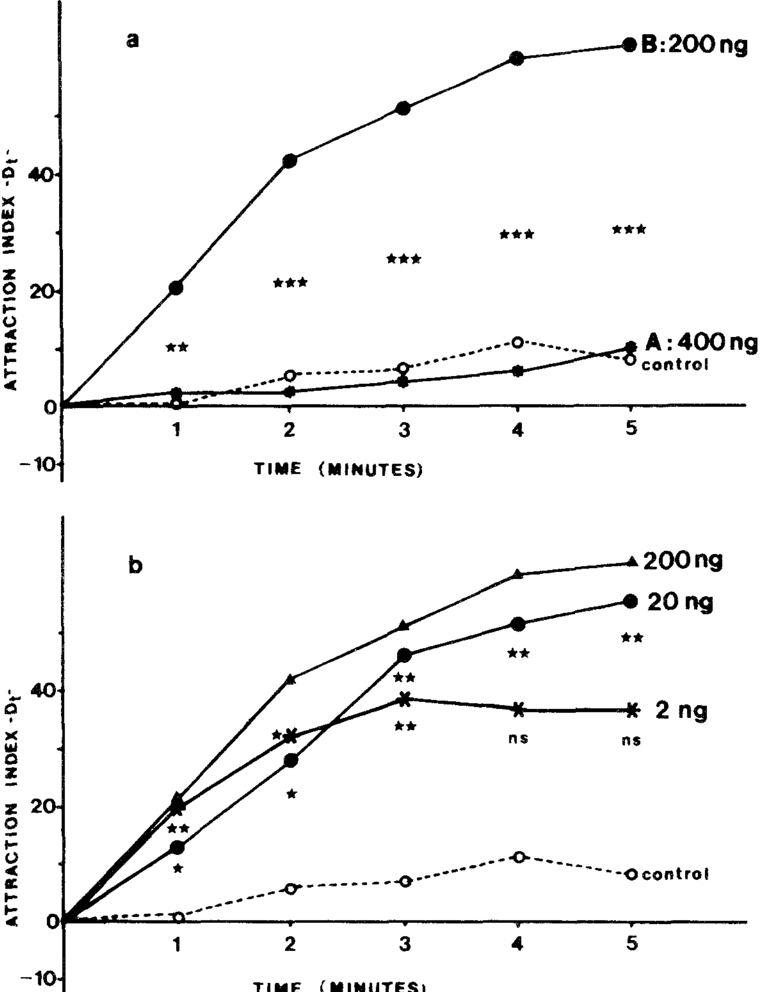

FIG. 6. (a) Attraction indices for male *C. cephalonica* moths to compound A (400 ng; 67 males), compound B (200 ng; 67 males), and hexane (5 μl; 64 males) as control (eight replicates). (b) Attraction indices for male *C. cephalonica* moths to compound B (20 ng, 30 males; 2 ng, 27 males) (four replicates). Statistical analyses by Mann-Whitney test: difference between treatment and control not significant, ns; significant at *P = 0.05; **P = 0.01; ***P = 0.001.

The synthetic compound B was used to investigate further the effect of the female pheromone on male behavior. Table 3 shows the results of exposing pairs of virgin males in small cages to compound B. Before the test, 50% of the males were essentially stationary and fanning their wings. The male pheromone is emitted during wing-fanning (Zagatti et al., 1987), and it is assumed

TABLE 3. EFFECTS OF SYNTHETIC PHEROMONE ON BEHAVIOR OF MALE *C. cephalonica*

	Observed behavior (%)[a]				
	Before stimulation		After stimulation		
Stimulant	Calling posture	Search for female	Calling posture	Search for female	Copulation attempts
100 ng (B)	50	0	0	87	53
1 μl hexane	57	0	47	0	0

[a]2 × 15 pairs of male moths observed within a 2-min period.

that this is "calling" behavior. In less than 2 min after stimulation with compound B, 87% of the moths tested started to walk sinuously while continuing their wing-fanning. This behavior seems to indicate searching for a mate, and homosexual copulation attempts were observed in 53% of the pairs. During control tests with pure solvent, the initial percentage of calling males decreased slightly, possibly due to the air disturbance. Neither searching for a mate nor copulation attempts were observed.

In the same experiment run with a dead female moth replacing one of the pair of live males, no copulation attempts were observed, showing the importance of visual and/or auditory cues in the courtship behavior.

DISCUSSION

Virgin female *C. cephalonica* moths have been shown to produce a pheromone that acts as a short-range attractant for the male moths. Volatiles collected from virgin female moths contain one component that elicits EAG responses from male moths, and the available chemical, chromatographic, and spectroscopic data on this component are consistent with that of synthetic 6,10,14-trimethyl-2-pentadecanol (B). In support of this identification, compound B causes an EAG response from male *C. cephalonica* moths and is highly active in the bioassays. Furthermore, the closely related compound, 5,9,13-trimethyl-2-pentadecanol (A) is biologically inactive.

No attempt has been made to determine which of the four diastereoisomeric pairs of enantiomers of B is (are) produced by the female moth, but there is chemical and biological evidence that only one or a few of the isomers is involved. Thus the GC peak width for the natural pheromone component is narrower than that for the synthetic mixture of isomers, and the natural phero-

mone causes a greater EAG response than that to an equal amount of the synthetic.

It is also possible that other pheromone components are present, although these must be present in much lower amounts and/or cause a smaller EAG response than the major component.

When this work was carried out, 6,10,14-trimethyl-2-pentadecanol (B) had not previously been found in an insect secretion. However, Burger et al. (1985) recently reported this compound to be a major component of the material produced in the male abdominal brushes of *Eldana saccharina*, another galleriid closely related to *C. cephalonica*. No biological function was reported.

In most lepidopterous species, the female produces a long-range attractant pheromone. It has also been demonstrated in many of these species that the male produces a short-range courtship pheromone. Butler (1967) used the general term "aphrodisiac" for these, but this is inadequate to describe the many roles they may play (Boppré, 1984). Short-range attraction of female *Grapholitha molesta* (Lepidoptera: Tortricidae) by the male was demonstrated by Baker et al. (1981), and the pheromone emitted from the male abdominal brushes was fully identified by Nishida et al. (1982). The bioassay was carried out over a distance of 2 cm.

Ephestia elutella (Lepidoptera: Pyralidae; Phycitinae) is more closely related to *C. cephalonica*. The female produces a long-range attractant, and the males have been shown to produce a pheromone from glands in the forewings that causes the female moth to stop calling, flex the abdomen downwards, and turn (Krasnoff and Vick, 1984). This pheromone was characterized chemically by Phelan et al. (1986) and shown to contain several γ-lactones and (*E*)-phytol [(*E*)-3,7,11,15-tetramethyl-2-hexadecen-1-ol], a compound not unrelated to the 6,10,14-trimethyl-2-pentadecanol (B) found to be produced by female *C. cephalonica* moths.

By contrast, in the Galleriinae it seems that the long-range pheromone is typically produced by the male moths, evoking a searching behavior in the females (Zagatti et al., 1987, and references therein). This necessitates a cue from the female for the male to change behavior from passive calling to courtship directed towards the female. In *Eldana saccharina* it was shown that this cue is auditory in nature (Zagatti, 1981). In *C. cephalonica* the female moths emit a courtship pheromone that acts over a short distance to attract the male and cause him to attempt copulation.

The site of production of the female pheromone has not been established, and histological examination has failed to show any glandular structures on the female abdomen. In the females of most Lepidoptera, the pheromone-producing glands are modifications of the intersegmental membranes between abdominal segments VIII and IX (Percy and Weatherston, 1974). In *C. cephalonica* it is possible that secretory cells are disseminated along the abdomen. Thus analyses

of female cuticular washes suggested that more pheromone was obtained when larger portions of the abdomen were taken, and collection of volatiles from female moths was found to be a better source of pheromone than cuticular washing.

It is uncertain how useful the synthetic pheromones will be in monitoring or control of *C. cephalonica*. The female-emitted pheromone is probably too short-range in action to be used alone. The male-emitted pheromone could be used to bait traps or to disrupt mating if it causes a directed, anemotactic response in the female moth, rather than a searching response as is the case with *E. saccharina*. Work to investigate this further is in progress.

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GALLERY INITIATION BY *Tomicus piniperda* (COLEOPTERA: SCOLYTIDAE) ON SCOTS PINE TREES BAITED WITH HOST VOLATILES

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Abstract—In a trapping experiment, wood chips from freshly cut Scots pine trees attracted flying adults of the bark beetle *Tomicus piniperda* (L.) Healthy Scots pine trees, which usually are not attacked by *T. piniperda*, were baited with chips from freshly cut trees. In other experiments trees were baited with a mixture of (−)-α-pinene, (+)-3-carene, and terpinolene; the individual monoterpenes; or with ethanol alone. All baited treatments were attacked by *T. piniperda*. Most of the attacks resulted in short egg galleries and in larval galleries which were only a few millimeters in length. In trees from which a new generation of beetles emerged, net reproduction was well below 1.0. Unbaited control trees remained unattacked or received a few isolated attacks.

Key Words—Gallery initiation, host volatiles, monoterpenes, ethanol, reproductive success, bark beetle, pine shoot beetle, *Tomicus piniperda*, Coleoptera, Scolytidae.

INTRODUCTION

The pine shoot beetle *Tomicus piniperda* (L.) is one of the most important forest insects of Scots pine, *Pinus sylvestris* L., in northern Europe. At high population levels of beetles their feeding in shoots of healthy trees can result in considerable growth reduction (Mattson-Mårn, 1921; Nilsson, 1974, 1975; Långström, 1980; Ericsson et al., 1985).

In early spring the beetles leave their hibernation sites in the bark at the base of living pine trees. Under favorable weather conditions the main flight period and the main period of gallery initiation last for only a few days (Bakke,

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1968; Eidmann, 1965, 1974). The beetles reproduce in logs, stumps, and pines that are windbroken, windthrown, or otherwise weakened. Field studies have failed to demonstrate the occurrence of an aggregation pheromone in *T. piniperda*; instead, this species uses host volatiles to locate breeding substrate (Kangas et al., 1967; Perttunen et al., 1970; Jordal, 1979; Byers et al., 1985). Byers et al. (1985) demonstrated that *T. piniperda* is strongly attracted to several major monoterpenes isolated from Scots pine: (+)-α-pinene, (−)-α-pinene, (+)-3-carene, and terpinolene. Subsequently, Vité et al. (1986) showed that the addition of ethanol to a mixture of α-pinene and terpinolene strongly increased the attraction of flying *T. piniperda*.

The influence of host volatiles on gallery initiation by *T. piniperda* has not been demonstrated earlier. The biodegradation product ethanol has been shown to induce attacks by some bark and ambrosia beetles (Kerck, 1972; Roling and Kearby, 1975; McLean and Borden, 1977; Moeck, 1981). In this study we addressed four questions: (1) Do naturally occurring blends of host volatiles induce gallery initiation? (2) Do the same monoterpenes that attract the beetles also induce gallery initiation? (3) Can single monoterpenes have this effect? (4) Can ethanol induce gallery initiation?

METHODS AND MATERIALS

All experiments were conducted in Scots pine stands in the province of Uppland in central Sweden except for the induction-of-attack test with monoterpenes, which was conducted in the province of Dalarna.

Attraction to Pine Chips. Attraction of beetles to traps with and without chips from freshly cut Scots pine trees was monitored by means of sticky traps during 1983. The beetles were caught on wire net cylinders (6.5 mm mesh, 50 cm high, 30 cm diam.) coated with Stickem-Special®. The traps were attached to plastic tubes at a height of 1.5 m. Inside each wire net cylinder one metallic cylinder (20 cm high, 8 cm diam.) covered on both ends with wire-netting was attached. In seven traps the cylinders were filled with chips of Scots pine, and in the seven control traps empty cylinders were used. Every 24 hr the cylinders were refilled with fresh chips. The traps were placed in two parallel rows, every other trap being a control. The distance between traps was 15 m while that between rows was 40 m. Captured beetles were collected after each day of flight, and catches were pooled for each trap in the analysis.

Induction of Attack with Pine Chips. Healthy trees in a 55-year-old pine stand with a mean height of 13 m were baited with chips from freshly cut Scots pine trees during 1983. Twelve trees were selected and four of these were randomly assigned to the treatment. During the first day of beetle flight (April 16), these four trees were provided with fresh pine chips in cylinders of the same model as described above. Fourteen cylinders were attached around the trunk

TABLE 1. MEAN NUMBER OF ATTACKS BY *T. piniperda* ON SCOTS PINE TREES BAITED WITH CHIPS AND ON UNBAITED CONTROL TREES

Treatment	Replicates (No.)	Tree diam. (cm)		Attacks per tree (No.)	
		$\bar{X}$	Range	$\bar{X}$	Range
Controls	8	14.3	11.6–18.0	1.9	0–6
Chips April 16	4	13.9	12.6–17.0	197.5[a]	98–279
Chips April 20	4	13.6	9.0–16.3	35.3[a]	24–54

[a]Treatment is significantly different from control ($P = 0.05$, Mann-Whitney test).

of each tree, seven at a height of 0.5 m and seven at a height of 1.2 m. The eight remaining trees served as controls and were left untreated. The following day (April 17), boring dust was found on the four baited trees. To obtain more replicates, four additional trees were baited with chips on the second day of beetle flight (April 20). The distance between selected trees was at least 4 m. Mean diameters at 1.3 m are given in Table 1.

In September 1983, all 16 trees were felled. The trunks were then totally debarked and inspected for bark beetle attacks. A *T. piniperda* attack was defined as any penetration through the bark that reached the phloem, even if no gallery was formed, with the exception of the penetrations at ground level caused by hibernating beetles during previous years. Attacks just reaching the phloem could be identified as *T. piniperda* attacks by the dimension of the entry holes and by the localization of the attacks to the rough bark.

In this and the following experiments we expressed our data as number of galleries per tree since entire trees were inspected for attacks. Attack densities expressed, e.g., as galleries per square meter, are of minor interest in the present context.

Induction of Attack with Monoterpenes. Healthy trees in a 130-year-old pine stand with a mean height of 12 m were baited with monoterpenes during 1984. On a 0.5-hectare plot, eight groups of five trees each were selected. The distance between selected trees was at least 5 m. Mean diameters at 1.3 m are given in Table 2. The trees in each group were randomly assigned one of five treatments. On the first day of beetle flight (April 28), three trees in each group were baited individually with commercial samples of either (−)-α-pinene (Fluka 99.5%, $[\alpha]_D^{20}$ −42 ± 1°), (+)-3-carene (Fluka 99%, $[\alpha]_D^{20}$ +17 ± 0.5°), or terpinolene (92% GC), and one tree in each group was baited with all three chemicals (Table 2). The remaining tree in each group was left as an unbaited control. Monoterpene baits were selected with respect to their attractivity to *T. piniperda* (Byers et al., 1985).

The chemicals (50 μl) were individually released from open conical vials

TABLE 2. MEAN NUMBER OF ATTACKS BY *T. piniperda* ON SCOTS PINE TREES BAITED WITH MONOTERPENES AND ON UNBAITED CONTROL TREES[a]

	Tree diam. (cm)		Attacks per tree (No.)	
Treatments	$\bar{X}$	Range	$\bar{X}$	Range
Control trees	11.7	10.3–13.4	0	0
(−)-α-Pinene	12.3	8.9–14.4	174.5[b]	121–237
(+)-3-Carene	11.6	9.5–14.2	52.4[b]	8–89
Terpinolene	12.5	11.0–14.2	116.5[b]	0–312
(−)-α-pinene, (+)-3-carene, and terpinolene	12.2	10.4–13.5	208.1[b]	121–298

[a] Eight replicates in each treatment
[b] Significantly higher number of attacks than control ($P = 0.05$, Dunnett's test, one-tailed).

(upper diam. 5 mm) which were attached to the trunk at 1.2 m height. Each tree was baited with two vials containing the same compound, one on each side of the trunk. The release rates of the compounds during the experiment were not measured. In the laboratory at 15°C the monoterpenes were released at about 5 μl/hr from each vial. The sequence of application was α-pinene, 3-carene, and terpinolene with about 30 min application time for each chemical. Because of this time lag in application and since the beetle flight had already started by the time the chemicals were applied, no comparison between baited treatments was made in the analysis. On the morning of the following day (April 29), boring dust was found on each of the 32 baited trees except for one of the eight trees baited with terpinolene, while none of the eight control trees had apparently been attacked.

During September 1984, all trees were felled and inspected for bark beetle attacks in the same way as described above. The data were subjected to a Friedman's test followed by a nonparametric Dunnett's test (one-tailed) (Zar, 1984). To evaluate reproduction with regard to attack densities and to compare our results to those of other authors, the data from this experiment were converted from galleries per tree to galleries per surface area. The surface area was estimated as square meters rough bark on each tree, since *T. piniperda* usually does not attack the smooth bark found higher up on Scots pine trees.

The number of offspring was estimated from the number of emergence holes, since in *T. piniperda* every beetle makes its own emergence hole, i.e., very few beetles emerge through already existing holes (Salonen, 1973; Saarenmaa, 1983).

Induction of Attack with Ethanol. Healthy trees in a 40-year-old natural regeneration of pine with a mean height of 8.5 m were baited with ethanol or

TABLE 3. MEAN NUMBER OF ATTACKS BY *T. piniperda* ON UNBAITED SCOTS PINE TREES AND ON TREES BAITED WITH ETHANOL OR (−)-α-PINENE[a]

Treatments	Tree diam. (cm)		Attacks per tree (No.)	
	$\bar{X}$	Range	$\bar{X}$	Range
Control trees	10.6	9.5–11.5	0[b]	0
(−)-α-Pinene	10.4	7.3–13.8	10.8	1–19
96% ethanol	9.9	8.6–11.5	4.2	0–11

[a] Six replicates in each treatment.
[b] All treatments were significantly different (P =0.05, Newman-Keuls test).

α-pinene during 1985. Eighteen trees (diameters are given in Table 3) were randomly assigned one of three treatments: (1) unbaited controls, (2) 96% ethanol, and (3) (−)-α-pinene (Fluka 97%, $[\alpha]_D^{20}$ −42 ± 3°). The distance between selected trees was at least 3 m. The α-pinene-baited treatment was included to ascertain whether responsive beetles were present in the area at the time of the test. The ethanol (2 ml) and α-pinene (100 μl) were released from open vials (9 and 5 mm diam., respectively). Each tree was baited with two vials as described in the previous experiments. The beetle population in the area was apparently low. Therefore the trees were baited daily from May 8 to May 13, except for one day when no beetle flight occurred. Vials were supplied with fresh compounds daily just as the beetle flight started. In the afternoon, after having been open for about 4 hr, the vials were refilled. The release rates during the experiment were not determined. In the laboratory at 15°C, ethanol was released at about 75 μl/hr, and α-pinene was released at about 10 μl/hr.

Late in June 1985 all trees were felled and inspected for bark beetle attacks as described above. Data were subjected to a Kruskal-Wallis test with tied ranks followed by a nonparametric multiple comparison similar to the Newman-Keuls test (Zar, 1984).

RESULTS

On traps baited with chips from freshly cut Scots pine trees, a mean of 13.1 *T. piniperda* (range 6–28) was caught while a mean of 1.1 *T. piniperda* (range 0–3) was caught on unbaited control traps. The difference is statistically significant (P = 0.001 level, Mann-Whitney test).

When healthy Scots pine trees were baited with chips, significantly more *T. piniperda* attacks were found on these trees than on unbaited control trees, which only received a few isolated attacks (Table 1). Only in one of the baited

trees did the attacks result in emergence of a new generation of beetles. In all other baited trees and in the control trees, attacks resulted in short egg galleries with an average length of 2.3 cm and, in the few cases where progeny developed, in larval galleries which were only a few millimeters in length. In two of the trees we also found a few larvae of *Pissodes* spp. (Curculionidae).

All trees baited with either α-pinene, 3-carene, terpinolene, or all three compounds were attacked by *T. piniperda* except for one tree baited with terpinolene (Table 2). No attacks were found on the control trees.

Reproduction success was low. In 23 of the baited trees, the attack density was higher than 100 galleries/m^2 ($\bar{X}$ = 167; range 101–302). In six of these trees, a new generation of beetles emerged. The mean attack density in these trees was 194 galleries/m^2 (range 153-221), and the net reproductivity (number of offspring per parent beetle) was 0.27 (range 0.05–0.47). In all other attacked trees, the attacks resulted in short egg galleries with an average length of 3.8 cm and in larval galleries which were only a few millimeters in length. In 14 of the baited trees we also found larvae of *Pissodes* spp.

Of the six trees baited with ethanol, five were attacked by *T. piniperda* (Table 3). The trees baited with α-pinene in the same experiment received a higher number of attacks than the trees baited with ethanol. All attacks were unsuccessful and resulted in short egg galleries with an average length of 1.3 cm. No attacks were found on the control trees. No attacks of other insect species were found on the trunks.

DISCUSSION

Host volatiles released by chips from freshly cut Scots pine trees both attracted *T. piniperda* and induced gallery initiation in healthy trees (Table 1). Attraction of beetles to host volatiles from pine logs was also observed by Byers et al. (1985), who demonstrated that several Scots pine monoterpenes, (+)- and (−)-α-pinene, (+)-3-carene, and terpinolene were strongly attractive to *T. piniperda*.

Trees baited with any one or all of these monoterpenes [(+)-α-pinene not tested] were attacked (Table 2). This induction of attack by several compounds may have resulted as an adaption to the great variation between pines in monoterpene composition (Tobolski and Hannover, 1971; Thorin and Nommik, 1974; Hiltunen, 1975).

The attacks of *Pissodes* spp. found on some of the baited trees should not have influenced gallery initiation by *T. piniperda* since the flight period of these species occurs later in the season than the flight period of *T. piniperda* (Saalas, 1923; Eidmann, 1974; Löyttyniemi and Uusvaara, 1977).

Trees baited with ethanol were also attacked by *T. piniperda* (Table 3). Since anaerobic metabolism in deteriorating tree tissue can result in the for-

mation of ethanol (Graham, 1968; Cade et al., 1970; Moeck, 1970; Ikeda et al., 1980), its presence may indicate a weakened tree that would provide suitable breeding substrate for *T. piniperda*.

A new generation of *T. piniperda* emerged from only six of the 31 monoterpene-baited trees that were attacked, and net reproductivity was well below 1.0. Reproduction failed despite the relatively high attack densities in most of the trees: in 23 of these trees the densities were higher than 100 galleries/m^2. These attack densities correspond well with those generally found in suitable breeding substrate, and good reproduction is often observed in weakened trees with attack densities lower than 100 galleries/m^2. The highest densities found in nature are usually less than 300 galleries/m^2 (Salonen, 1973; Saarenmaa, 1983, 1985; Långström et al., 1984). From this the following conclusions can be drawn: (1) gallery initiation induced by host substances does not necessarily lead to successful reproduction; and (2) *T. piniperda* is adapted to breed in dead or severely weakened trees and is usually unable to reproduce in trees of good vigor.

Our study demonstrates that monoterpenes as well as ethanol, eventually together with terpenes, can induce gallery initiation by *T. piniperda* on apparently healthy trees, which generally are not suitable for reproduction. Responses to the two different classes of compounds may reflect the various kinds of breeding substrate utilized by this species. In trees broken or windthrown during winter, ethanol production may not have reached levels high enough to be detected by the beetles during their flight period in early spring; however, high amounts of terpenes may be released from resin exuding from injuries on these trees. In contrast, weakened and dying pine trees lacking external injuries (e.g., suppressed trees) would not be expected to release high amounts of monoterpenes. Instead, metabolic processes resulting in detectable quantities of ethanol may guide beetles to attack these trees.

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IDENTIFICATION OF FEMALE-PRODUCED SEX PHEROMONE FROM BANDED CUCUMBER BEETLE, *Diabrotica balteata* LECONTE (COLEOPTERA: CHRYSOMELIDAE)[1]

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Abstract—A sex pheromone produced by female banded cucumber beetle adults, *Diabrotica balteata* LeConte, was isolated from volatiles trapped on Porapak Q and identified as 6,12-dimethylpentadecan-2-one. The structure was elucidated by spectroscopic analyses and confirmed by synthesis. The synthesized racemic compound was equal to the purified natural pheromone in eliciting responses by banded cucumber beetle males to field traps. A dose-response characteristic was demonstrated for the racemic material formulated on filter paper or rubber septa and placed in field traps. The absolute configuration at the C-6 and C-12 positions was not established.

Key Words—Sex pheromone, 6,12-dimethylpentadecan-2-one, ketone, banded cucumber beetle, *Diabrotica balteata*, Chrysomelidae, Coleoptera, attractant.

INTRODUCTION

The banded cucumber beetle (BCB), *Diabrotica balteata* LeConte, an economic pest of vegetable and field crops, particularly sweet potato and seedling

[1]This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or the recommendation for its use by USDA.
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cucurbits (Pitre and Kantack, 1962; Saba, 1970; Teng et al., 1984; Young, 1961), occurs from southern United States to Colombia, Venezuela, and Cuba (Krysan, 1986).

Since the use of broad-spectrum insecticides to control BCB often causes outbreaks of secondary pests, a number of studies on host performance of BCB adults have been conducted in efforts to develop noninsecticidal methods to manage this pest (Melhus et al., 1954; Cuthbert and Jones, 1972; Overman and MacCarter, 1972; Risch, 1976). The presence of a BCB female-produced sex pheromone, which should be useful in monitoring and possibly in controlling this species, was reported by Cuthbert and Reid (1964). Later Schwarz et al. (1971) reported that the BCB pheromone molecule included *n*-dodecenyl, ethylene epoxy, and methyl ketone moieties, but they were unable to complete the structure elucidation.

We report here the isolation, identification, and synthesis of a sex pheromone, collected from BCB female-produced volatiles, that attracts males of the species in the field.

METHODS AND MATERIALS

Pheromone Collection and Bioassay

The insects were from a laboratory colony maintained at the Northern Grain Insects Laboratory, Brookings, South Dakota, by the method of Jackson (1985). The colony was begun in Brookings, in August 1981, with eggs obtained from a laboratory colony at the USDA, ARS, Vegetable Insects Research Laboratory, Charleston, South Carolina. Virgin females were isolated from males within 24 hr of emergence and held in screened cages (30 × 30 × 30 cm) for three days before being placed in the pheromone collection chamber.

The pheromone collection system and methods were the same as those used to collect volatiles from the southern corn rootworm (SCR), *Diabrotica undecimpunctata howardi* Barber (Guss et al., 1983b). The collection was performed at 24°C with a 12-hr photophase and the pheromone was collected on Porapak Q (Byrne et al., 1975).

During the initial isolation stages, a laboratory bioassay similar to that used for the SCR (Guss et al., 1982) was employed. In this array, four or five virgin male beetles were placed in a Petri dish (150 × 15 mm) and allowed to acclimate for 15 min. A test extract or fraction in 1–5 μl of hexane was applied to a 5-mm^2 piece of filter paper, the solvent was evaporated for about 10 min, and then the paper was placed into the dish. Responses of beetles toward the treated paper, antennal waving, and copulatory behavior were considered evidence of the presence of the pheromone.

Field bioassays were conducted with synthesized putative pheromone and crude and purified natural material from the Porapak Q collectors. Wing traps (Pherocon 1C-type, Trece, Inc., Salinas, California) were baited with various doses of these materials applied (in 200 μl of hexane) to 5.5-cm filter paper circles or to methylene chloride-extracted rubber septa (A.H. Thomas, Philadelphia, Pennsylvania, No. 8753-D22).

The first test was conducted in a field of sweet potato in the vicinity of Homestead, Florida, on November 6, 1985. On the morning of the test, the air temperature was about 18°C at 9:00 AM with winds about 6–10 mph and increasing. The afternoon temperature was about 20°C with winds 10–15 mph.

The traps were baited with filter paper formulations of 1 μg of the purified natural pheromone, a crude extract that contained 1 μg of the natural pheromone, and 1, 4, 10, 100, and 1000 μg of synthetic racemic 6,12-dimethylpentadecan-2-one and a hexane blank. Treatments were assigned randomly to traps within each replicate. Traps were placed 25 m apart in a row across the field perpendicular to the prevailing wind. Two replicates (16 traps in a row) were conducted beginning at 8:45 AM and terminating at 11:30 AM. The experiment was then moved 400 m upwind in the field and two replicates were conducted with rebaited traps in a new random order from 1:40 PM until 9:00 AM the next morning. An observer was assigned to each replicate, and the cumulative total males captured in each trap was recorded at 15-min intervals from 8:45 to 9:30 AM (morning test), from 1:40 to 2:30 PM, at 5:00 PM, and from 8:30 to 9:00 AM the next morning. Tests were conducted during the day because preliminary trapping observations indicated that night temperatures during this period were too cool for BCB flight.

The second test was conducted in a field of mature yellow crook-neck squash in the vicinity of Gainesville, Florida, from November 14 to November 20, 1985. Traps were placed 50 m apart in a row across the field and perpendicular to the prevailing wind. Treatments were 0.03, 0.1, 0.3, 1.0, 3.0, 10, and 30 mg of racemic 6,12-dimethylpentadecan-2-one, and a hexane blank formulated on rubber septa. The baits were not renewed during the test. The traps were checked daily and captured males were removed and counted, at which time the trap and bait were moved to the next trapping location in the line.

Release rates of synthetic pheromone were measured by collecting volatiles released by the septa and analyzing them by gas–liquid chromatography (GLC). Septa were loaded with the same doses used in the field tests and aired at room temperature for 24 hr prior to release-rate measurement. Then a septum was placed in a 15-mm (ID) × 20-mm-long stainless-steel tube, and compressed air, purified by passage through a charcoal filter, was passed over it at 1 liter/min for 1 hr. Volatiles entrained in the airstream were trapped on a small charcoal filter that was subsequently extracted with methylene chloride. After addition of internal standards, the extract was analyzed by capillary GLC.

Pheromone Purification

Volatiles were extracted from Porapak Q filters with 50 ml of ether–hexane (60:40) by agitation for 24 hr. The extract was concentrated with a stream of N_2, and the concentrate was subjected to preparative GLC without further treatment.

Micropreparative GLC was performed with a Varian model 1400 gas chromatograph equipped with a flame ionization detector. The pheromone was purified using two packed columns: 10% OV-101 on 60–80 mesh Chromosorb W (glass column 2 × 2 mm ID) and 7.5% Carbowax 20 M on 60–80 mesh Chromosorb W (glass column 2 m × 2 mm ID). The injection port and column temperatures were both 200°C when operating the OV-101 column and 180°C when operating the Carbowax 20 M column. Detector temperature was 250°C. The carrier gas (He) flow rate through each column was 20 ml/min.

The chromatograph was modified to accommodate a 90:10 effluent splitter and an external, Dry Ice–acetone-cooled fraction collector (Brownlee and Silverstein, 1968). Fractions were collected in 1.5-mm (ID) × 305-mm glass capillary tubes and eluted with about 10 μl of hexane.

Synthesized pheromone was purified by preparative high-performance liquid chromatography (HPLC) using a 4.6-mm (ID) × 25-cm stainless-steel column packed with Adsorbosphere C18 (5 μm) (Alltech/Applied Science, Deerfield, Illinois). The methanol–water (90:10) mobile phase was pumped through the column at 1.0 ml/min, and the eluting components were detected with a Waters model R401 differential refractometer. The 6,12-dimethylpentadecan-2-one eluted from this system in 17.5 min.

Pheromone Analysis and Identification

Natural and synthesized pheromone was analyzed on 50-m × 0.25-mm-ID fused silica capillary columns in a Hewlett-Packard 5790 gas chromatograph equipped with a split–splitless injector system (30-sec split delay) at a carrier gas (He) linear flow rate of 18 cm/sec. The OV-101 column was operated at 60°C for 1 min after injection, temperature programmed at 30°C/min to 200°C, and then operated isothermally. The Carbowax 20 M column was operated at 60°C for 2 min after injection, programmed at 30°C/min to 210°C and then operated isothermally.

Mass spectra were obtained with a Nermag R10-10 mass spectrometer equipped with a chemical ionization (CI)/electron impact (EI) source and interfaced with a Varian Vista model 6000 gas chromatograph equipped with a split–splitless injection system. Samples were introduced into the mass spectrometer source through a 50-m × 0.25-mm-ID fused silica OV-101 column operated at 60°C for 2 min, then programmed at 32°C/min to 230°C and operated isothermally. The linear flow velocity of the carrier gas (He) was 18 cm/

sec. The spectrometer was interfaced with a Digital PDP 11/23 computer for collection and analysis of the data.

Hydrogenoloysis in the inlet of the GC interfaced to the mass spectrometer was performed by the method of Beroza and Sarmiento (1963, 1964). About 6 cm of a glass insert (1 mm ID) was filled with 1% neutral Pd catalyst on Gas-chrom W and placed in the injection port ahead of the OV-101 column. The catalyst was maintained at 285°C for the hydrogenolysis, and H_2 was used as the carrier gas at a flow rate of 20 ml/min.

PMR analysis was performed with a Nicolet 300 MHz Fourier transform NMR spectrometer interfaced to a Nicolet model 1280 data system (16K data points, 10 μsec pulse). Samples purified by micropreparative GLC (about 3 μg total pheromone) were transferred from the glass capillary, using benzene-D6, into an NMR tube, the top of which was 5 mm (OD), with a 50 × 2-mm (OD) coaxial extension on the bottom (Wilmad Glass Co., Buena, New Jersey, catalog No. 507 with WGS-5BL stem).

Synthesis

1-Bromo-4-methyl-3-heptene (1). This was reported previously by Guss et al. (1983b), and its preparation has been described in detail by Kulesza et al. (1969). It was prepared in 79% yield; bp 68–70°C at 7 mm (Kuleza et al. reported bp 51–52°C at 2 mm). Its IR and NMR spectra were in complete agreement with its assigned structure.

1-Bromo-4-methylheptane (2). This was prepared from (1) in 70% yield by hydrogenation over platinum oxide in glacial acetic acid (Guss et al., 1983b); bp 67–70°C at 8.5 mm. Its IR and NMR spectra were in agreement with the assigned structure and reported values (Guss et al., 1983b).

8-Methyl-undecan-2-one (3). The lithium reagent was prepared from 9.66 g (0.05 M) of bromide (2), and 0.853 g (0.123 M) of lithium metal in 50 ml of dry ether at −150°C and 100 μl of methyl iodide was used to start the reaction. This lithium reagent was converted into an organocopper reagent and reacted with freshly distilled methyl vinyl ketone in the presence of tri-*n*-butylphosphine by the method of Suzuki et al. (1980). Distillation afforded 80% yield of product; bp 58–60°C at 0.5 mm; CI-MS (CH_4) *m/z*, 185 (M + 1); IR (CCl_4), 2960(s), 2940(s), 2880(s), 2860(s), 1723(s), 1468(m), 1380(m), 1358(m), 1160(m); NMR ($CDCl_3$), 0.84 (6H, m), 1.1–1.4 (11H, m), 1.57 (2H, m), 2.12 (3H, s), 2.41 (2H, t).

4-Oxo-pentane Ketal Triphenylphosphonium Bromide (4). The phosphonium salt was prepared in 60% yield by the method of Sonnet et al. (1986); IR ($CHCl_3$), 2950(s), 1438(s), 1110(s), 1030(m), 690(m), 660(m), NMR ($CDCl_3$), 1.22(3H, s), 1.58 (2H, bds), 1.75(2H, m), 2.05(2H, t), 3.9(4H, s), 7.8(15H, m).

6,12-Dimethyl-5-pentadecen-2-one Ethylene Ketal (5). A hexane solution

of *n*-butyllithium (19.0 ml, 0.038 M) was added dropwise at 0°C to a suspension of 20 g (0.038 M) ketal phosphonium bromide (4) in dry THF and stirred for 30 min. The reaction mixture was brought to room temperature, and 3.6 g (0.02 M) of 8-methylundecan-2-one (3) in ether was added dropwise and the mixture was stirred for 6 hr. The reaction mixture was poured into ice water, and the mixture was extracted with hexane. The hexane solution was washed with water and saturated NaCl and dried over Na_2SO_4. Distillation furnished 870 mg (15% yield) of 6,12-dimethyl-5-pentadecen-2-one ethylene ketal (5); bp 120–130°C at 0.7 mm Hg; CI-MS (CH_4) (*m/z*), 297(M + 1); IR(CCl_4), 2960(s), 2940(s), 2880(s), 1467(m), 1380(m), 1240(m), 1160(m), 1220(m), 1090(m), 1060(m), 950(m), 900(m); NMR ($CDCl_3$), 0.85 (6H, m), 1.1–1.7 (17H, m), 1.68 (3H, s), 2.05 (2H, m), 2.13 (3H, s), 3.95 (4H, bds), 5.10 (1H, m).

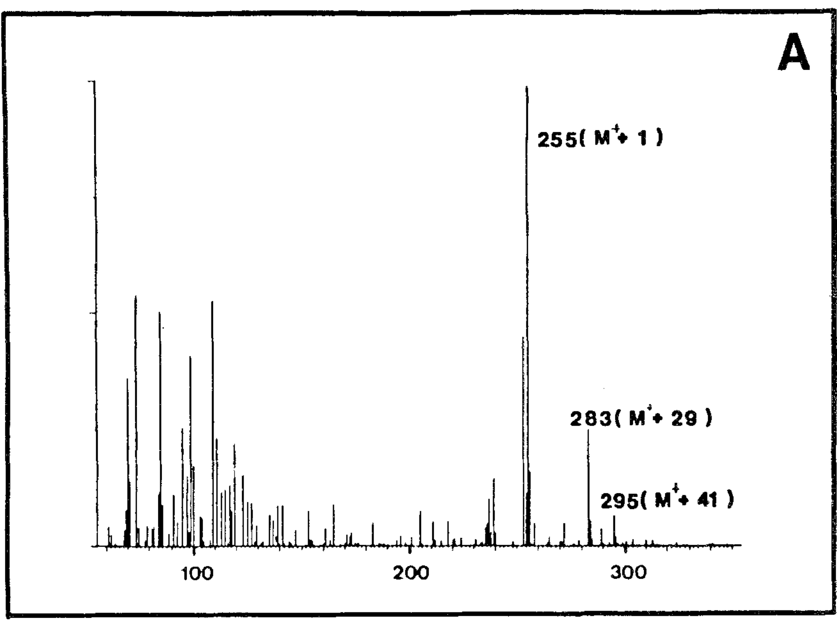
6,12-Dimethylpentadecan-2-one Ketal (6). The unsaturated ketal (5) (870 mg, 0.003 M in absolute ethanol) was hydrogenated over 50 mg of palladium on charcoal to yield 832 mg of 6,12-dimethylpentadecan-2-one ketal (6); CI-MS (CH_4) *m/z*, 299(M + 1).

6,12-Dimethylpentadecan-2-one (7). A solution of 832 mg (0.003 M) of ketal (6) in acetone–10% H_2SO_4 was stirred for 3 hr. It was then extracted with hexane and the extract washed with saturated aqueous NaCl, dried over Na_2SO_4, and concentrated in vacuo to yield 695 mg (98%) of 6,12-dimethylpentadecan-2-one (7); CI-MS (CH_4) *m/z*, 255(M + 1); EI-MS (*m/z*), 43(100), 58(82), 59(38), 71(42), 85(24), 95(13), 97(8), 109(10), 110(15), 123(3), 137(2), 151(1), 152(0.5), 153(0.5), 165(1), 196(3), 211(2), 236(6), 254(M+, 0.9); IR (CCl_4), 2960(s), 2930(s), 2850(s), 1735(s), 1460(m), 1375(m), 1360(m); NMR (C_6D_6), 0.875 (3H, d, J = 8.1 Hz), 0.895 (3H, d, J = 8.1 Hz), 0.905 (3H, t, J = 6.2 Hz), 1.1–1.4(20H, m), 1.64(3H, s), 1.91(2H, t, J = 7.3 Hz).

RESULTS AND DISCUSSION

The hexane–ether extracts of Porapak Q filters from the collection chamber elicited behaviors from BCB males in the laboratory bioassay that indicated the presence of the female sex pheromone. Laboratory assay of the fractions obtained by preparative GLC indicated that the pheromonal activity was confined to a single peak eluting at 11 min on OV-101 under the experimental conditions. Further micropreparative chromatography on the Carbowax 20 M-packed column yielded a compound with a purity of greater than 99.5% by analysis on the OV-101 and Carbowax 20 M capillary columns. The retention indices (Kovats, 1965) of this compound, relative to paraffin hydrocarbons, on the OV-101 and Carbowax 20 M capillary GLC columns were 1787 and 2114, respectively.

The methane CI mass spectrum (Figure 1A) established that the molecular weight of the compound was 254 with diagnostic peaks at *m/e* 253(M − 1),



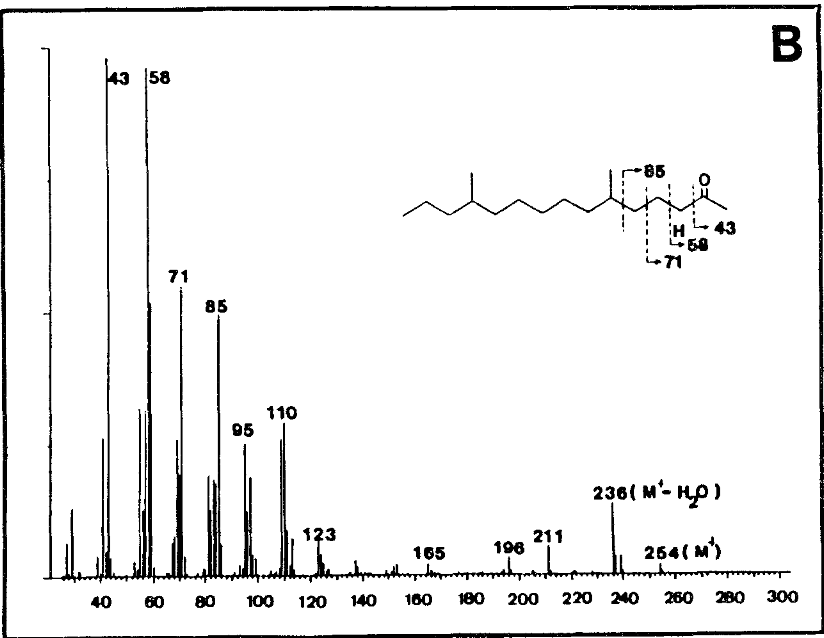


FIG. 1. (A) Chemical ionization (CH_4) and (B) electron impact mass spectra of *D. balteata* pheromone.

255(M + 1), 283(M + 29), and 295(M + 41). In the EI mass spectrum (Figure 1B), the peak at *m/e* 254(M+) confirmed the molecular weight. Furthermore, the EI mass spectrum of this pheromone is very similar to that of the SCR pheromone, 10-methyl-2-tridecanone (Guss et al., 1983b) with a peak at *m/e* 236 indicating the loss of H_2O, and the strong peaks at *m/e* 43 and 58, the weak peak at *m/e* 57, and the moderately strong peak at *m/e* 71 strongly suggesting a methyl ketone with no substitution on the carbons α or β to the carbonyl (Budzikiewicz et al., 1967).

The [^{1}H] NMR spectrum of the purified natural pheromone (Figure 2) supported the above assignment. The signals at δ 1.64 ($\underline{CH}_3CO-$; 3H, s) and 1.92 ($-CO\underline{CH}_2$-CH_2-; 2H, t) confirmed the presence of a 2-oxobutyl moiety in the pheromone molecule. Furthermore, when expanded, the group of peaks from δ 0.86 to 0.91 appeared to consist of three overlapping signals, δ 0.875 (3H, d, J = 8.1 Hz), 0.895 (3H, d, J = 8.1 Hz), and 0.905 (3H, t, J = 6.2 Hz) which suggests the presence of two methyl branches and a terminal methyl in the molecule.

Hydrogenolysis of the pheromone in the GC injector leading to the mass spectrometer source and EI mass spectral analysis of the product yielded the

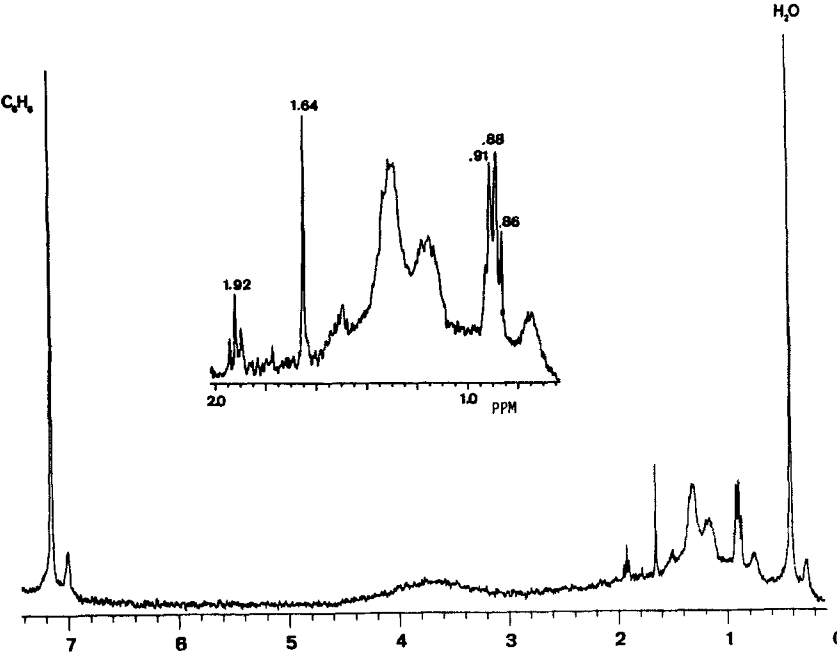


FIG. 2. 300-mHz proton magnetic resonance spectrum of approximately 3 μg of the purified natural *D. balteata* pheromone.

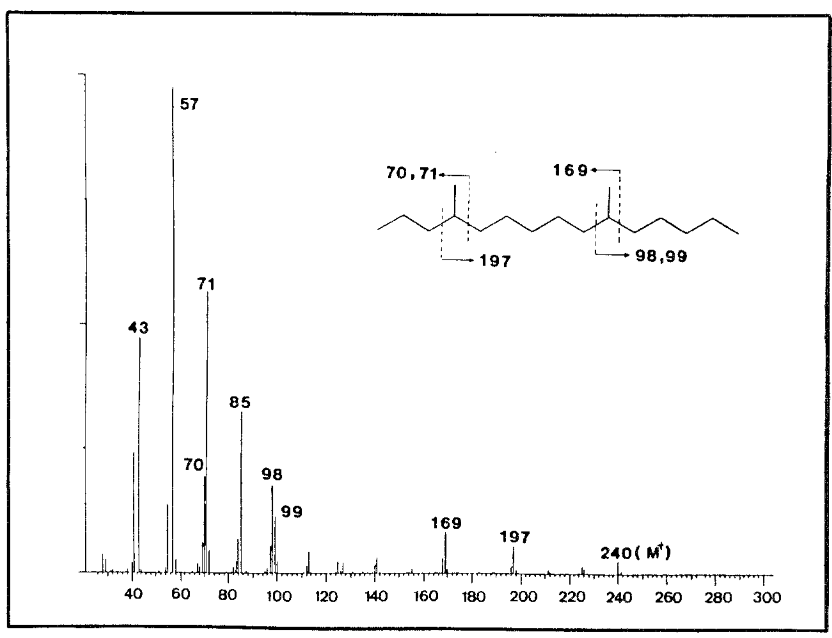


FIG. 3. Electron impact mass spectrum of hydrogenolysis product of *D. balteata* pheromone.

spectrum shown in Figure 3. The peak at 240(M+) confirms the 17-carbon skeleton indicated by mass spectra of the parent compound. The peaks at *m/e* 70, 71, and 197 and at *m/e* 98, 99, and 169 are of greater intensity than would be expected in a normal hydrocarbon and represent preferred cleavage on either side of two methyl branches. These data support a structure with methyl branches on carbons four and ten as indicated in Figure 3. The only other possible structure, with methyl branches on carbons 4 and 6, was ruled out because of the relative intensities of the peaks at *m/e* 98 and 99. The greater intensity of the peak at 98 is consistent with cleavage of the bond between carbons 9 and 10 in 4,10-dimethylpentadecane, with the fragment ion containing only one methyl branch. Cleavage between carbons 6 and 7 of 4,6-dimethylpentadecane yielding a fragment ion containing two methyl branches would be expected to produce peaks at *m/e* 98 and 99, but the peak at 99 should be of greater intensity (Nelson et al., 1972; Nelson and Sukkestad, 1970, 1975; Pomonis et al., 1980; Pomonis and Hakk, 1984). Thus the structure of the hydrogenolysis product was established as 4,10-dimethyl pentadecane. Since the EI mass and PMR spectra of the pheromone indicated that the carbons α and β to the carbonyl must be unsubstituted, the only possible structure is 6,12-dimethylpentadecan-2-one.

Synthesis. The synthesis of racemic 6,12-dimethylpentadecan-2-one (Figure 4) was analogous to that for the sex pheromones of the western corn rootworm (WCR), 8-methyl-2-decanol propanoate, *D. virgifera virgifera* LeConte (Guss et al., 1982), and the SCR (Figure 5) (Guss et al., 1983b). Methylcyclopropyl ketone was allowed to react with *n*-propylmagnesium bromide. The intermediate tertiary carbinol was isomerized to the homoallylic bromide (1) with hydrobromic acid (Julia, 1961). The allic bromide (1) was reduced to 1-bromo-4-methylheptane (2) by hydrogenation with PtO_2. The saturated bromide (2) was converted to the corresponding organocopper reagent which was reacted with methyl vinyl ketone in the presence of tri-*n*-butyl phosphine (Suzuki et al., 1980) to produce 8-methylundecan-2-one (3). A Wittig reaction between the C-12 methylketone (3) and ethylene ketal pentan-2-one phosphonium salt (4), produced a mixture of the *Z* and *E* isomers of the ethylene ketal of 6,12-dimethyl-5-pentadecen-2-one (5) (15% yield). The unsaturated ethylene ketals were hydrogenated and then subjected to acid hydrolysis to produce 6,12-dimethylpentadecan-2-one (7). The final product was purified (>99%) by HPLC and GLC.

The CI and EI mass spectra and the PMR spectrum of pure synthetic racemic 6,12-dimethylpentadecan-2-one were identical with those of the isolated natural pheromone. Additionally the synthetic and natural pheromones cochromatographed, producing only one peak, on each of the two capillary GLC columns.

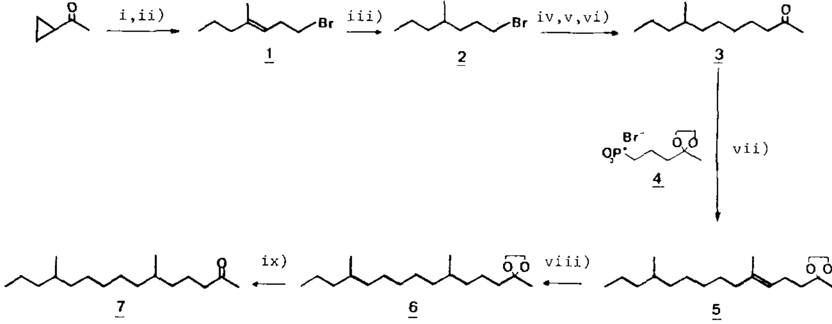


FIG. 4. Synthesis of racemic 6,12-dimethylpentadecan-2-one. (i) C_3H_7MgBr; (ii) HBr; (iii) $H_2/PtO_2/CH_3COOH$; (iv) Li; (v) CuI; (vi) methylvinylketone/$(n\text{-Bu})_3P$; (vii) *n*-BuLi; (viii) H_7/Pd-C/ETOH; (ix) H_2SO_4.

	SPECIES	STEREOCHEMISTRY	STRUCTURES
Fucata species group	D. balteata	unknown	
	D. undecimpunctata howardi (SCR)[1]	10R(++)	R
	D. undecimpunctata undecimpunctata[1]	10R(++)	
	D. undecimpunctata duodecimnotata[1]	unknown	
Virgifera species group	D. virgifera virgifera (WCR)[2,3]	2R,8R(++), 2S,8R(+)	R R OPr
	D. virgifera zeae (MCR)[2,3]	2R,8R(++), 2S,8R(+)	
	D. barberi (NCR)[2,4]	2R,8R(++), 2S,8R(--), 2S,8S(-)	
	D. longicornis[2,5]	2S,8R(++), 2R,8R(--)	R S OPr
	D. lemniscata[5]	2S,8R(++), 2R,8R(+)	
	D. porracea[2,3]	2S,8R(++)	
	D. cristata[6]	2S,8R(++)	R S OAc

FIG. 5. Structures and stereochemistry of *Diabrotica* sex pheromones or attractants. (1) Guss et al. (1983b); (2) Guss et al. (1982); (3) Guss et al. (1984); (4) Guss et al. (1985); (5) Krysan et al. (1986); (6) Guss et al. (1983a). Structures shown are the main attractants of the corresponding species. (+ +), strong attraction; (+), weak attraction; (− −), strong inhibition; (−) weak inhibition.

Field Tests. The mean number of males captured in traps baited with filter paper formulations of natural and synthetic materials are summarized in Table 1. Since the purified natural pheromone attracted more males into traps than the crude natural material, the crude material may have included substances that either reduced the release rate or inhibited the response of the males. The captures for 1 μg natural, 4 μg synthetic, and 10 μg synthetic were not statistically different, but were different from all other means. While the number of males captured increased with increasing dose for synthetic pheromone treatments from 1 to 10 μg, linear regression analysis yielded correlation coefficient values slightly less than required for significance at the 5% level of probability (nontransformed data $r = 0.9945$, 1 *df*; the coefficient did not increase with log-transformation). The responses of males to doses > 10 μg was greatly reduced.

The observed captures for the first 2 hr of each replication of the test with filter paper formulations are shown in Table 2. The captures were not dose related for all periods and reveal a complex pattern that is probably related to nonlinear release rates from the filter papers coupled with trap interaction and the depletion of males available to react to each trap. This field was infested heavily with BCB, and reaction to the baits was nearly instantaneous. Males were seen immediately in the vicinity of the 100- and 1000-μg-baited traps but did not enter these traps immediately as they did the other treatments. Again, a dose-response relationship is evident, and regression analysis yields very high correlations for the synthetic treatments 1-10 μg ($r = 0.983$ for the total captures at 2 hr). The increase in captures at the 100-μg level, beginning 30 min after the traps were baited, suggests a decreasing release rate with time.

Apparently the 10-μg dose on filter paper provided a near optimum release

TABLE 1. MEAN CAPTURE OF MALE *Diabrotica balteata* IN TRAPS BAITED WITH NATURAL OR RACEMIC SYNTHETIC 6,12-DIMETHYLPENTADECAN-2-ONE EVAPORATED FROM FILTER PAPERS

Amount (μg)	Treatment	Mean No. males captured per trap, $\bar{X} \pm$ SE ($N = 4$)[a]
0	Hexane blank	0.0 ± 0.0d
1	Natural crude	32.2 ± 7.1b
1	Natural purified	81.7 ± 20.0a
1	Synthetic	65.5 ± 19.1b
4	Synthetic	82.0 ± 18.6a
10	Synthetic	104.2 ± 13.0a
100	Synthetic	37.2 ± 12.4b
1000	Synthetic	8.7 ± 2.9c

[a] Means followed by a common letter are statistically equivalent ($P = 0.05$, data subjected to $\sqrt{x}$ transformation) in Duncan's multiple-range test.

TABLE 2. NUMBER OF MALE *Diabrotica balteata* CAPTURED PER TIME INTERVAL IN TRAPS BAITED WITH FILTER PAPER FORMULATIONS OF NATURAL OR RACEMIC SYNTHETIC 6,12-DIMETHYLPENTADECAN-2-ONE

Treatment						
Natural		Synthetic				
Crude (1 μg)	Pure (1 μg)	1 μg	4 μg	10 μg	100 μg	1000 μg
0–15 min after baiting						
11	71	32	77	86	10	2
15–30 min after baiting						
11	26	23	29	31	1	4
30–45 min after baiting						
4	24	18	15	23	39	6
45 min–2 hr after baiting						
37	145	83	76	138	42	7
Total						
63	266	156	197	278	92	19

rate of the racemic synthetic material. The effect of the stereoisomers cannot be determined clearly at this time, but the similar responses to the 1 μg pure natural and the 4 μg and 10 μg synthetic material suggest that one of the stereoisomers of 6,12-dimethylpentadecan-2-one is a pheromonal constituent.

The response of males to traps baited with rubber septa formulations are summarized in Table 3. Again, a relationship of increasing capture with in-

TABLE 3. MEAN NUMBER OF *Diabrotica balteata* MALES CAPTURED IN TRAPS BAITED WITH RUBBER SEPTA FORMULATIONS OF RACEMIC 6,12-DIMETHYLPENTADECAN-2-ONE

Treatment (μg)	Mean number males captured per trap per replication (±SE)	Release rates from rubber septa (ng/hr)[a]
0	0	
30	5.1 ± 0.5	14
100	13.4 ± 1.9	23
300	24.8 ± 4.3	39
1000	14.5 ± 2.1	124
3000	16.8 ± 1.9	476
10000	9.2 ± 1.1	1322
30000	5.8 ± 1.2	4369

[a]Means of two measurements; flow rate 1 liter/min, ca. 25°C.

creasing dose is evident for traps baited with 30–300 μg of synthetic racemic pheromone ($r = 0.998$ for linear regression of log dose vs. log response). Trap captures declined for treatments above 300 μg. This is the same response pattern observed with the filter paper formulations (Table 1).

The results of the study of release rates of the synthetic pheromone from rubber septa (Table 3) indicate that the release rates continue to increase as the dose is increased over the dose range studied. Thus, the leveling off and decline of trap captures at doses higher than 300 μg/septum cannot be attributed to a leveling off or decrease in the release rate. A similar phenomenon was observed for captures of *D. barberi* Smith and Lawrence, the northern corn rootworm (NCR), in traps baited with racemic 8-methyl-2-decanol propanoate (Figure 5) (Guss et al., 1982). Later this was explained when it was discovered that the NCR is attracted to the 2*R*, 8*R* isomer but that attraction is inhibited by the 2*S*, 8*R* and 2*S*, 8*S* isomers (Guss et al., 1985). On the other hand, the dose–response relationship reported here may be more like that of the WCR, in which rubber septa loaded with 0.25–1000 μg of the 2*R*, 8*R* isomer produced highly correlated captures of males; however, many WCR males ceased their upwind response to the 1-mg bait and attempted to copulate with nearby males (Guss et al., 1984). Thus, BCB males respond positively to one or more of the stereoisomers of 6,12-dimethylpentadecan-2-one and are either inhibited by other stereoisomers of the same compound or, with higher doses, their response to the locus of the active material is diminished. The use of stereoisomers to achieve specificity in their chemical signals is common among the *Diabrotica* studied thus far (Krysan et al., 1986).

The SCR was present in the test areas, and a pheromone of this species, 10-methyl-2-tridecanone, was evaporated from rubber septa baits using doses similar to those formulated in the BCB baits. Neither BCB nor SCR were captured in traps baited with the synthetic racemic pheromone of the other species.

We conclude from the congruence of chemical analytical data and demonstrated field activity in traps with doses equivalent to that of natural material that 6,12-dimethylpentadecan-2-one is a sex pheromone of female BCB. The active enantiomer(s) and the complete role of this chemical in premating communication remain to be determined.

This identification adds to our knowledge of the pheromonal communications systems of the *Diabrotica*: a pattern of chemical structures is beginning to emerge that appears to be related to the taxonomy of this genus. The species of *Diabrotica*, for which the chemical structures of sex attractants are known, fall into two groups (Wilcox, 1972). Those in the *fucata* species group have pheromones characterized by the methyl ketone functionality and a methyl branch on the fourth carbon from the hydrocarbon end of the chain. By contrast, all those taxa is included in the *virgifera* species group are attracted to one or more stereoisomers or analogs of 8-methyl-2-decanol propanoate (Figure 5).

The genus *Diabrotica* includes some 338 species (Wilcox, 1972). It will be interesting to see if these patterns prevail as pheromones from additional species are identified and knowledge accrues on phylogenetic relationships within the genus.

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THEORETICAL RANGE OVER WHICH BACTERIA AND NEMATODES LOCATE PLANT ROOTS USING CARBON DIOXIDE

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Abstract—A theory used to estimate the smallest relative chemical gradient that is potentially detectable is given approximately by $G > (DCRv^2t^3)^{-1/2}$ where D is the diffusion constant and C the concentration of the stimulus chemical, R is the effective radius of the receptor, v the velocity of the organism, and t the time period within which the organism measures the concentration. Of these factors, time has the strongest influence. Combining this result with available information on gradients of carbon dioxide around roots and behavior of bacteria and nematodes leads to several conclusions. Bacteria can potentially detect carbon dioxide gradients in the layer of water in contact with the root but not further away. In contrast, plant-parasitic nematodes can potentially detect gradients one meter from a single long root fiber and over 2 m from a plant root mass using klinokinesis. A direct approach using klinotaxis can start a few centimeters from a single root fiber and half a meter from a root mass. These differences are due to differences in the time available to measure the concentrations. Increasing the length of a bacterium could increase its ability to detect gradients by reducing its rotational diffusion. Collimating stimuli that serve to maintain a straight path may provide a means of improving chemotaxis by permitting concentration to be measured for longer times during klinokinesis. More accurate predictions can be made when more precise data are available. The analysis is applicable to a wide variety of other organisms and stimuli.

Key Words—Theory, chemotaxis, rhizosphere, soil, klinokinesis, klinotaxis, nematode, bacteria, carbon dioxide, root.

INTRODUCTION

An important question in soil ecology is how animals find their way in this complex environment. A specific question of particular importance to plant

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ecology and agriculture is how plant-parasitic nematodes and root-colonizing bacteria locate plant roots. Carbon dioxide is an attractant for several phytoparasitic nematodes (reviewed by Prot, 1980). Some are very sensitive to it (Pline and Dusenbery, 1987). While CO_2 is quantitatively the most important root exudate, its relative importance as an attractant under natural conditions is debatable. The suggestion has been made that CO_2 may attract nematodes toward host roots, but that its influence does not extend further than 1 or 2 cm from the roots (Klinger, 1963; Prot, 1980). It has recently been demonstrated that the bacterium *Pseudomonas* can migrate 1 cm toward seeds (Scher et al., 1985).

Because it is very difficult to observe the behavior of organisms in soil and to measure concentration gradients over small distances, an experimental approach to the problem is difficult. Consequently, a theoretical approach was taken. A relatively simple theory is presented that can place more specific limits on what is physically possible. Using existing data, some rough estimates are made.

METHODS AND MATERIALS

Berg and Purcell (1977) present a theoretical treatment of the sensory limits of bacterial chemotaxis. Their theory is based on the fact that for organisms the size of bacteria, the forces of viscosity completely dominate those of inertia, i.e., the Reynolds number is very low. In these circumstances, diffusion limits the rate at which molecules can be detected. The same considerations should apply to plant-parasitic nematodes, which move at about the same speed as bacteria and have sensory openings about the same size as bacteria.

Assume a receptor has an effective radius of R, that it measures the concentration of a stimulus chemical with diffusion constant D for a time t, and that the true concentration is C. Berg and Purcell provide several arguments that, ignoring factors of order unity, the relative error (noise) will be given approximately by $\Delta C/C \sim (tDCR)^{-1/2}$ where C is in units of molecules per cubic centimeter. For a 1 M solution, C is $6.0 \times 10^{20}/\text{cm}^3$. For a pure gas at standard temperature and pressure, C is $2.5 \times 10^{19}/\text{cm}^3$.

If an organism is moving with a velocity v along a relative concentration gradient $G = (1/C)\ (dC/dx)$ for a time t, the change in true concentration or signal will be $\Delta C/C \sim vtG$. In order to be detected, the signal must be larger than the noise. Thus, $vtG > (tDCR)^{-1/2}$. Rearranging this gives $t > [DCRv^2G^2]^{-1/3}$.

This provides a minimum time required to detect a gradient. Note that any gradient can be detected if sufficient time is allowed, including the lifetime of the gradient and adaptation time. The optimal orientation to the gradient was assumed since the result will be used to estimate the maximal range of attraction. If the organism responds by klinokinesis (Fraenkel and Gunn, 1940), the

limit on the time available is the time within which the organism maintains a straight course. If the organism responds by klinotaxis, the limit is the time the receptor is held to one side of its path.

If an estimate of t is available, the minimum gradient that can be detected is approximated by $G > [DCRv^2t^3]^{-1/2}$. Note that there is a relatively strong dependence on the time taken for measurement and somewhat less on the velocity.

RESULTS

Carbon Dioxide. To describe the distribution of CO_2 around roots, simplifying assumptions must be made. Close to a root fiber, the situation can be modeled as a line source in which the root is assumed to be infinitely long and straight. In practice, the root need only be long compared to the distance from the root to the point of interest. At distances that are large compared to the average separation between individual root fibers but small compared to the separation between plants, the situation can be modeled as a point source. In this case, the CO_2 produced by the entire root mass of one plant is treated as emanating from a small volume. This approach should be reasonably accurate for distances that are several times the diameter of the volume occupied by the root mass. In all cases, the effect of the soil–atmosphere surface will be ignored. This approximation will have little effect if the distance to the root is small compared to the distances to the surface. Where roots are shallow compared to the distance under consideration, the surface will cause distortions, and the calculations will only provide rough indications of the CO_2 distribution.

Nye (1981, Appendix) provides data for CO_2 production from roots in a typical case. The relevant parameters are: C is the concentration of CO_2 in the air space of soil (typically 0.5% v/v $= 1.3 \times 10^{17}$ mol/cm^3); $a = 0.037$ cm, the radius of a typical root; $F = 1.59 \times 10^{-6}$ ml/cm^2/sec, rate of CO_2 produced per unit of root surface; $L_v = 1.0$ cm of root length/cm^3 of soil volume; and D is the diffusion constant of CO_2 and is equal to 0.065 cm^2/sec in soil, 0.163 cm^2/sec in gas phase, and 1.66×10^{-5} cm^2/sec in water.

In soil, carbon dioxide can diffuse through both gas and liquid phases. Since the diffusion constant differs by a factor of 10^4 between the two phases, the appropriate value must be used. Over distances larger than the gas spaces between soil particles, diffusion is dominated by movement through the gas phase. In this case, the gaseous value is used but corrected for the volume of soil occupied by gas. Conversely, on a size scale of a bacterium, diffusion in the liquid phase is more appropriate.

In the case of the line source, in the steady state, the rate at which CO_2 diffuses across a cylinder centered on the source must equal the rate of production. Thus, in Nye's terms $2\pi rD\ (dC/dr) = 2\pi aF$ or $dC/dr = aF/rD$.

Substituting in the above values with $r = a$ and using D for water, the

maximum gradient in the film of water on the root is 9% CO_2/cm. Since this is several times larger than the typical concentration in soil, the relative gradient $(1/C)(dC/dr)$ will be > 1/cm. More precise estimates require knowledge of the thickness of the water film.

Beyond this thin layer, diffusion takes place mostly through the gas phase and the gradient is much shallower. The maximum gradient, near the root surface, is 0.0025% CO_2/cm or a relative gradient of 0.005/cm. The gradient is extremely shallow because of rapid diffusion through the gas phase—so shallow that at a distance of 10 m the concentration drops only one part per thousand. Thus, in determining relative gradients, the average concentration can be used in the denominator.

The relative gradient for a line source with the above parameters is $G = (1/C)(dC/dr) = aF/(CDr)$, where C is in units consistent with F. Using the above values for bulk soil, the maximum range of chemotaxis can be determined if the minimum detectable gradient is known: $r = 1.8 \times 10^{-4}/G$.

At greater distances, the gradient around the root mass of an isolated plant is better modeled as a point source. The rate of CO_2 production (P) by the root mass must equal the rate of diffusion through any surrounding sphere. Thus, $F2\pi aL_vV = P = 4\pi r^2D(dC/dr)$, where V is the volume of soil occupied by the root mass. The relative gradient is $G = (1/C)(dC/dr) = aFL_vV/(2r^2DC)$. Assuming a typical root mass occupies 1000 cm^3 of soil, 3.7×10^{-4} ml/sec of CO_2 are produced and $r = 9.1 \times 10^{-2}/G^{1/2}$. Thus, far from the root mass, the square root of the threshold gradient is inversely proportional to the range rather than the first power of the gradient.

Bacteria. The chemotaxis of *Escherichia coli* and *Salmonella typhimurium* have been studied extensively. These bacteria respond to chemical gradients by klinokinesis (Carlile, 1980). They swim in relatively straight paths for about a second. These runs are separated by tumbles in which the orientation of the bacterium is altered erratically but is not biased with respect to the gradient (Berg and Brown, 1972). A change in stimulus concentration alters the length of the runs in such a way that runs up a gradient of attractant, or down a gradient of repellent, are longer than those in the opposite direction. Thus, the organism works its way along the gradient by a biased random walk. In this case, the time for sampling the concentration is limited to the length of a run, since directional information is lost in a tumble. There is also a physical limitation on the length of time a direction can be maintained due to rotational diffusion. For ordinary rod-shaped bacteria like *E. coli*, the period is a few seconds. Thus, the time available for measuring the concentration is about 1 sec (Berg and Purcell, 1977).

The speed with which the bacteria swim during a run is about 15 μm/sec

or 1.5×10^{-3} cm/sec. The effective radius of the receptor is estimated as 0.8 μm (Berg and Purcell, 1977). Using these values for bacteria, the concentration of CO_2 for bulk soil, and the diffusion constant for liquid water, the minimum detectable gradient is estimated as 0.06/cm. Since the gradient in the bulk soil near the root is less, bacteria cannot use this mechanism to move toward roots from distances beyond the thin film of water on the root.

In an actual experiment, bacteria responded to a relative gradient of 0.7/cm (Berg and Purcell, 1977). Thus, there is sufficient sensitivity to respond to the much larger gradient in the thin film of water on the root surface.

Nematodes. The major chemosensory organs in nematodes are a pair of amphids. In infective juveniles of *Meloidogyne incognita*, the opening of the amphids to the outside is about 0.2×0.8 μm (Wergin and Endo, 1976). Let $R = 5 \times 10^{-5}$ cm for the effective radius of the receptor. The question then arises as to the appropriate value of the diffusion constant. Since nematodes are covered by a thin layer of water, the stimuli must be in water at the time of detection. However, if the layer is sufficiently thin, diffusion through it will be rapid compared with diffusion through soil. The characteristic time for diffusion is L^2/D. For a distance, L, of 40 μm the time for diffusion in water is about 1 sec. This time is sufficiently short so that no significant behavior occurs in plant-parasitic nematodes (Goode and Dusenbery, 1985). *Meloidogyne* infective juveniles are only 15 μ in diameter (Bird, 1979) and the layer of water covering them should be much less than their thickness. In addition, movement of the nematode will have the effect of stirring the media in its vicinity, which is equivalent to an increased diffusion rate. Since the effect of stirring will not be considered elsewhere, the rate of diffusion limiting measurement of concentration is assumed to be that of bulk soil: $D = 0.065$ cm^2/sec.

Like bacteria, nematodes are capable of responding by klinokinesis (Dusenbery, 1980; Goode and Dusenbery, 1985). The rate of locomotion of *Heterodera schachtii* (Wallace, 1958) is about 10 μm/sec and that of *Meloidogyne incognita* is about the same (Pline and Dusenbery, 1987). Free-living nematodes generally move about 10 times faster (Goode and Dusenbery, 1985). From tracks left by *Meloidogyne javanica* (Prot, 1978), the distance over which they maintain a straight course in the apparent absence of external stimuli can be estimated as 0.1 cm. Thus, the time available to measure concentration is on the order of 100 sec. These values yield a minimal detectable gradient of about 2×10^{-6}/cm. The gradient falls to this level at a distance of 90 cm from the single root fiber and 210 cm from the root mass.

Nematodes are also thought to be capable of chemotaxis via klinotaxis (Klingler, 1963; Prot, 1978). In this mechanism, the nematode moves its head (carrying the receptors) from side to side as it moves along and thus samples the gradient with each swing of the head. When a concentration difference is detected, the nematode turns in the appropriate direction. In this case, the time available for measuring the concentration is the time the head is bent to one

extreme and the velocity is that of swinging the head from one extreme to the other. Tethered *M. incognita* immersed in water undulate with a period of 5 sec (Goode and Dusenbery, 1985). However, movement is usually less rapid when nematodes are in a thin film of water than when immersed. From published tracks (Prot, 1978), the wavelength appears to be about 0.01 cm. At a speed of 0.001 cm/sec, the undulations would occur with a priod of about 10 sec. Let us take 10 sec as a maximum time for testing the concentration at each extreme of head motion. During locomotion, the velocity of head swinging will be about the same as the forward velocity. Thus, the main difference between the two mechanisms is that klinotaxis allows about 10-fold less time to sample the concentration. Since the minimal gradient depends on $t^{-3/2}$, the threshold gradient is increased by a factor of $0.1^{-3/2} = 32$. For the single root fiber, the maximum range of detection is proportional to the threshold gradient, so it will be reduced to about 3 cm. For the root mass, the range is reduced by the square root of the threshold gradient and is about 40 cm.

DISCUSSION

The analysis indicates that the time available for measurement of concentration is the most important factor limiting response. Shallower gradients can be detected if an organism can maintain a straight course for longer periods. For example, a bacterial cell that is elongated would have a larger rotational diffusion time, which would permit a longer time for collecting the signal. Another possibility is the use of collimating stimuli. It has been suggested that nematodes might use thermal gradients simply as a guide for maintaining a straight path (Diez et al., 1987). Using such a stimulus, a nematode could maintain a straight course over longer distances than without such a guide. Thus, the presence of a thermal gradient might aid chemotaxis. Another possible example would be motile algae. They can follow the direction of light (Foster and Smyth, 1980), and this could permit them to measure concentrations over longer times than their rotational diffusion would otherwise permit. Nematodes sometimes cease locomotion and wave their heads back and forth (Green, 1977). This may provide a mechanism of extending the sampling time without losing direction.

This analysis explains different ranges for klinokinesis and klinotaxis. A number of observations have indicated that klinotaxis occurs when a nematode gets sufficiently close to a target, and it has been proposed that klinokinesis occurs when the stimulus is weaker. The analysis developed above explains this difference in sensitivity on the basis of the time available to determine concentration. The theory predicts that the ratio of threshold ranges for the two mechanisms is the ratio of available times to the 3/2 power. This is roughly estimated

to be about 30-fold for plant-parasitic nematodes. Burr (1984) has previously pointed out the difference in threshold for klinokinesis and klinotaxis, emphasizing differences in sample distances instead of sample time.

The analysis suggests that gradients of carbon dioxide emanating from roots in soil can potentially be detected at much greater distances than previously believed possible. It remains to be seen whether nematodes actually have this level of sensitivity. Recent experiments with *M. incognita* demonstrated responses to gradients that were lower than 0.01% CO_2/cm or a relative gradient of 0.01/cm (Pline and Dusenbery, 1987). Shallower gradients could not be produced in the apparatus used. The threshold demonstrated seems very low, but it is about 10^4 times larger than the limiting threshold predicted by the theory.

Several of the assumptions used in the above calculations are subject to modification. The value of $D = 0.065$ (Nye, 1981) is at the high end of the range of commonly measured values (Wesseling, 1962), and a value of 0.03 may be more typical. Using the latter value, the ranges of detection would increase by about 50%. The concentration of CO_2 found in soil also varies a great deal. Values from 0.1 to 5% are common (Boynton, 1941; Wesseling, 1962). On this basis, the range for nematode klinokinesis to an isolated root fiber could theoretically vary between 5 and 500 cm.

Another potential limitation is that the time required to establish a gradient over such large distances might be limiting. Gradients will be disrupted by rainwater percolating through the soil. The characteristic time, L^2/D, for diffusion equals a day for a distance of 75 cm in soil. Thus, gradients form relatively rapidly, and this limitation is not likely to be important.

The theory assumes that the root source is surrounded by soil without other roots for distances greater that those of interest. For the large distances of potential attraction determined here, these assumptions will not normally hold in real situations because of the proximity of other roots and the soil surface. However, the assumptions may apply well to recently planted fields where young plants are often well separated and vulnerable to nematodes. In any case, the calculations demonstrate that the sensory abilities of nematodes need not be limiting. If nematodes have the theoretically possible sensitivity, they can follow the gradients normally present in soil to the nearest source.

It is an open question whether soil organisms actually utilize CO_2 gradients to locate plant roots. In the natural soil environment, there are many sources of CO_2 besides plant roots. If CO_2 were the only stimulus utilized, these sources could lead to dead ends. On the other hand, there is certainly a concentration of sources at the root surface. Dead ends might be sufficiently rare so that such losses would not interfere with effective infection. Other chemical stimuli are probably also utilized, especially by parasites with a narrow host range. In support of this view, the species of phytoparasitic nematodes that have been reported as attracted to CO_2 mostly have a wide host range.

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INTRASPECIFIC PHEROMONE DISCRIMINATION AND SUBSTRATE MARKING BY ATLANTIC SALMON PARR

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Abstract—Attraction and preference behavior patterns shown by Atlantic salmon (*Salmo salar* L.) parr towards intraspecific odors were measured. Salmon parr were attracted to tank water in which relatives of their own strain were held but were also attracted to tank water containing another strain of their species. When given a choice between the two types of tank water, water containing their own strain odor was significantly favored over water containing conspecific but nonfamiliar odor, revealing that pheromones are present at intraspecific levels in salmonid juveniles. Competitive experiments with various extracts suggest that the active compounds are most likely produced in the liver and are voided via the intestinal tract, as determined by presence of attractants in intestinal contents and bile. Intraspecific discrimination was not detected with extracts from either skin surface mucus or blood plasma. Extracts from gravel that had been kept below the fish in their rearing tanks, however, induced a strain-related preference behavior. This suggests an ability for substrate marking by salmonid fishes, presumably mediated by deposition of fecal material. Strain discrimination and substrate marking are discussed in relation to stationary behavior and homing of fishes within discrete populations in natural systems.

Key Words—*Salmo salar*, olfaction, pheromones, substrate marking, feces, bile, skin mucus.

INTRODUCTION

Salmonid fishes living in streams demonstrate a substantial degree of regional stability throughout their life, (Miller, 1954; Saunders and Gee, 1964; Edmundson et al., 1968; Hesthagen, 1978; Cargill, 1980; Harcup et al., 1984).

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When moved from their "home area," most of the fishes will return to that area whether displaced upstream or downstream. Downstream displacement, however, was found by Miller (1954) to result in a more precise homing in cutthroat trout (*Salmo clarki* L.). Accordingly, it was suggested that the sense of smell was the most important factor in homing since fish displaced downstream could smell their home area continuously.

Regional stability and homing after displacement in streams have also been demonstrated in several nonsalmonid species of fishes (Gerking, 1953, 1959; Stott, 1967). In sensory deprivation experiments, using longear sunfish (*Lepomis megalotis megalotis* Raf.), Gunning (1959) demonstrated that fishes with an impaired olfactory sense moved at random in the stream, whereas blinded individuals homed as quickly and accurately as controls. The precision of intact fish homing in the upstream direction was also found to be greater than that of those moving downstream. Homing was therefore concluded to be maintained by olfactory mechanisms.

The above summary indicates that fishes within streams search for their home range through rheotactic behavior mediated by olfaction, and they apparently recognize their "home area" by some characteristic odor. The question then arises: what is the origin of the characteristic odors used by fishes when homing and when maintaining regional stability?

Hasler and Wisby (1951) suggested that because of local differences in soil and vegetation of the drainage basin, each stream has a unique chemical composition. During smolt transformation and seaward migration, the young salmon were presumed to become "imprinted" to the distinctive odor of their home stream (see also: Hasler et al., 1978; Hasler and Scholz, 1983). When returning as mature adult salmon, they would proposedly remember this "imprinted" odor and use it as a cue for homing when migrating through the homestream network. The "imprinting" hypothesis, however, has been closely associated with the process of smolt transformation (Hasler and Scholz, 1983) and does not, therefore, explain the phenomena of regional stability and homing after displacement in juvenile fishes (Stabell, 1984).

The pheromone hypothesis for anadromous salmonids (Nordeng, 1971, 1977) proposes that homing migrants return to their home area by detecting population-specific pheromones secreted by downstream migrating smolt and young relatives resident in the stream. The hypothesis has been supported through behavior studies with mature anadromous char (*Salvelinus alpinus* L.) by Selset and Døving (1980), demonstrating that char have the ability to detect the odor of young relatives and that they prefer the odor of relatives to that of nonrelated conspecifics.

By positive rheotaxis olfactometry, it has recently been demonstrated that Atlantic salmon (*Salmo salar* L.) parr are also able to discriminate between substances secreted by strains of conspecifics (Stabell, 1982). Accordingly, it was suggested that intraspecific odors could be responsible for the regional sta-

bility of juveniles in streams and that these odorants could well be identical to those attracting mature migrants to the spawning grounds. Studies of positive rheotactic behavior in parr towards conspecific odors could therefore provide insight into adult homing behavior.

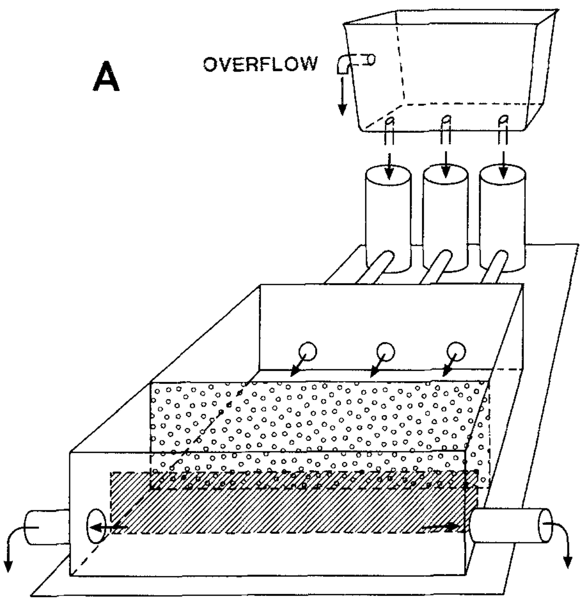
The intention with the present study was to further investigate the preference and attraction phenomena of Atlantic salmon parr towards conspecific odors. In particular, it was of interest to evaluate possible pathways for the secretion of the intraspecific signal substances and to test for substrate marking by fishes related to regional stability and homing.

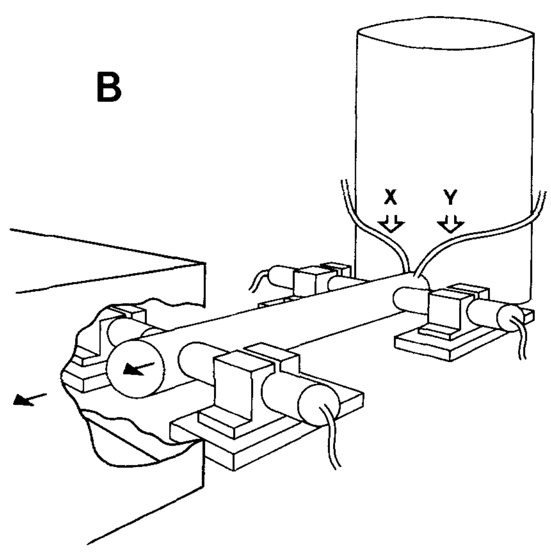
METHODS AND MATERIALS

Study Animals. Atlantic salmon (*Salmo salar* L.) parr were used in the study. The parr originated from the river Sandvikselv, Akershus County, southeastern Norway; the river Imsa, Rogaland County, southwestern Norway; and from the river Altaelv, Finnmark County, northern Norway. After artificial fertilization, the eggs were hatched and the fish raised at the Research Station for Freshwater Fish, belonging to the Directorate for Wildlife and Freshwater Fish, at the Imsa River. The fishes were subsequently transported to aquarium facilities at the University of Oslo, where the experiments were conducted. The Altaelv parr were all siblings of the brood year 1981 and were transported to Oslo in May 1982. The parr from Sandvikselv and from Imsa were of the brood year 1982 and arrived at the University in September 1982. Of these fish, the Imsa parr belonged to one sibling group, while the Sandvikselv parr came from a group where ova and milt from several spawners had been mixed. The three strains of fish were reared in separate tanks supplied with tap water and fed with commercially available dry pellets from automatic feeders.

Test System. A fully automated test system was used in the experimental series to measure preference behavior and upstream movement aroused by chemical stimuli. The aquarium part of the system was of the two-choice, parallel-entrance type, as described by Selset and Døving (1980). In the current study the aquarium part was slightly modified with regard to water-flow characteristics and test sample injections, according to the description by Stabell (1982).

The system involves a main chamber with three freshwater inlets, of which the fish were allowed to choose between the two outermost (Figure 1A). The middle inlet was used to ensure good separation of water flow within the main chamber, and fish were prevented from entering that inlet by a plastic net cover. Tap water was introduced from an overflow reservoir into three separate chambers in the upstream end of the system, ensuring equal flow from each of the inlets (3×3 liters/min). A water level of 8 cm was used in the system, giving a main chamber volume of 28.3 liters.


A
OVERFLOW


B
X
Y

The test substances were introduced to the system at the upper end of the inlet tubes, as shown in Figure 1B, and added by hydrostatic pressure to the background flow through solenoid valves at an approximate ratio of 1:60 in volume. When a test sample (X) was introduced to one of the inlets, a reference sample (Y) was introduced to the other and vice versa. Movements of fish into the inlets were registered by photocells (IR light) located at the lower and upper end of each inlet (Figure 1B). Test performances were controlled and monitored by a microcomputer connected to an interface with in–out ports and a printer.

Odor Sampling. Fish odors were sampled by siphoning water from fish rearing tanks (volume: 500 liters, flow: 10 liters/min) containing approximately 100 fish that had been undisturbed for more than 12 hr. Skin mucus was sampled from the surfaces of the fish using an aspirator flask, according to the description by Stabell and Selset (1980). Skin mucus, intestinal contents, blood plasma, and bile were all sampled from the same fish. Each type of material was pooled from five fish and subsequently divided for use in two 4-hr test series. Material not used for immediate testing was frozen and stored at −20°C.

Test solutions from skin mucus as well as intestinal contents were prepared by adding 20 ml of tap water to each of the sampled materials. After careful mixing, the solutions were centrifuged and the supernatant pipetted off. Another 20 ml of tap water was added to the residue and the procedure repeated. The supernatants from both water extracts were then pooled for use in the tests. Ethanol extracts from each type of material were prepared in a similar manner to that described for water extracts. Before using in the tests, however, ethanol was removed in a rotary evaporator and the residues redissolved in tap water.

Gravel retained by a 2-cm sieve and washed in water was introduced under the fish in the rearing tanks upon arrival of the 1982 brood years. Approximately 20 liters of gravel were used in each tank. After two months, the gravel was removed from the tanks and washed carefully with running water to remove food residues and feces. It was then spread out on water-absorbing paper on the laboratory bench to dry. After a week, methanol extracts were made from the

FIG. 1. (A) Overview of the aquarium part of the test system; flow directions are shown by solid arrows. City tap water is introduced to the upper overflow tank, ensuring equal flow from each of the three inlets. Entrance by the fish into the middle inlet is prevented by a grid. A perforated wall in the downstream end of the main chamber prevents fish from moving downstream, and an overflow wall (hatched) secures a constant level of water in the system. (B) Close-up of a freshwater inlet showing positions of photocells in the downstream and upstream ends. Positions of tubes for test sample injections, denoted X and Y, are shown by open arrows in the upstream end of the inlet. Test samples are introduced through solenoid valves, and test performance is controlled and monitored by a microcomputer.

material covering the gravel surface. Five liters of methanol was used to wash the gravel from each tank. Methanol extracts were subsequently treated in the same manner as that previously described for the ethanol samples.

Experimental Procedures. Test samples were introduced to the inlet tubes for periods of 20 min followed by pauses of 10 min. Every half hour the samples were switched between the inlets in order to balance possible preferences of the fish for a particular side of the aquarium. Because of possible side preferences, two subsequent half-hour test periods were treated pairwise in the evaluation of data.

Four hours of testing, consisting of four pairs of half-hour test periods, were run as a series. This time limitation for each test series was because of the storage capacity of the test-sample reservoirs. Several 4-hr test series were usually necessary to obtain a satisfactory number of choices within each type of test. Ten fish were used simultaneously in the test system, and no fish were ever used in more than two successive 4-hr test series.

Data Evaluation. The two photocells used for each inlet allowed two different events to be evaluated: (1) search: only the beam of the downstream photocell is broken, i.e., the fish enters the lowermost part of the inlet and then backs out; (2) ascent: the beam of the downstream photocell is broken followed by that of the upstream cell, i.e., the fish moves all the way up through the inlet tube.

In random choices, the fish will choose equally between the two inlets (Selset and Døving, 1980; Stabell, 1982), and the binomial model can therefore be used for probability calculations (Siegel, 1956). The activity score (AS) obtained for each event (ascents or search) is given by the expression:

$$AS = \frac{N_t - N_r}{N_t + N_r} \times 100$$

where N_t and N_r are the number of choices in favor of the test substance and the reference substance, respectively (Olsén, 1985). The data are presented as column graphs for the two events, each representing the activity score for the total number of trials performed.

Probabilities for random choice are given in the figures. The probabilities are given as the lowest score obtained for each experiment at the 5, 1, or 0.1% levels. All probabilities given are one-tailed. The results were regarded to be within significant levels of attraction or repulsion when the probability of being generated by random choice was found equal to, or less than, 5%.

RESULTS

Fish Odors in Tank Waters. The results obtained from tests with fish odors in tank waters are presented in Figure 2. Fish from the Sandvikselv strain were

used as test fish in all three experiments of this type, which took place from October 10 to November 3, 1982. When water from the tank containing fish of the Sandvikselv strain was tested against tap water without any salmon odor, the fish significantly preferred the tank water (Figure 1A). The results refer to both parameters tested. Of 93 ascents performed, 55 were in favor of the tank water, giving an activity score (AS) of +18.3. This result represents a significant level of attraction ($P = 0.0495$). For search behavior, 468 of 820 trials performed were in favor of the tank water containing the related fish, giving an AS of +14.2, which is highly significant ($P < 0.00003$).

The test fish also demonstrated a preference for tank water over tap water when water from a tank containing an unfamiliar strain of salmon parr was used (Figure 1B). Of 122 ascents recorded, 77 were in favor of the tank water, giving an AS of +26.2 ($P = 0.0025$); from a total of 1333 searches, 725 were also in favor of the tank water (AS = +8.8; $P = 0.007$).

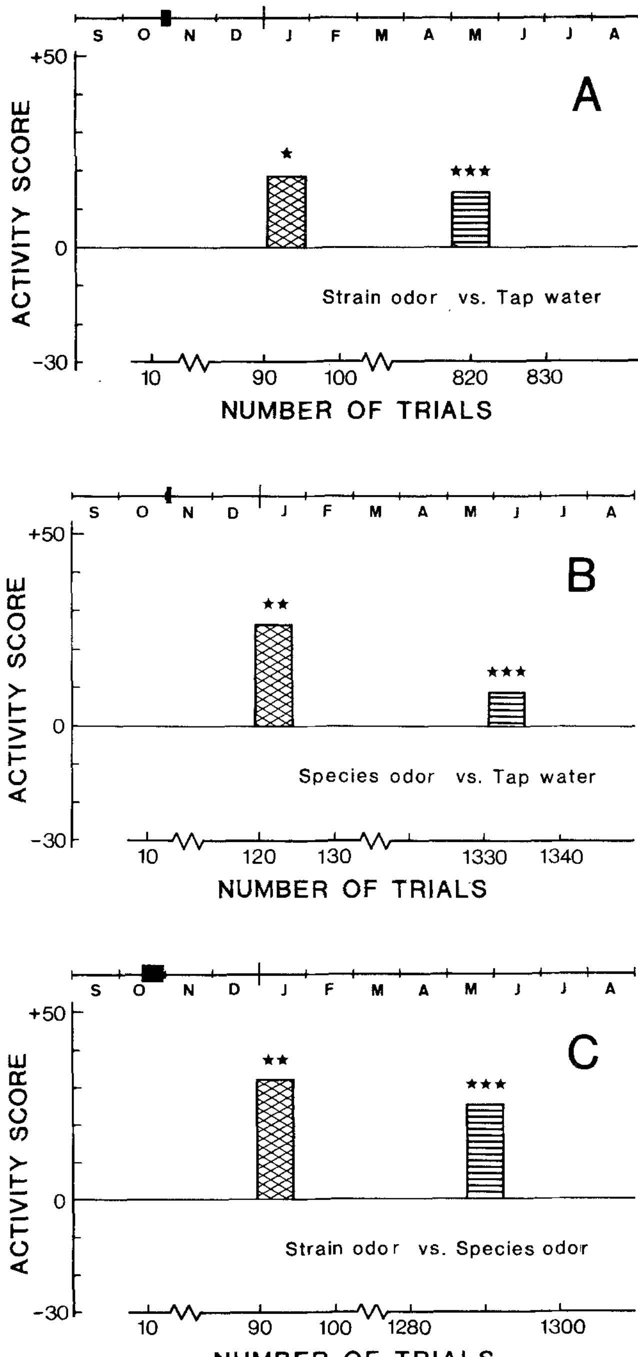
When tank water containing the related Sandvikselv strain of fish was tested against tank water containing the unfamiliar Imsa strain that had previously been found attractive, the Sandvikselv fish highly favored water from the related strain to that from the unfamiliar one (Figure 2C). Of 92 ascents recorded altogether, 61 were in favor of the familiar odor (AS = +32.6; $P = 0.0013$). For search, 806 of 1290 trials were in favor of the familiar odor (AS = +25.0), demonstrating that this result was also at a highly significant level ($P \ll 0.00003$).

Odorants in Excretion Products from Fish. Competition experiments with extracts from skin mucus of the Sandvikselv strain versus similar extracts of the Imsa strain are presented in Figure 3A. Fish from the Sandvikselv strain were used as test fish. In the middle of November 1982, fish from the Sandvikselv strain were found to discriminate between water extracts for the search parameter only. Of 70 ascents recorded, only 32 were in favor of the related strain (AS = −8.6; $P = 0.274$), whereas 470 of 880 searches were directed towards the familiar strain (AS = +6.8; $P = 0.023$).

In the middle of June 1983, however, no significant preferences for either strain was found in competition experiments with water extracts from skin mucus (Figure 3A). Fish from the Imsa strain were used as test fish this time. Sixty-one ascents from a total of 128 were in favor of the related strain (AS = +4.7; $P = 0.33$), and 258 of 514 searches were similarly directed (AS = +0.4; $P = 0.484$).

Ethanol extracts from skin mucus of the two strains mentioned above, tested in July 1983 with the Sandvikselv strain as test fish, elicited neither of the preference behaviors (Figure 3A). Only 33 ascents were registered in total, of which 15 were in favor of the material from the related strain (AS = −9.1; $P = 0.36$). Of 199 searches, 96 were in favor of the related strain (AS = −3.5; $P = 0.334$).

Experiments with extracts from intestinal contents are presented in Figure


S O N D J F M A M J J A
+50
0
-30
ACTIVITY SCORE
A
Strain odor vs. Tap water
10 90 100 820 830
NUMBER OF TRIALS
B
Species odor vs. Tap water
10 120 130 1330 1340
C
Strain odor vs. Species odor
10 90 100 1280 1300

3B. When water extracts from intestinal material were run in the middle of November 1982 with fish from the Sandvikselv strain as test fish, no preference behavior was detected (Figure 3B). Seventy-seven ascents were recorded; 35 of them were in favor of the related strain (AS = −9.1; P = 0.248). Of 963 searches, 464 were in favor of the related strain (AS = −3.6; P = 0.138).

When tested in June 1983, however, an ability among the Sandvikselv parr for intraspecific discrimination of water extracts from intestinal material was revealed (Figure 3B). Although a significant level was not found among ascents, 28 of 45 trials were in favor of the familiar odor (AS = +24.4; P = 0.068). For the search parameter, 124 of 205 trials were in favor of the related strain, revealing a highly significant level of preference (AS = +21.0; P = 0.0017).

Ethanol extracts from intestinal contents also elicited preference behavior of the Imsa fish towards material from the familiar strain (Figure 3B). Twenty-eight ascents of 42 were found in favor of the familiar strain, giving a significant level of preference (AS = +33.3; P = 0.022). For the search parameter, however, a repellent effect from the familiar strain was found. Of 1460 trials, only 633 were directed towards the native material, revealing a highly significant repellent effect (AS = −13.3; $P \ll 0.00003$).

Origin of Odorants. Results from competition experiments using bile from two genetic strains of parr are presented in Figure 4A. Fish from the Sandvikselv strain were used as test fish. Very few ascents were recorded during the experimental period which took place in June 1983. Only 28 trials were registered in total, and 16 were in favor of the extracted material from the familiar strain (AS = +14.3; P = 0.284). For search, however, 254 trials were recorded, of which 142 were directed towards the familiar material (AS = +11.8; P = 0.034). This result suggests that the strain-specific substances demonstrated in tank water and intestinal contents of fish may originate from the liver.

←

FIG. 2. Activity scores for ascents (cross-hatched bars) and search (horizontal lined bars) in test series with water from fish-rearing tanks, presented at the total number of trials recorded for each event. (A) Test with tank water from the Sandvikselv strain against tap water as reference. (B) Test with tank water from the Imsa strain against tap water as reference. (C) Test with tank water from the Sandvikselv strain against tank water from the Imsa strain as reference. Fish from the Sandvikselv strain were used as test fish in all three test series. Ascents are the movements of fish up through the inlet tubes. Search is the entrance into the downstream part of inlets only. Activity scores for each event are given as the difference between trials in favor of the test sample and trials in favor of the reference sample, expressed as percent of the total number of trials. Probabilities for random choice are given in the figures. The lowest probability obtained among the 5, 1, and 0.1% levels are in each case given by one, two, or three asterisks, respectively. Black vertical bars in the upper part of each figure indicate experimental periods in 1982.

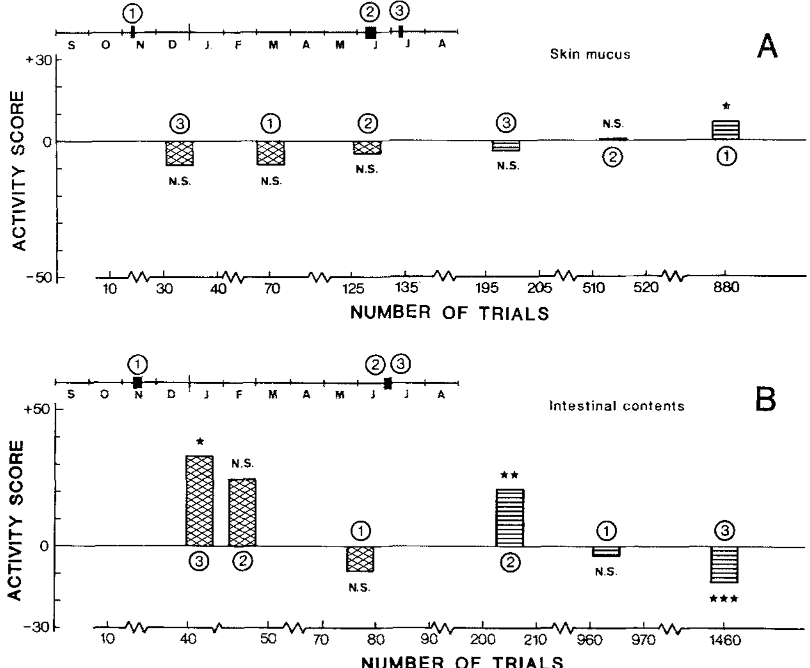
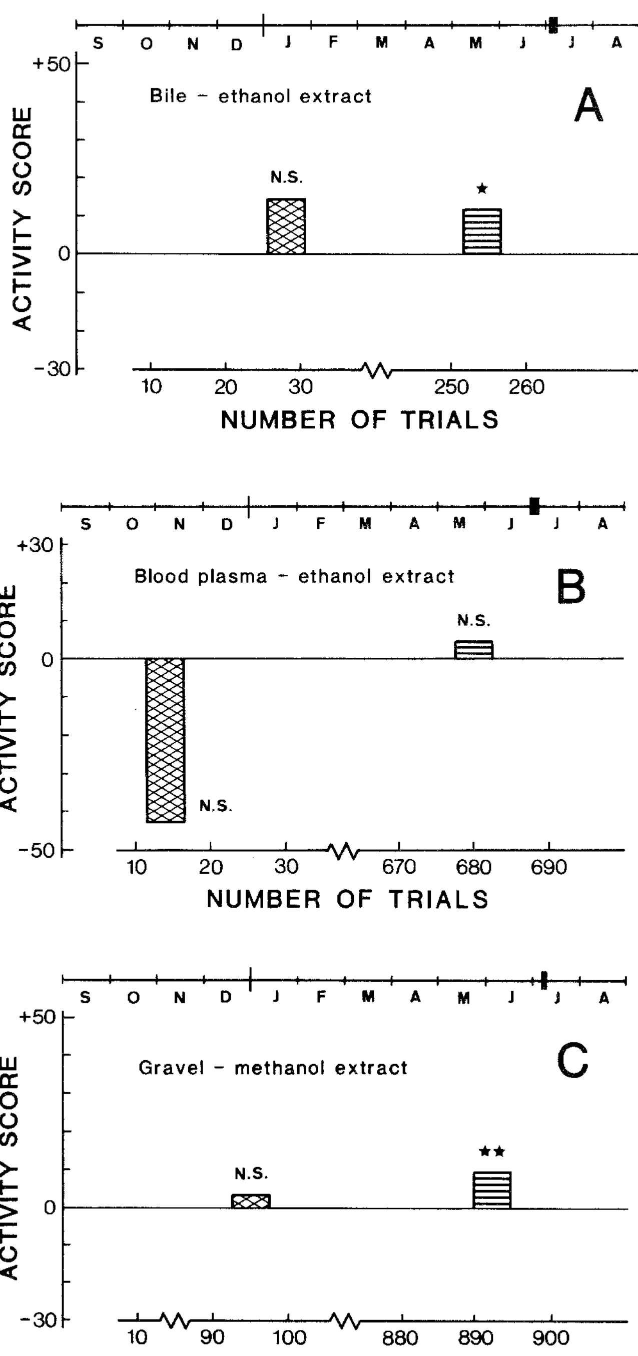


FIG. 3. Activity scores for ascents (cross-hatched bars) and search (horizontal lined bars) in test series with extracts from skin mucus and intestinal contents. The tests were performed with extracts from a familiar versus an unfamiliar strain and are presented at the total number of trials recorded for each event. (A) Tests with extracts from skin mucus. (B) Tests with extracts from intestinal contents. Encircled number one represents experiments performed in 1982 with water extracts from crude material. Encircled number two represents experiments performed in 1983 with water extracts from crude material. Encircled number three represents experiments performed in 1983 with ethanol extracts from crude material. The strain of test fish used in each case and experimental design for each type of test performed are given in Table 1. General figure explanations are given in the legend to Figure 2.

→

FIG. 4. Activity scores for ascents (cross-hatched bars) and search (horizontal lined bars) in test series with ethanol or methanol extracts from various sources. The tests were performed with extracts from familiar versus an unfamiliar river strain. (A) Competitive test with extracts from bile. (B) Competitive test with extracts from blood plasma. (C) Competitive test with extracts from gravel which had been retained for two months in rearing tanks below fish from two separate river strains. Strains of test fish used in each case and experimental design for each type of test performed are given in Table 1. General figure explanations are given in the legend to Figure 2.


S O N D J F M A M J J A
+50
0
-30
ACTIVITY SCORE
Bile - ethanol extract
A
N.S.
★
10 20 30 250 260
NUMBER OF TRIALS
S O N D J F M A M J J A
+30
0
-50
ACTIVITY SCORE
Blood plasma - ethanol extract
B
N.S.
N.S.
10 20 30 670 680 690
NUMBER OF TRIALS
S O N D J F M A M J J A
+50
0
-30
ACTIVITY SCORE
Gravel - methanol extract
C
N.S.
★★
10 90 100 880 890 900
NUMBER OF TRIALS

Experiments with extracts from blood plasma elicited no preference behavior of the fish from the Imsa strain (Figure 4B). Only 14 ascents were recorded in total in June 1983, with four in favor of the familiar strain (AS = −42.9; P = 0.092); 355 searches of 680 were directed in a similar manner (AS = +4.4; P = 0.134). This suggests that the attractant material found in the bile is secreted through the intestinal tract and not transported in the blood to be secreted elsewhere.

The Imsa parr demonstrated preference behavior when extracts from gravel were tested in June 1983 (Figure 4C). Of 892 searches recorded, 487 were in favor of the extract from gravel kept below their strain (AS = +3.2; P = 0.0034). For ascents, 49 of 95 trials were in favor of the gravel extract from the tank containing familiar fish (AS = +9.2; P = 0.417). Since the gravel used in the tanks originated from the same source, the results suggest that each strain of fish is able to mark the substrate of its living environment.

Activity of Fish. All experiments presented in this paper were performed within the relatively narrow temperature range of 7.3–9.3°C (Table 1). Activity of the fish, however, as shown by the data, appears to be independent of temperature. This suggests that variations obtained in activity of the fish must be related to parameters other than temperature.

When tank waters were used, the activity per hour of ascents ranged from 7.7 to 15.3, and for search from 107.5 to 166.6 (Table 1). Activity was found to be at the highest level when water containing an unfamiliar strain of fish was used. When waters from tanks containing fish were introduced to both entrances in competition tests, the activity was found to be at the lowest level. Regardless of the test type performed with tank waters, the ascents-to-search ratio remained relatively stable.

In tests with water extracts from skin mucus and intestinal contents (Table 1) a higher activity in search seems to have been present in November than in June. In general, test samples made from extracts also resulted in a lower activity than tank waters in the tests. For skin mucus, tests with ethanol extracts appear to have reduced the overall activity compared to tests with water extracts, whereas similar extraction from intestinal contents apparently had the opposite effect. Ethanol extracts from bile and blood plasma (Table 1) induced a very low activity in ascents, while search did not deviate apparently from that found for other organic solvent extracts. For gravel extracts, the fish demonstrated a behavior that did not deviate from normal activity.

DISCUSSION

The data presented in this paper reveal that Atlantic salmon parr demonstrate preference behavior towards, and are attracted to, water conditioned by conspecifics. In competition experiments they favor water conditioned by their

TABLE 1. SUMMARY OF PREFERENCE TESTS PERFORMED AND ACTIVITY RELATIONSHIP FOR BEHAVIORAL PARAMETERS MEASURED[a]

Figure	Sample origin	Type of test	Test Fish	Experimental Period	Activity per hour		A/S ratio	T (°C)
					Ascents	Search		
2A	Tank water	S vs. TW	S	Oct. 28–Nov. 11	13.3	117.1	0.11	9.0
2B	Tank water	I vs. TW	S	Nov. 02–Nov. 03	15.3	166.6	0.09	8.6
2C	Tank water	S vs. I	S	Oct. 15–Oct. 27	7.7	107.5	0.07	9.3
3A	Skin mucus	S vs. I	S	Nov. 09–Nov. 11	5.4	67.7	0.08	8.1
3A	Skin mucus	I vs. S	I	June 09–June 16	6.4	25.7	0.25	7.3
3A	Skin mucus, ethanol extract	S vs. I	S	July 09–July 11	1.7	10.0	0.17	8.0
3B	Intestinal content	S vs. I	S	Nov. 11–Nov. 17	3.9	48.2	0.08	8.0
3B	Intestinal content	S vs. I	S	June 25–June 27	2.3	10.3	0.22	7.8
3B	Intestinal content, ethanol extract	I vs. S	I	June 28–June 29	2.3	81.1	0.03	7.7
4A	Bile, ethanol extract	S vs. I	S	July 04–July 07	1.4	12.7	0.11	7.9
4B	Blood plasma, ethanol extract	I vs. S.	I	July 01–July 04	0.7	34.0	0.02	7.9
4C	Gravel, methanol extract	I vs. A	I	July 07–July 09	4.8	44.6	0.11	7.9

[a] S = Sandvik River strain; I = Imsa River strain; A = Alta River strain; TW = tap water.

related strain to that conditioned by another genetic strain of their species. The findings reveal that salmon parr secrete substances specific for their strain and that they are able to discriminate between these chemical cues.

The secretion of signal substances seems restricted to the gastrointestinal tract and appears to originate from the liver, the largest "chemical factory" in the vertebrate body. Evidence has also been provided for substrate marking by salmonid fishes, presumably mediated by deposition of fecal material. In the following sections, the behavior towards chemical signals will be considered, together with aspects of physiology and ecology related to the ability of strain discrimination and substrate marking in salmonid fishes.

Determination of Chemically Mediated Rheotaxis. Fish denied olfaction demonstrate a preference for directing the body towards water currents (Høglund and Åstrand, 1973), i.e., positive rheotaxis is maintained in the absence of olfactory cues. In blind tests related to preference experiments, fish will also move upstream in the absence of any known chemical stimulus (Selset and Døving, 1980; Stabell, 1982). It is therefore difficult to give a direct measure for attractive responses due to chemical stimuli in the determination of fish behavior. The problem has been solved indirectly in behavior experiments by comparing the rate of activity of the fish to stimulants added simultaneously. In the current study, it has been possible to separate the behavior towards chemical stimuli into two events by using a double set of photocells in each tube (Figure 1B).

When held under abnormal and crowded conditions, it has previously been demonstrated that salmon parr secrete substances to which their relatives demonstrate an avoidance response when given an alternative and unscented choice of water (Stabell, 1982). Furthermore, confronted with a choice of odors from two stressed groups of fish, the parr demonstrated a preference for chemical cues secreted by their related strain. Both search and ascents were found within significant statistical levels of preference in the report mentioned, even when "repellent" substances were present in the samples tested. Tests with sample extracts in the current study, however, apparently produced a shift in preference behavior towards the search parameter. When organic solvents were used for sample extractions, ascents were found to work only once as a functional parameter in the tests. The shift in preference behavior was also found to coincide with a general decrease in activity when sample extracts were used, the decrease being greatest after treatment with organic solvents, while the ratio of ascents to search was not found to be systematically affected. A decrease in attractive responses in mature char, probably similar to the one obtained here, was also reported by Selset and Døvig (1980) when test samples had been treated by chemical means. Therefore, the extraction procedure rather than the presence of repellents seems responsible for the decrease in activity and the shift in preference behavior found in this study.

All pretreated samples in the present study were dissolved in small quantities of water before testing. In order to function as substrate markers, as will be discussed in a subsequent section, water solubility of the topical signal substances must be expected to be very low. The small volumes of water used for extraction of the active material may, therefore, have been a determining factor for the observed shift in behavior because of the low concentrations of the applied stimulus. Additional "trapping" of active compounds within precipitated material when organic solvents were used for extraction may possibly explain the special behavioral effects found following such treatment. Accordingly, both ascents and search must be viewed as parameters suitable for detecting preference behavior. While the ascent parameter is probably that which most strongly expresses attractive properties, it is suggested that search may work alone as a functional parameter in determination of chemically mediated rheotaxis when dealing with low stimulus concentrations.

Production and Secretion of Odorants. Preference tests with tank waters, each containing a separate genetic group of salmon parr, demonstrate that the fish secrete odorants specific for their strain. The observation that salmonid juveniles secrete intraspecific pheromones supports the results from several earlier reports in the field (Nordeng, 1971, 1977; Selset and Døving, 1980; Quinn and Busack, 1985; Quinn and Tolson, 1986; Olsén, 1985, 1986). Skin mucus was initially suggested to be the source of salmonid pheromones (reviewed by Stabell, 1984), but the origin of the attractive chemical components was questioned by Stabell and Selset (1980) because of a demonstrated contamination by intestinal juices of mucus collected in the customary way. Selset and Døving (1980), who found no behavioral response in mature Arctic char to "pure" skin mucus, also questioned skin mucus as a source for salmonid pheromones.

In the current study, ascents were never found below the 5% probability level in tests with water extracts from skin mucus. As for the search parameter, on one occasion only, in late autumn, an intraspecific preference for skin mucus was obtained. Since intestinal contents gave no behavioral responses at that time of the year, the result is difficult to interpret. Comparative physiologic indicators for presence of attractive substances in vertebrate skin mucus are, however, given in the literature. In female garter snakes (*Thamnophis sirtalis parietalis*), blood plasma has been shown to contain pheromones arousing sexual behavior in males (Garstka and Crews, 1981). As in this study, the pheromones from the mentioned reptiles are produced in the liver but are transported in the blood and secreted by mucus glands present between the surface scales. Extracts from blood plasma in the current study, however, revealed no preference or attractant properties. If mucus cells in salmonid fishes secrete attractant substances, the production must consequently be suggested to take place within the same cells. Possible attractant properties of skin mucus in salmonid fishes obviously await further investigation. Altogether, however, the data reported here appear to sup-

port the preliminary conclusion that the topical signal substances do not originate from skin mucus.

Tests with water extracts from intestinal contents of different salmon strains presented here demonstrate that the fish are able to discriminate among various compounds found in the gastrointestinal tract. The observed variation in odorants secreted cannot be attributed to food ingested, since the different strains of fish were fed by food pellets of common origin. Secretion of intraspecific pheromones through the intestinal tract by juvenile Atlantic salmon is in accordance with earlier observations with Arctic char (Selset and Døving, 1980). The experiments performed with ethanol extracts from intestinal contents in the present study were found to result in a significant preference level for ascents, while repellent effects were obtained for the search parameter. This was the only time during the study that contrary results were found for the parameters used, and an interpretation would therefore appear futile. Ethanol extracts from the bile, however, gave significant preference levels in search towards the familiar strain, supporting the conclusion by Selset (1980) that salmonid pheromones may originate from the liver and could be of a steroid nature.

Substrate Marking and Regional Stability. Atlantic salmon parr apparently mark their living environment with scents of an intraspecific nature, as demonstrated in this study. The odorants in question are most probably secreted within fecal material and may be deposited passively on the substrate by the bottom-dwelling parr. The chemical compounds representing the specific odors have been demonstrated to withstand dried-up conditions for at least a period of time, but they must also hold properties of low solubility due to the continuous flow of water in natural environments. Since the holding tanks in the current study were continuously exposed to flowing water, and the gravel washed by water before drying, a low solubility of the compounds in question seems to have been confirmed.

It has been suggested by some authors that salmonid fishes may leave residual scent on the spawning site long enough to secure homing by returning migrants (Larkin, 1975; Kristiansen, 1980; Selset, 1980; reviewed by Stabell, 1984). Foster (1985) also demonstrated in tank experiments that lake trout (*Salvelinus namaycush*) would preferently spawn on artificial reefs scented with material taken from eggs and excretion products of young fish. It would seem unlikely, from the point of view of energy costs, that juvenile fish have developed several parallel systems of pheromone production. The above reports, therefore, together with the data presented here, strongly indicate a common chemical basis for regional stability in juveniles and the mechanisms underlying homing of adult migrants to specific spawning grounds.

Ecological Aspects of Olfactory Recognition. Intraspecific recognition among juvenile siblings of Atlantic salmon, originating from different river strains, was demonstrated by Stabell (1982). It was suggested that the events

observed among the parr were based on the same mechanisms of recognition as the one attracting mature migrants to the spawning grounds. Quinn and Busack (1985) also demonstrated an ability among juvenile coho salmon (*O. kisutch*) to recognize and prefer chemical cues secreted by siblings. They suggested that the most obvious adaptive values of kin recognition were to encourage schooling and thereby improve predator avoidance. Facilitated inbreeding avoidance among adults was also suggested as a possible resulting effect. The conclusions reached by Quinn and Busack (1985), however, appear to contradict the events generally observed in nature, since bottom-dwelling, territorial behavior in juvenile stream-living salmonids, together with specific return to native spawning grounds by adult maturing fish, are well documented phenomena. In the current study, fish other than pure sibling groups were used, indicating that strain recognition may work on additional levels to that of siblings and suggesting that a hierarchic order of chemical recognition may be at work within discrete populations of salmonids.

The fish in this study were able to discriminate between intestinal water extracts in June but not in the middle of November. This finding may result from a late autumn decline of the behavioral response, the olfactory sensitivity, or the secretory activity of the fishes. Since, however, high behavioral activities were still recorded in November, and olfactory sensitivity in electrophysiological recordings have been found present also during the winter (G. Thommesen, Department of General Physiology, University of Oslo, personal communication), it is suggested that the results are due to a decline or absence of pheromone secretion outside the migrating season.

Selset and Døving (1980) reported an ability of mature Arctic char to discriminate between conspecific odorants secreted within the intestinal contents of juvenile fish. The present study reveals that juvenile salmonids also have the ability to discriminate between conspecific odorants of intestinal origin from juveniles. An olfactory-dependent intraspecific recognition in young fish, together with substrate marking in the "home" area by juveniles, thus extends the view of the functional role of pheromones in the ecology of salmonid migration.

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DIET MANIPULATION AFFECTS SOCIAL BEHAVIOR OF CATFISH:
Importance of Body Odor

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Abstract—Diet manipulation, an habituation test, and chemical analysis of urinary free amino acids were used to demonstrate that bullhead catfish (*Ictalurus nebulosus*) naturally detect the body odors of conspecifics and respond to them in a predictable fashion. These signals are used in dominance and territorial relationships and lead to increased aggression toward chemical "strangers." The results support the general notion that nonspecific metabolites, as well as specific pheromones, are important in chemical mediation of social behavior.

Key Words—Pheromones, body odors, catfish, diet, *Ictalurus nebulosus*, social behavior, amino acids, urine.

INTRODUCTION

Body odor is the sum of all perceivable compounds in excreta and secreta. Pheromones represent a specialized portion of this odor complex and play an important role in the formation and maintenance of many vertebrate social relationships (Müller-Schwarze, 1974; Doty, 1976). They represent the social chemical "sign stimuli" (Tinbergen, 1951; Brown, 1975) which tend to be of discrete chemical composition and are usually associated with stereotyped physiological or behavioral responses (Wilson and Bossert, 1963; Wilson, 1975). These latter responses are generally thought not to be dependent upon learning. Commonly, pheromone synthesis is glandular and release is under voluntary control (Albone, 1984; Liley, 1982). Body odors, on the other hand, are typi-

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cally chemically complex (Müller-Schwarze, 1969; Beruter et al., 1973; Evans et al., 1978; Preti et al., 1977) and consist of general metabolic waste-products rather than specifically synthesized compounds. It has been suggested that at least in mammals, chemical complexity in social stimuli may be common (Beauchamp et al., 1976). In addition, these types of stimuli tend to be used in behavioral contexts which allow or depend upon considerable plasticity or variation in response (Beauchamp et al., 1976).

In fish, a role for social chemical stimuli has been demonstrated primarily in behavior leading to spawning (Liley, 1982). These chemical stimuli function as sex- or species-specific attractants (Keenleyside, 1955; Kendle, 1968; Gandolfi, 1969; Mainardi and Rossi, 1968; Newcombe and Hartman, 1973; Chen and Martinich, 1975; Ingersoll and Lee, 1980; Teeter, 1980) or as indicators of reproductive status (Tavolga, 1956; Partridge et al., 1976; Crow and Liley, 1979; Honda, 1979, 1980a, Ingersoll and Lee, 1980; Crapon de Carpona, 1980). In addition, population-specific odors are implicated in home stream recognition (Selset and Døving, 1980). Equally important and less well studied are those chemical stimuli associated with pair-bonding (MacGintie, 1939) or dominance and territorial behavior (Todd, 1968; Todd et al., 1967). MacGintie (1939) demonstrated that mated blind gobies (*Typhlogobius californiensis*) chemically perceive gender, evicting nest intruders of the same sex. Similarly, in dominance and territorial behavior, the recognition of traits such as gender, individuality, or physiological or social status may also be mediated by chemical stimuli.

Todd et al. (1967) and Richards (1976), using sensory ablation (cautery) and behavioral conditioning methods, inferred that olfaction is important in the dominance and territorial behavior of several ictalurid catfish species. Their experiments, while demonstrating sensory capabilities, do not demonstrate which stimuli or biological traits the fish actually respond to during normal unconditioned behavior. In addition, sensory ablation can cause confounding effects by affecting aspects of behavior that are not normally mediated by the ablated sense, i.e., general motivation (Alberts, 1974). An attractive alternative to manipulation of the sensory system is to manipulate the stimulus and observe unconditioned responses to it. By altering a portion (chemical, visual, etc.) of a stimulus complex, it is possible to determine if and how that portion contributes to behavior.

We were interested in determining the importance of nonpheromonal metabolites in social behavior. To this end, we examined the chemosensory basis of bullhead catfish social behavior by changing the chemical output of one of a pair of socially interacting fish by diet manipulation. Behavioral observations indicated that changing the diet of one of the fish resulted in altered behavior of the other fish. That the observed change in behavior was due to chemical stimuli was supported by two lines of evidence. First, results from a behavioral bioassay based on habituation comparisons indicated that urine from donor fish,

a potential source of social stimuli in fish (Barnett, 1981; Richards, 1974) was perceived as different following a change in diet. Second, diet-related changes in urinary amino acids, which are known to be effective behavioral (Little, 1977, 1981; Atema, 1977; Holland and Teeter, 1981) and electrophysiological stimuli (Caprio, 1975, 1977; Suzuki and Tucker, 1971), suggested that amino acids may act as social chemical stimuli in catfish. Together, these results indicate that nonpheromonal chemical stimuli are important in the natural territorial and dominance behaviors of bullhead catfish.

METHODS AND MATERIALS

Animals. Brown bullheads, *Ictalurus nebulosus*, were caught in local (Cape Cod, Massachusetts) ponds and maintained in 680-liter tanks in groups of five to eight fish. The fish were held for one to four months before use, during which time they were fed Purina Trout Chow twice weekly. These fish were used in the two behavioral experiments and as urine donors.

Diet Manipulation. Pairs of males were kept in 680-liter (1.2 × 0.9 × 0.6-m) tanks for two to four weeks before behavioral observations began. The fish, matched to a weight difference of 20% or less, had been maintained on Purina Trout Chow for at least one month and were fed every three days before the experiment. Individual fish were identified by unique coloration patterns and fin and barbel morphology.

At the start of the experiment, most pairs of fish had established in the home tank either a territorial or dominance relationship in which the larger fish was dominant and/or held a larger territory. During the establishment of the relationship, the dominant fish frequently displayed at or nipped the subordinate fish. This aggressive behavior was not returned by the subordinate fish which avoided the dominant one. This resulted in restricted access by the subordinate fish to shelters and most areas of the tank.

In this experiment, the diet of the smaller fish was manipulated. After two days of preliminary behavioral observations, the smaller fish was transferred from the home tank to a 38-liter tank (Figure 1). Each fish was fed 2% of its body weight with trout chow. Thirty-six hours later, the smaller fish was returned to the home tank. This manipulation is referred to as the control (C) introduction. Observation of the pair was resumed. Two days later, the smaller fish was again removed and this time fed beef liver (2% body weight) while the resident fish received trout chow. Thirty-six hours later, the smaller fish was returned to the home tank. This is referred to as the experimental (E) introduction. To control for possible serial effects of reintroduction, two, rather than one, control introductions preceded the experimental introduction in seven experiments. Thus, experiments 1 to 6 (Table 1) are C followed by E introductions (C-E), while experiments 7 to 13 are denoted C-C-E.

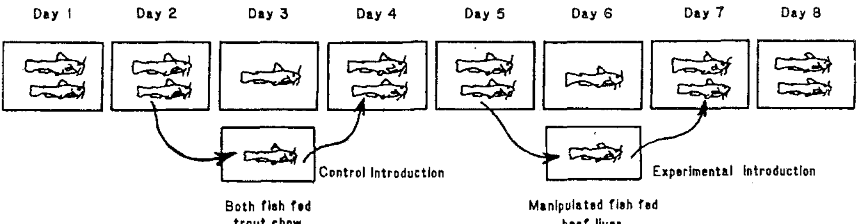

FIG. 1. Schedule of experiment. After two days of observation, the smaller fish was removed to a 38-liter tank and both fish were fed trout chow; 36 hr later the smaller fish was returned to the resident tank. This is a control introduction. Two days later the smaller fish is again removed and fed beef liver; the larger fish received trout chow. Thirty-six hours later, the smaller fish was returned to the resident tank. This is an experimental introduction. Behavioral observations were conducted in both the morning and the afternoon.

Data Collection and Analysis The location and behavior of each fish was recorded verbally on tape during observation periods of 30 min (daily morning and afternoon). Behavioral data consisted of a repertoire of discrete behavioral displays and acts modified after Todd (1968). For behaviors which lasted for more than a few seconds, only the onset was recorded. The location of each behavior was recorded by noting in which of eight areas in the tank it occurred. Activity of each individual was defined as the total number of behavioral acts regardless of type, occurring in an observation period.

Analysis of aggressive behavior and activity was based on frequencies of behavioral acts by the resident (dominant) fish during the introduction periods. Territorial behavior was analyzed by comparing the frequencies of territorial intrusions during the two observation periods preceding and following feeding. Aggressive behavior was considered to have changed between introductions only if there was either a change in the intensity of behaviors observed (e.g., "nips" escalating to "bites") or in the frequency of any aggressive act (chi-square, $P < 0.05$). Similarly, a change in the pattern of territorial behavior was registered if there was a significant change (Friedman Two-way ANOVA, $P < 0.05$) from one introduction to the next, in the frequency of intrusion by the resident fish.

Chemical Analysis of Urine. To demonstrate that the diet manipulation affected the chemical composition of urine, urine from male fish was collected and the free amino acid composition determined. Urine was obtained from chronically placed catheters which emptied into rubber sacs. Up to 15 ml of urine was collected every 24 hr from isolated fish which were fed either trout chow or beef liver. Urine samples were centrifuged at 1000 g for 15 min and stored at $-10°C$. Urinary free amino acids were determined either using a Beckman amino acid analyzer or as N-heptafluorobutryrl isobutyl esters by gas

TABLE 1. DIFFERENCES IN AGGRESSIVE BEHAVIOR, TERRITORIALITY, AND ACTIVITY, COMPARING SUCCESSIVE INTRODUCTION PERIODS[a]

		Control introduction followed by another control (C-C)			Control introduction followed by experimental introduction (C-E)				Summary, significant change in at least one of the measures (+)	
Expt.	Aggressive behavior[b]	Aggressive behavior	Territorial behavior	Activity	Aggressive behavior	Territorial behavior	Activity		C-C	C-E
1	a	ND[c]	ND	ND	+			1	ND	+
2	a	ND	ND	ND	+	+		2	ND	+
3	a	ND	ND	ND	+	+		3	ND	+
4	c	ND	ND	ND			+	4	ND	+
5	c	ND	ND	ND			−	5	ND	+
6	a	ND	ND	ND	+	+	−	6	ND	+
7	c						+	7		+
8	a							8		
9	c						+	9		+
10	c							10		
11	b				+	+		11		+
12	b	+		−			+	12	+	+
13	b	+					+	13	+	+

[a] + = significant increase ($P < 0.05$, chi-square, test); − = significant decrease ($P < 0.05$, chi-square, test). Experiments 1–6 had a C followed by an E introduction. Experiments 7–13 had a C followed by another C followed by an E introduction.
[b] Type of aggressive relationship—highest intensity of behavior observed during any observation period. a = more than two nips or bites per period; b = two nips per period or aggressive displays only; c = no aggressive behavior.
[c] Key: ND = introduction not done.

chromatography (Pearce, 1977). In several cases, the catheter remained patent for eight days, allowing collection or urine samples corresponding to each day of the diet manipulation experiment (Figure 1). These cases included both trout-chow and beef-liver feedings.

Habituation Test. To demonstrate that urine was perceived as different following the dietary manipulation of a urine donor, urine samples taken before and after manipulation were compared using a habituation test. Urine samples were collected during the 24 hr after trout-chow and beef-liver feedings (corresponding to control and experimental introductions) using the method described above. Fifty-microliter aliquots of post-trout-chow urine (i.e., urine corresponding to "day 4" of the behavioral experiment) were introduced into the tanks of individually held male fish every 3–7 mins. This was continued until the swimming response returned to control (tank water) levels. To test the similarity of post-trout-chow urine and post-beef-liver urine, the response to 50 μl of post-beef-liver urine (corresponding to "day 7"), was then measured.

RESULTS

Diet Manipulation Experiment. Of the 13 pairs of fish observed, five pairs displayed intense aggressive behavior (actual biting and damage to one of the fish), five pairs were moderately aggressive (aggressive displays only), and three pairs were nonaggressive (Table 1). Of the 10 pairs displaying aggressive behavior, nine pairs established clear territorial and/or dominance relationships.

Since the particular set of behaviors displayed by each pair of fish varied considerably, direct comparison of specific behavioral acts across all pairs was impossible. However, for each pair, comparisons could be made of the frequencies of specific acts within the general classes of behavior (aggressive, territorial, or activity) occurring during successive introduction periods. These comparisons are shown in Table 1 and selected examples of the time course of several of the behaviors are illustrated in Figure 2. Eleven of the 13 pairs showed significant differences in at least one of the behavioral categories between control and experimental introductions (chi-square tests, $P < 0.05$). Of these, two pairs also showed significant differences between successive control introductions. For five pairs of fish, the intensity of resident aggression increased from post-trout-chow (C) to post-beef-liver (E) periods as indicated by a significant increase ($P < 0.05$, chi-square test) in either frequency of aggressive acts (dislays, nips, or bites) or escalation (displays and nips during the control period being replaced by bites and actual damage during the experimental introduction period). In addition, the activity of both the resident and manipulated fish increased significantly during the experimental period in five pairs. This was accompanied by increased "approach" behavior by the resident (pair 12) or an increase in the behavior "fast swim" for both fish (pairs 4 and 7). Significant

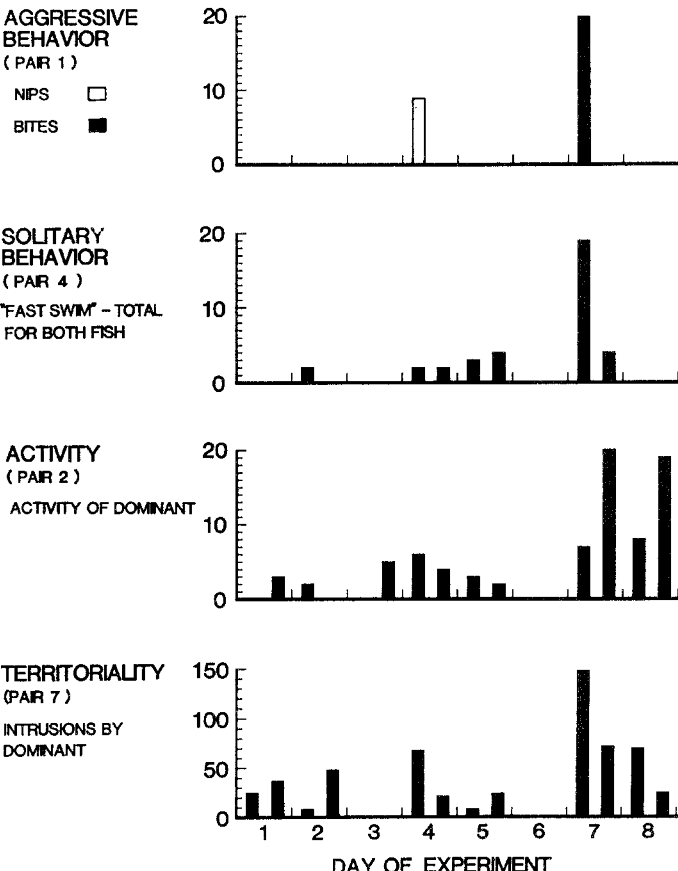

FIG. 2. Frequencies of selected behaviors observed during the course of the experiment. Behavioral observations were conducted in the morning and afternoon. Fish were fed on days 3 and 6. The smaller fish was reintroduced into the resident tank on day 4 following trout-chow feeding (control introduction) and on day 7 following beef-liver feeding (experimental introduction).

decreases in activity of the resident fish occurred in two pairs, one of which (pair 5) was a complete cessation of behavior, both social and solitary, during only the experimental introduction period.

In four instances during or following the experimental introduction period, the manipulated fish lost territory as measured by the number of intrusions, either aggressive or not, by the resident fish. In no cases did the resident fish lose territory or did the size of the smaller fish's territory increase. In those experiments in which a control introduction was followed by another control

introduction, an increase in aggressive behavior during the second introduction occurred twice (pairs 12 and 13).

Other than the loss of territory and changes in activity, no significant changes in the frequency of any specific behaviors of the smaller fish were detected.

To summarize, a comparison of experiments 1 through 6 (C followed by E introductions) with experiments 7 through 13 (C followed by C introductions) (Table 1), indicates that an effect of diet manipulation, regardless of the type of effect observed, is associated with the E introduction (Fisher exact probability test, $P < 0.02$).

Urine Chemistry. Analysis of urinary free amino acids (UFAA) indicated that concentrations of most UFAA increased following feeding. Both beef-liver (Figure 3) and trout-chow meals cause a net increase in amino acid excretion. The relative amino acid composition of urine, when each amino acid was expressed as moles per 100 moles of total FAA, was also affected by feeding. This is summarized in Table 2. Although there appeared to be no diet-related pattern of change which was common to all fish, individual fish did exhibit diet-

TABLE 2. CHANGES IN URINE COMPOSITION FOLLOWING TROUT-CHOW AND BEEF-LIVER MEALS[a]

	Trout chow						Beef liver				
	Expt. P	Expt. Q	Expt. R	Expt. S	Expt. T	Expt. U	Expt. P	Expt. Q	Expt. R	Expt. V	Expt. W
Asp						−					
Thr	−		+		−		+				
Ser		+	+				+				
Glu	−		+				+				
Pro	+							+			
Gly							+			+	+
Ala		+	+		+		+				+
Val		+	+		+	+	+		−		+
Met	−	−			+		−				
Ile	+						+				
Leu	+		+		+	+	+				
Tyr	+	+		+			+				
Phe	+	+			+		+		−	−	−
Lys		+	+	+	−	−	−	−			
Arg				−	+		+			−	−
Hyp					−	−					−

[a] Changes in mole percentage of each amino acid are registered if the change from the prefeeding urine sample to the postfeeding urine sample is greater than a twofold increase or decrease. + or − indicates the sign of the change.

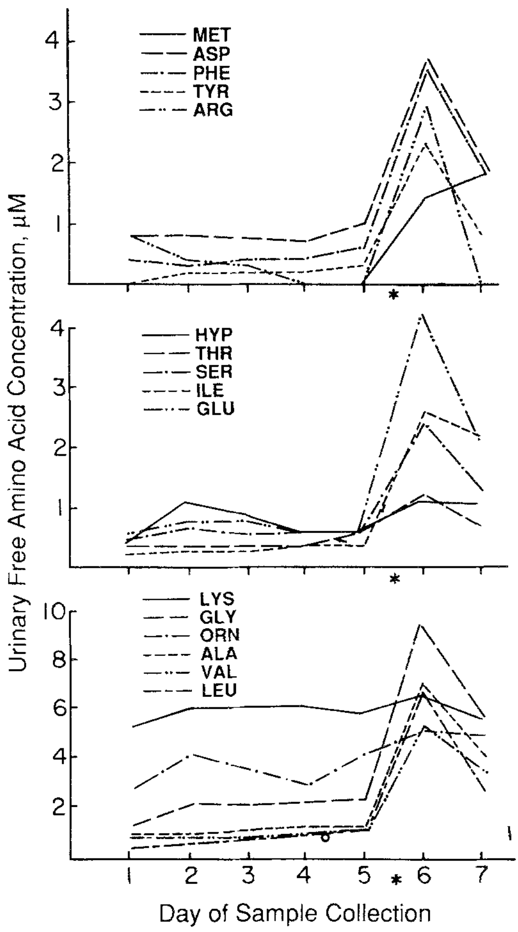

FIG. 3. Effect of feeding on urinary free amino acid (UFAA) concentration. Urine was collected from isolated fish and amino acids determined as described in Methods and Materials. The fish were fed beef liver (2% body weight) after the urine collection on day 5. Asterisk indicates the feeding.

related changes in UFAA. A selected example is shown in Figure 4. Although several amino acids varied randomly (not shown), many showed changes in composition which were directly related to feeding. The mole percentage of several amino acids increased after both types of feedings (isoleucine, serine and histidine), while others (alamine, valine, leucine, and tyrosine increased only after beef liver feedings. Because dietary amino acids affect plasma amino acid levels in carp (Kaushik and Luquet, 1979) and trout (Nose, 1972), the difference in amino acid composition between trout chow (higher L-glutamine

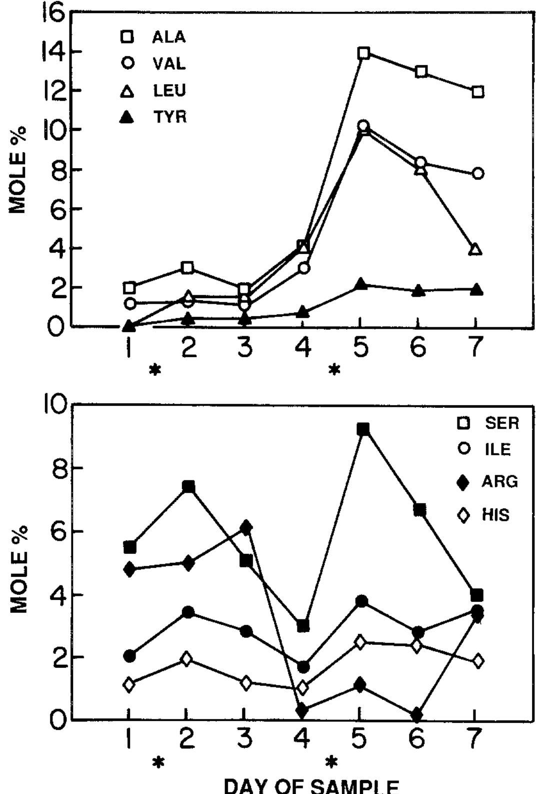

FIG. 4. Effect of trout chow and beef liver meals on urine composition. Urine was collected from isolated fish and amino acids determined as described in Methods and Materials. The fish were fed trout chow (2% body weight) on day 1 following urine collection and beef liver (2% body weight) on day 4 following urine collection. Asterisks indicate meals.

+ L-glutamatic acid and L-proline) and beef liver (higher L-methionine and L-aspartic acid) (Table 3) might be expected to be directly reflected in the UFAA composition following feeding. However, these amino acids did not increase differentially following each type of feeding.

Comparison of Urines by Habituation Test. Individual fish responded to the introduction of post-trout-chow urine by moving from a resting position and swimming around in the tank. Depending on the individual, between 14 to 33 introductions of post-trout-chow urine were required to habituate the swimming response. During the last 13 introductions of post-trout-chow urine, the mean duration ($\pm$SE) of the swimming response decreased from 69 ($\pm$16) sec to 5 ($\pm$4) sec (Figure 5). The subsequent introduction of post-beef-liver urine caused

TABLE 3. AMINO ACID COMPOSITION OF TROUT CHOW AND BEEF LIVER[a]

	Moles per 100 moles FAA	
	Trout chow	Beef liver
Asp	5.74	9.24*
Thr	4.61	4.72
Ser	7.75	6.11
Glu	20.35*	12.75
Pro	8.75	6.98
Gly	10.01	11.68
Ala	7.20	8.80
Val	3.89	5.22
Met	0.00	1.18*
Ile	3.31	3.47
Leu	9.59	10.24
Tyr	2.43	2.31
Phe	3.14	3.91
Lys	10.26	10.32
Arg	1.63	1.76
Hyp	1.34*	.34

[a] Samples of food were hydrolyzed in 6 N HCl, 110°C, 18 hr, and amino acids were determined as described in Methods and Materials. The values represent the mean of two determinations. Asterisks indicate amino acids in which there is at least a 50% difference between the two foods.

a significant increase ($P < 0.05$) in the mean duration ($\pm$SE) of the swimming response to 75 $\pm$ 16 sec.

DISCUSSION

This study demonstrates that a change in the diet of one of a pair of socially interacting catfish results in changes in the behavior of the other fish. Because these social interactions are sensitive to a relatively minor manipulation, such as a change in diet, these results provide support for the hypothesis that the stimuli used during dominance and territorial behavior are not specialized pheromones.

The social relationships which develop within different pairs of fish are not identical. Therefore, responses to experimental manipulation can take different forms. Indeed, the particular type of change in behavior that occurred varied from pair to pair but can be categorized as being either increased aggression on the part of the resident fish or else a change in activity, possibly searching behavior, of the resident fish. The aggression observed during the control introductions shows that a one-day separation (Figure 2) causes an increase in

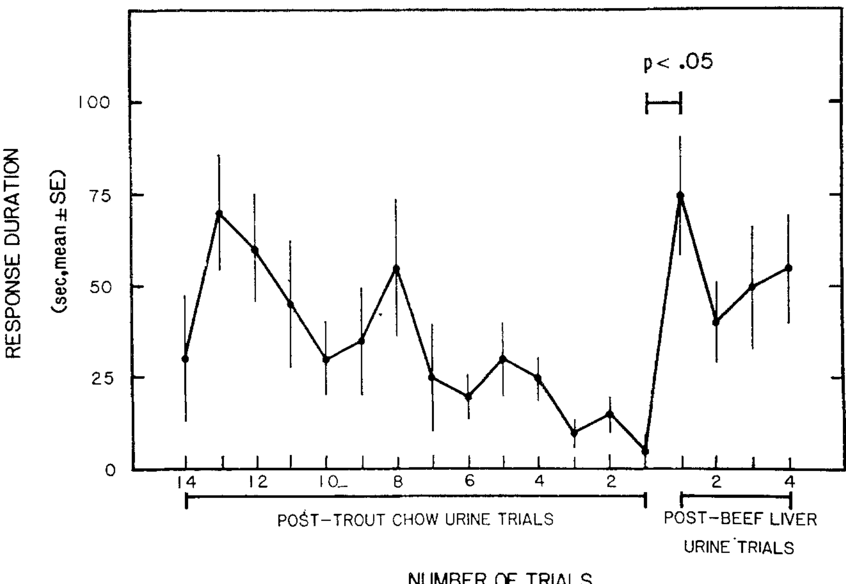

FIG. 5. Behavioral comparison of urines collected before and after diet manipulation. Individual fish were tested for their response to repeated introductions into their tanks of conspecific urine collected after donors had fed on Purina Trout Chow. After the responses had waned to that of control introduction (tank water), aliquots of urine collected after a beef-liver feeding were introduced and the response observed.

activity or aggressive behavior in some resident fish. A similar effect of separation has been demonstrated in such territorial and visually dominated fish as sticklebacks (Iersel, 1958) and cichlids (Peeke et al., 1971) and is interpreted to be the loss of recognition of previously familiar territory holders. In catfish, such territorial responses are transient and differ from those observed when the effect of separation is compounded with diet manipulation. The more intense territorial response following diet manipulation suggests that some of the cues serving territorial recognition have changed and that the manipulated fish is perhaps no longer recognized as the same fish.

Urine stimuli taken before and after diet manipulation were shown to be different using both chemical analysis and behavioral assay. Consequently, the simplest hypothesis is that diet manipulation changes the chemical output of the manipulated fish and thus its chemical identity. This change in identity disrupts a previously stable dominance or territorial relationship which is normally maintained by continuing chemosensory familiarity. Because the observed effects on behavior were specific to the beef-liver feeding, the fish may not have been responding only to quanititative changes in chemical output associated with

feeding on any diet, such as the observed increase in amino acid excretion. Since the fish were maintained on trout chow, they had already experienced the type of change in chemical output associated with trout-chow feeding and presumably had habituated to such changes. Instead, the fish may be responding either to the presence of specific compounds in the new body odor or to relative changes in an overall body odor complex caused by the beef-liver feeding.

A more complex hypothesis is that behavioral rather than chemical cues from the fish whose diet was manipulated caused the increase in resident aggression or activity. A change in diet might affect the behavior of the subordinate either directly, by affecting neurotransmitter levels (Lajtha et al., 1963), or indirectly, through a perceived difference in its own odor. While it is not possible to rule out the possibility of behavioral cues, there are some data which favor the simpler hypothesis: behavioral acts (territorial intrusions and sudden flight movements) by the subordinate which are known to induce attack by a dominant (Todd, 1968; Atema and Labeyrie, unpublished) occurred infrequently and did not increase after the diet manipulation.

Amino acids are good candidates as nonspecific social signals in catfish. Importantly, they are present in urine at concentrations that are suprathreshold using both behavioral (Atema, 1977; Holland and Teeter, 1981; Little, 1977) and electrophysiological (Caprio, 1975, 1977) criteria. Because there were changes in amino acid composition which are specific to beef-liver feeding, it is possible that specific amino acids contribute to the perceived chemical difference following diet manipulation. The possibility remains, however, that other compounds specific to beef liver were responsible for both the chemical and the perceived differences in body odor and the concomitant changes in behavior.

As in other vertebrates, fish territorial behavior is based on recognition of neighboring territory holders (Keenleyside, 1979). Such recognition allows for the discrimination between familiar neighbors and strangers, thereby reducing aggression between neighboring territory holders. Strange catfish are attacked by resident dominant fish in aquaria (Todd, 1968) and by nest holders in the field (Blumer, 1982). In these cases, the fish are discriminating resident from nonresident and mate from all other conspecifics, respectively. The results of the present study, together with these examples, are also consistent with the hypothesis that the fish are responding to chemically unfamiliar cues rather than specific aggressive stimuli. Social recognition in catfish may thus be based in large part on chemosensory habituation to tank- or nestmates. As is observed in visually mediated aggressive responses in sunfish (Colgan et al., 1977), aggression or searching behaviors in catfish may result when other, chemically different conspecific stimuli appear. Similarly, the unconditioned preference of a fish for its own odor (Richards, 1976) may result from habituation to one's own odor along with avoidance of strange conspecific odor.

Experimental changes in diet have been demonstrated to have an effect on social investigating behavior (Beauchamp, 1976) and on material odor imprinting (Galef, 1981; Porter, 1977; Leon, 1975). Although it is doubtful that natural changes in maternal diet would perturb maternal recognition in any animal, daily variation in diet may make individual recognition based on diet impossible except for those species that are specialist feeders. Chemosensory recognition of traits other than individuality may be influenced less by such daily variation. It is well known, for example, that catfish of different sizes eat different diets (Raney and Webster, 1940; Moore, 1972; Imamura, 1975; Keast, 1985). Because of this and size-related differences in metabolism (Gerking, 1955), size-related odors may be produced that are based in part on diet. In catfish aggressive encounters, size is an important determinant of the outcome (Bryant and Atema, unpublished). While visual and mechanical stimuli are more likely to be used by fish to determine size at close range (Partridge and Pitcher, 1980; Dijkgraaf, 1963), odor could be a useful indicator of size at greater distances, particularly in turbid water. Thus, size-related odors may be used during the establishment and maintenance of territories.

These experiments demonstrate that general body odor, as well as specific pheromone signals, must be considered important in the regulation of social behavior. Indeed, it is possible, perhaps likely, that many so-called pheromones in vertebrates will turn out to be rather nonspecific metabolites, exerting their influence by virtue of chemical habituation and familiarity.

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PLANT-DERIVED SYNERGISTS OF ALARM PHEROMONE FROM TURNIP APHID, *Lipaphis (Hyadaphis) erysimi* (HOMOPTERA, APHIDIDAE)

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Abstract—The turnip aphid, *Lipaphis (Hyadaphis) erysimi*, responds weakly to (E)-β-farnesene, the main component of the alarm pheromone, but the response is substantially increased by incorporating plant-derived isothiocyanates, identified in aphid volatiles by coupled gas chromatography–single-cell recording.

Key Words—Aphid, pheromone, alarm pheromone, plant components, electrophysiology, single-cell recording, isothiocyanate, *Lipaphis erysimi*, *Hyadaphis erysimi*, Homoptera, Aphididae, (E)-β-farnesene.

INTRODUCTION

The turnip aphid, *Lipaphis (Hyadaphis) erysimi*, has been reported to respond well to its own alarm pheromone but not to the pheromones from a series of other aphids, even though these responded well to pheromones from each other (Nault and Bowers, 1974). Pheromone from these aphids, including *L. erysimi*, contained the sesquiterpene (E)-β-farnesene (I, Figure 1). This compound elicited a high response for each aphid except *L. erysimi*. It was proposed that the alarm pheromone from *L. erysimi* contained other components, and the presence of another sesquiterpene was suggested (Nault and Montgomery, 1979; Nault and Phelan, 1984). The objective of the present study was to identify the compounds necessary for full alarm response by *L. erysimi*.

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FIG. 1. Structures of (*E*)-*β*-farnesene (EBF) (I), 4-pentenyl cyanide (II), allyl isothiocyanate (III), 2-butyl isothiocyanate (IV), 3-butenyl isothiocyanate (V), 4-pentenyl isothiocyanate (VI), and 2-phenylethyl isothiocyanate (VII).

METHODS AND MATERIALS

Compounds. Farnesene, prepared by the method of Dawson et al. (1982) containing 40% of the active isomer, (*E*)-*β*-farnesene (EBF), was employed at a concentration of 4 μg/ml EBF in ether, or 1 μg/ml in water, and stored in glass ampoules under N_2. 4-Pentenyl cyanide (II), allyl isothiocyanate (III), 2-butyl isothiocyanate (IV), 3-butenyl isothiocyanate (V), 4-pentenyl isothiocyanate (VI), and 2-phenylethyl isothiocyanate (VII) were obtained commercially or synthesized by conventional methods. When tested in behavioral bioassays, the concentrations of these compounds were 0.2 μg/ml in ether or 1 mg/ml in water.

Insects. Lipaphis (Hyadaphis) erysimi (Kaltenbach), obtained on shepherd's purse [*Capsella bursa-pastoris* (L.) Medic.] in Hertfordshire in 1981, were maintained on turnip (*Brassica campestris* var. *rapifera* Metz.) unless otherwise stated, at 16 hr daylength and 20 ± 5°C.

Isolation of Volatiles. L. erysimi were extracted with ether and the extract dried and concentrated down from a fivefold dilution to give a solution equivalent to 1 g aphids/ml ethereal extract. The extract was then vacuum distilled as described previously (Pickett and Griffiths, 1980).

Aerial parts of turnip, Chinese cabbage [*Brassica campestris* var. *chinensis* (L.) Makino], and shepherd's purse, grown under glass, were cooled under N_2, broken up finely, covered, and allowed to stand for 85 min so that conversion of glucosinolates to volatile metabolites could take place. The volatiles were then isolated by condensation as described previously (Pickett and Stephenson, 1980) and the condensate extracted into ether to give a concentration equivalent to 20 g of plant material/ml ethereal extract. Solutions were sealed in glass ampoules under N_2 and stored at −20°C.

Bioassays. Colonies of third- to fourth-instar *L. erysimi* were established on leaves of Chinese cabbage by confining adults in clip cages on the plants for

three days. The adults were then removed and the young allowed to develop for a further two to three days before testing. Single aphids were reared in a similar manner, excess nymphs being removed at the same time as the adults. Numbers of aphids responding by moving from their feeding sites were recorded 60 sec after application of the test material (colonies of 50–60 insects, five replicates; single aphids, ten replicates).

Test materials were either air (20 ml) from above *L. erysimi* crushed in a glass syringe (20 aphids containing a total amount of ca. 1 ng EBF by GC), blown slowly (ca. 10 sec) from the syringe, or ethereal or aqueous solutions (0.2 μl) applied as a single droplet to the leaf on which the aphids were settled. Thus, EBF was applied at 0.8 ng in ether or 0.2 ng in water and the isothiocyanates at 0.04 ng in ether or 0.2 μg in water. Ethereal *L. erysimi* extract and volatiles (1 μl) were also applied as single droplets to leaves at ca 0.3 ng of EBF.

Gas Chromatography (GC). GC employed fused SiO_2 columns, 24 m $\times$ 0.3 mm, with a bonded OV-101 stationary phase at 30°C (2 min), 20°/min to 100°C, 6°/min to 200°C. Confirmation of identity was by coinjection with authentic compounds.

Gas Chromatography-Mass Spectrometry (GC-MS). Ionization was by electron impact at 70 eV, 200°C, with the GC capillary column directly coupled to the source of an MM 70-70F mass spectrometer and the integrated data system 2025 (VG Analytical, Altringcham, U.K.). Spectral enhancement by means of the data system was used to produce mass spectra for regions of the chromatogram associated with electrophysiological activity but where peaks in the total ion current were not clearly discernible. Tentative identification was by comparison with published spectra (Kjaer et al., 1963).

Electrophysiology. Recordings from the cells associated with the olfactory receptors on the primary rhinaria of *L. erysimi* alates were made using tungsten microelectrodes (Boeckh, 1962). The indifferent electrode was placed in the first antennal segment, and the recording electrode was then brought into contact with the multiporous plate of the rhinaria until impulses were recorded. Permanent copies of the action potentials generated by the receptor cells were obtained by standard methods (Wadhams et al., 1982).

Most recordings showed the presence of a number of cells since there are up to 14 olfactory cells in the rhinarium (Bromley et al., 1979). Only those recordings in which the responding cells could be readily distinguished were used in these experiments.

Stimulation. The stimulus (2 sec duration) was delivered into a purified airstream (800 ml/min) which flowed continuously over the preparation. The delivery system, employing a filter paper strip in a disposable Pasteur pipet cartridge, has been described previously (Wadhams et al., 1982). Compounds III–VI in pentane (10 μl) were applied to the filter paper and, after evaporation of solvent, were presented twice to each preparation at intervals of 2–15 min;

the exact interval was dependent upon the concentration of the previous stimulus. Impulse frequency was determined as the number of impulses elicited during the first 1 sec after stimulus initiation.

Gas Chromatography-Single-Cell Recording (GC-SCR). The coupled GC-SCR system has been described previously (Wadhams, 1982). After each experiment, records of the flame ionization detector response and of the action potential frequency were obtained by detecting the impulses with a level discriminator and plotting them by means of a voltage/frequency converter.

RESULTS AND DISCUSSION

Alarm pheromone released by crushing turnip aphids, *L. erysimi*, caused most aphids to move away from feeding sites (Table 1). The presence of EBF was confirmed by GC and GC-MS studies, but no other major terpenoid component was detected. However, synthetic EBF elicited only a weak response at a level comparable to the amount produced by the aphid. Ethereal extract of *L. erysimi* gave rise to a good response, and the activity was found to be largely in the volatile fraction obtained by vacuum distillation.

The alarm pheromone is thought to be perceived principally by the rhinaria on the fifth (proximal) and sixth (distal) antennal segments (Nault et al., 1973; Wohlers and Tjallingii, 1983), and Bromley and Anderson (1982) implicated the former in host-plant volatile reception. Although olfactory cells which responded strongly to EBF were found on the distal rhinarium, it was not possible to elicit a similar response from the receptors associated with the proximal rhinarium.

Cells on the proximal rhinarium responded strongly to *L. erysimi* volatiles. Coupled GC-SCR of the *L. erysimi* distillate revealed the presence of a number of active components which, with the exception of VIII, were perceived by one cell type A in this rhinarium (Figure 2). These compounds were identified by

TABLE 1. ALARM BIOASSAY WITH COLONIES OF TURNIP APHIDS, *Lipaphis (Hyadaphis) erysimi*

Treatment[a]	Aphids moving (% ± SE)
1. Crushed *L. erysimi*	99 ± 0.9
2. *L. erysimi* extract	90 ± 3.2
3. *L. erysimi* volatiles	74 ± 5.5
4. *L. erysimi* residue	23 ± 10.5
5. EBF	20 ± 8.4

[a]Treatments 4 and 5 differ from 1-3 at $P < 0.01$, based on paired t tests.

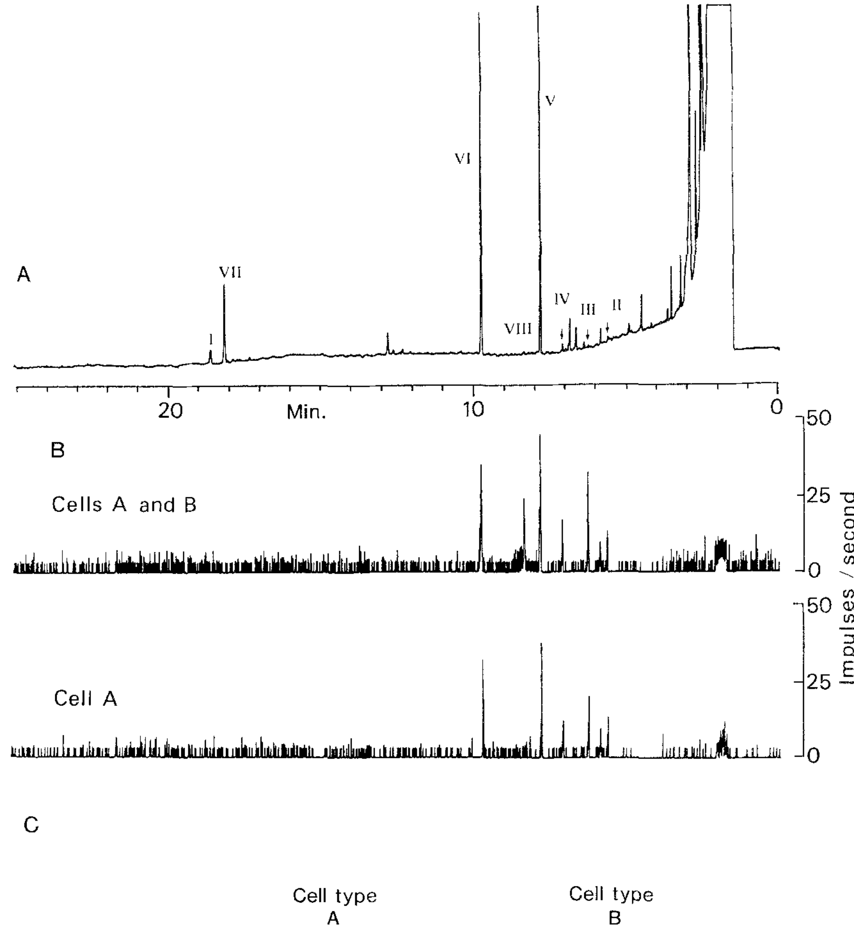

FIG. 2. GC-SCR: (A) Gas chromatogram of the vacuum distillate of *L. erysimi* extract. (B) Corresponding impulse frequency responses of two olfactory cells to stimulation with the *L. erysimi* extract. (C) Typical spontaneous activity of the cells recorded from the primary rhinarium on the fifth antennal segment.

GC and GC-MS as 4-pentenyl cyanide (II), allyl isothiocyanate (III), 2-butyl isothiocyanate (IV), 3-butenyl isothiocyanate (V), and 4-pentenyl isothiocyanate (VI) (Figure 1). 2-Phenylethyl isothiocyanate (VII), a major component of the extract, was inactive, and peak VIII, which was detected by cell type B, was not identified. Isothiocyanates are well-known components of cruciferous plants, including the turnip; they arise from enzymic decomposition of glucosinolate precursors (Ju et al., 1982) and were therefore considered likely to have arisen from the host plant.

To investigate further the activity of the newly identified compounds, the bioassay was modified to employ single aphids, thus avoiding release of isothiocyanates through leaf damage caused by large numbers of feeding aphids. Results (Table 2) again showed that the pheromone and the volatiles isolated from *L. erysimi* were more active than synthetic EBF alone. It was also demonstrated that the pheromone from *L. erysimi* bred on Chinese cabbage was equally active. 3-Butenyl isothiocyanate alone elicited only a weak response, but when it was applied together with EBF, all the test aphids responded. Allyl isothiocyanate and 2-butyl isothiocyanate gave a similar increase in the alarm response when applied in admixture with EBF. Dose–response data for these compounds were established using the electrophysiological bioassay and showed that, at the receptor level, allyl and 3-butenyl isothiocyanates were significantly more active than the 2-butyl and 4-pentenyl isothiocyanates (Figure 3). The

TABLE 2. ALARM BIOASSAY WITH SINGLE TURNIP APHIDS, *Lipaphis (Hyadaphis) erysimi*

Treatment	Aphids moving (%)[a]
Crushed *L. erysimi* from turnip	100a
Crushed *L. erysimi* from Chinese cabbage	100a
L. erysimi volatiles	100a
EBF	20b
3-Butenyl isothiocyanate	20b
EBF + 3-butenyl isothiocyanate	90a
Allyl isothiocyanate	0b
EBF + allyl isothiocyanate	100a
EBF + 2-butyl isothiocyanate	90a
Turnip volatiles	20b
EBF + turnip volatiles	100a
EBF + shepherd's purse volatiles	100a
EBF + Chinese cabbage volatiles	90a
EBF (aqueous)	0b
EBF + allyl isothiocyanate (aqueous)	70a

[a]Difference a from b, $P < 0.05$, based on chi-square test.

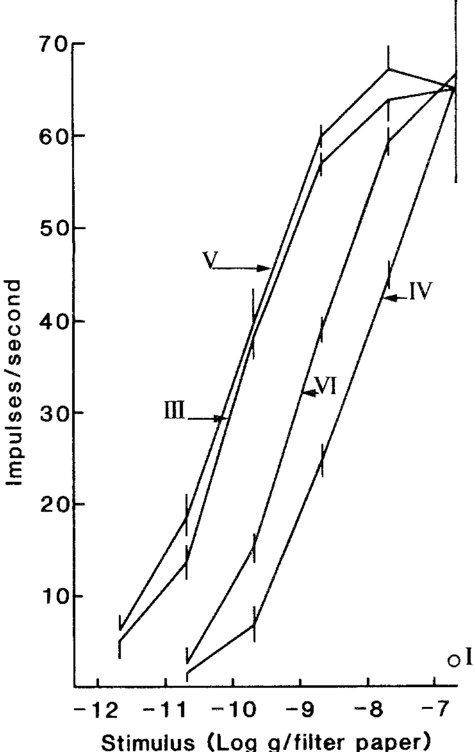

FIG. 3. Dose–response curves of *L. erysimi* olfactory cells to (*E*)-β-farnesene (I), allyl isothiocyanate (III), 2-butyl isothiocyanate (IV), 3-butenyl isothiocyanate (V), and 4-pentenyl isothiocyanate (VI). The cells were recorded from the primary rhinarium on the fifth antennal segment. Each point is the mean response (five preparations) ± SE. Where standard errors overlap only half the SE bar is shown.

olfactory receptor exhibits considerable selectivity towards these compounds: no response was observed to either EBF or the common leaf volatiles (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, and linalol when tested at high stimulus concentrations (2×10^{-7} g per filter paper).

Although the volatiles obtained from turnip, Chinese cabbage, and the weed host of *L. erysimi* in the U.K., shepherd's purse, increased activity of EBF (Table 2), only the turnip volatiles contained relatively large amounts of 3-butenyl and 4-pentenyl isothiocyanates. However, the relative proportions of these compounds obtained from *L. erysimi* bred on Chinese cabbage were similar to those from *L. erysimi* bred on turnip. This suggested that *L. erysimi* plays an active role in the production of these compounds. Indeed, when sinigrin, the glucosinolate precursor of allyl isothiocyanate, was added to an homogenate of

L. erysimi, allyl isothiocyanate was released at 37% of the theoretical amount after 5 hr. It is interesting to note that while the glucosinolate, sinigrin, serves as a feeding deterrent for non-Cruciferae feeding aphids, it serves as a powerful phagostimulant for *L. erysimi* (Nault and Styer, 1972). Thus, the glucosinolates or their isothiocyanate products play a dual key role in the biology of this species.

When sinigrin was added to an homogenate of *Myzus persicae*, a closely related aphid also in the Aphididae, release of allyl isothiocyanate was negligible. Also, the isothiocyanates III, IV, and V did not significantly increase the response of this aphid to the alarm pheromone.

EBF can be used to improve the efficiency of contact pesticides and biological control agents against aphids (Pickett et al., 1986), but the response of aphids to aqueous formulations of EBF is very weak. Indeed, only special formulations containing hydrocarbon propellents, applied using electrostatic spraying systems, have so far given good results (Pickett et al., 1984). However, the readily available allyl isothiocyanate, when applied together with EBF in water, causes a good response from *L. erysimi* (Table 2). Further studies on such alarm pheromone synergists may lead to the use of aqueous formulations of EBF against other aphids in the field.

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TOWARDS CHEMICAL CHARACTERIZATION OF WATERBORNE PHEROMONE OF AMPHIPOD CRUSTACEAN *Microdeutopus gryllotalpa*

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Abstract—Previous studies demonstrated the existence of a waterborne pheromone secreted by receptive females of the amphipod crustacean *Microdeutopus gryllotalpa* which attracts males. The data were obtained by using a bioassay apparatus based on a two-choice test paradigm. The present study reports the results of additional tests employing this apparatus which have shed some light on the chemical nature of the pheromone. The bioactive substance was isolated from receptive female waters with anion exchange resin columns, but not with C-18 reverse-phase columns. This suggests that the substance is polar. Another finding of the present study was that effluents from the green alga *Ulva lactuca* inhibit males' responses to the pheromone.

Key Words—Pheromone, crustacean, amphipod, *Microdeutopus gryllotalpa*.

INTRODUCTION

The pheromones of numerous terrestrial arthropods have been chemically characterized (reviewed by Blomquist and Dillwith, 1983). However, although recent studies have demonstrated the existence of waterborne sex pheromones in many Crustacea (summarized by Dunham, 1986), the nature of crustacean pheromones has not been demonstrated for any species.

Until recently, the existence of pheromones in amphipod crustaceans was

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disputed. For example, Dahl et al. (1970 a, b) reported that pheromones are produced by receptive female *Gammarus duebenii*, but Hartnoll and Smith (1980) could not confirm the former workers' behavioral observations. Dunham (1978) suggested that some confusion in the literature might be due to different workers' definitions of sex pheromones. He suggested that waterborne secretions should be considered sex pheromones if they serve one or more of the following distinct functions: (1) intersexual attraction; (2) stimuli for courtship; and/or (3) stimuli for mating.

With this in mind, the effects of receptive female secretions on male conspecifics' behavior was tested in two other amphipods (*Microdeutopus gryllotalpa* and *Gammarus palustris*, Borowsky, 1984, 1985, respectively), and each potential function of a pheromone was considered separately. It was learned that (1) pheromones do exist in the two species and (2) they attract males. However, subsequent work (Borowsky and Borowsky, 1985, and in preparation) showed that waterborne pheromones do not influence courtship or mating behavior after males have located females.

The effects of receptive female secretions as male attractants were demonstrated using Y-tube behavioral assay systems modified to mimic each species' habits. It was reasoned that the systems could facilitate the isolation and ultimate identification of the active substance(s). The present report presents the results of some experiments which employed the Y-tube apparatus to shed light on the general nature of the pheromone of *Microdeutopus gryllotalpa*.

METHODS AND MATERIALS

All animals were taken from the filter beds of the cold-water tanks of the New York Aquarium. They were supplied with *Ulva lactuca* thalli ad libitum for food and tube-building materials, and maintained in seawater of 29 ppt Cl^- taken from wells that supply the aquarium. All animals were maintained at 20°C. Females were maintained in individual 10-cm-diameter glass culture dishes and examined daily to determine the day they molted. Females six days past their molt were considered "receptive," and females two days past their molt were considered "nonreceptive" on the basis of previous studies (Borowsky, 1980). Males were isolated from females, and maintained communally in 20-cm-diameter glass culture dishes for one week until they were tested in the bioassay apparatus.

The bioassay apparatus is designed to mimic field conditions while testing animals' responses to waterborne substances in a standard two-choice paradigm (see Fig. 1). The apparatus is based on a glass Y-tube (Y). The base of the Y is connected to a 1-liter aspirator bottle (the departure bottle (DB) by Tygon tubing (7 mm ID), while each of the arms is similarly connected to two 2-liter aspirator bottles in series. The distal bottles (DAB) are elevated about 1 m

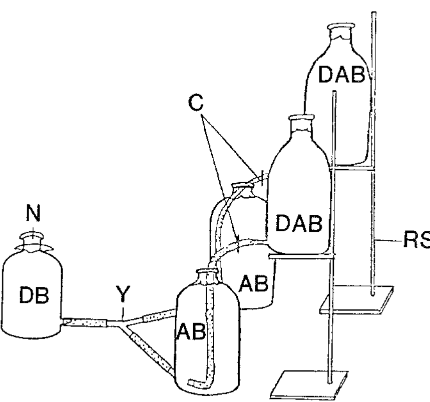

FIG. 1. Apparatus employed for testing *Microdeutopus gryllotalpa* males' responses to receptive females' secretions after the secretions were passed through columns containing different types of absorbents. Receptive females were placed in one of the distal aspirator bottles (DAB), and nonreceptive females in the other. Males were placed in the departure bottle (DB). Water was introduced into the distal aspirator bottles, passed through the absorbent columns (C), then entered the arrival bottles (AB), and finally entered the departure bottle. Males could leave the departure bottle, travel to the Y tube (Y), then pass freely to either arrival bottle. Censuses of the number of males in the arrival bottles and departure bottles at the end of each test indicated whether males responded to the receptive females' secretions after they were exposed to the absorbants. All Tygon tubing is stippled; N = nylon mesh netting, to prevent washing males out of the departure bottle RS = ring stand, to elevate distal aspirator bottles and establish a head of water to force secretions through the column. *C* = site of insertion of clamps (Experiments I and II) or absorbent columns (Experiments III and IV).

above the proximal, or arrival bottles (AB) on ring stands (RS). This establishes a pressure sufficient to force water through the columns containing the absorbents (placed at points C).

Thirty receptive females were placed in one of the distal aspirator bottles, and 30 nonreceptive females were placed in the other one. Fresh sea-water dripped into the tops of these bottles at the rate of 100 ml/hr each and continued out at the same rate through their spouts. Thus, water flowing out of these bottles had bathed the females. The water then passed through two 1-m lengths of tubing, which led into the spouts of the arrival bottles. The water flowed out of the arrival bottles' spouts through 8-cm lengths of tubing and continued into the arms of the Y-tube. A single 13-cm length of tubing carried the water from the base of the Y to the spout of the departure bottle. The water exited the system through the top of the departure bottle. To prevent individuals from

passing between the distal and arrival bottles, the ends of the connecting tubes were covered with nylon mesh netting, which did not impede the flow of water.

At the beginning of each test, the water was turned off, and the current halted. Then 15 males were placed in the departure bottle, and its mouth was capped with nylon mesh netting (N) to prevent males from leaving the apparatus. Each test was begun by starting the flow of water through the system. During a test, males could pass freely among the two arrival bottles and the departure bottle. Each test lasted 1 h. At the end of each test, the number of males left in the departure bottle as well as the number in each arrival bottle was counted. Four tests were conducted for each experiment. For each experiment receptive females were placed in the right distal aspirator bottle and nonreceptive females in the left one for two tests, and the females' sides were switched for the other two tests. Hypotheses were tested using χ^2 and binomial statistics. The overall significances of the experiments (each consisting of four tests) were obtained by combining probabilities from separate replicates using the method of Fisher (1946). Individual probabilities for each replicate were obtained from the binomial distribution (one-tailed test) and -2 times the sum of their natural logarithms was taken as χ^2 with eight degrees of freedom (referred to in the text as the aggregate χ^2).

The objective of the experiments was to learn whether the removal of polar or nonpolar substances from females' water by isolation techniques would eliminate the males' responses to the waters. It was reasoned that if the pheromone was a lipid, for example, then receptive female waters passed through a lipid adsorbent would remove bioactivity from the waters, and males would not be attracted to it.

Four experiments were conducted. In experiment I, *Ulva lactuca* thalli were provided as substrate for females in the distal bottles. In experiment II, conditions were identical to the first, but beach sand, boiled in distilled water and then dried, rather than *U. lactuca*, was used as a substrate. In experiment III, C-18 reverse-phase columns (Sep-Paks, Waters Associates, Milford, MA) were introduced into the tubes leading from the distal to the arrival bottles. (The columns were inserted at C in Figure 1; in experiments I and II clamps were inserted at these sites to reduce the flow to the rate of experiments III and IV). Experiment IV was identical to the third, except that columns containing anion exchange resin, rather than Sep-Paks were introduced into the tubes. The resin was a strongly basic quaternary ammonium (polystyrene type $RN(CH_3)_3^+Cl^-$; Mallinckrodt Chemical Works, Amberline CG-400, 100–200 mesh, chromatographic grade).

RESULTS

There was no significant difference between males' responses to receptive and nonreceptive females when the females were maintained on *U. lactuca* (Ta-

TABLE 1. NUMBERS OF MALES FOUND IN DIFFERENT BOTTLES AFTER EACH ONE-HOUR TEST IN BIOASSAY APPARATUS[a]

	Type of bottle		
	With receptive female secretions	With nonreceptive female secretions	Departure
Experiment I:	*Ulva* substrate, no columns		
Test 1	3	1	11
Test 2	5	1	9
Test 3	3	0	11
Test 4	5	2	8
$\bar{X} \pm$ SD	4 ± 1.2	1 ± 0.8	10 ± 1.5
Experiment II:	sand substrate, no columns		
Test 1	12	0	3
Test 2	7	0	8
Test 3	7	3	5
Test 4	11	0	4
$\bar{X} \pm$ SD	9 ± 2.6	1 ± 1.5	5 ± 2.1
Experiment III:	sand substrate, C_{18} reverse-phase columns inserted in tubes		
Test 1	7	1	7
Test 2	10	4	1
Test 3	10	1	3
Test 4	8	0	6
$\bar{X} \pm$ SD	9 ± 1.5	2 ± 1.7	4 ± 2.7
Experiment IV:	sand substrate, anion exchange columns inserted in tubes		
Test 1	0	4	11
Test 2	1	0	14
Test 3	4	1	10
Test 4	0	1	14
$\bar{X} \pm$ SD	1 ± 1.9	2 ± 1.7	12 ± 2.0

[a] $\bar{X}$ = mean, SD = standard deviation.

ble 1, Exp. I: aggregate $\chi^2_8 = 7.40$, $P > 0.05$). However, in the second experiment, which substituted sand for the *U. lactuca*, males traveled significantly more often to receptive than to nonreceptive females' waters (Table 1, Exp. II: aggregate $\chi^2_8 = 40.82$, $P < 0.001$). In addition, fewer males left their departure bottles in the first than in the second experiment (Mann-Whitney U test; U = 0.5, $P < 0.05$, two-tailed). This suggests that the presence of *U. lactuca* inhibits the males' response to female secretions.

The insertion of the C-18 reverse-phase columns in the flow of water from the females did not interfere with the attractiveness of the receptive females' secretions to males. Males traveled to receptive females' sides significantly more often than to nonreceptive females' sides (Table 1, Exp. III: aggregate χ^2_8 =

31.99, $P < 0.001$). This suggests that the pheromone was not removed from the water by this absorbent. In contrast, when the anion exchange columns were inserted, only a few males left their departure bottles, and those that did showed no preference for either type of female secretion (Table 1, Exp. IV: aggregate $\chi^2_8 = 3.34$, $P < 0.05$). Thus, the bioactive substance was isolated from the females' waters by the resin. This suggests that the pheromone is a polar substance.

DISCUSSION

Female waters exposed to anion exchange resins lost their ability to attract males, but female waters exposed to C-18 reverse-phase columns did not. These observations indicate that the anion exchange columns isolate the bioactive substances from female waters, and thus the pheromone is polar. The substance may be an amino acid or derivative or a charged organic conjugate.

These results are consistent with the results of most other investigations of the chemistry of crustacean pheromones. The pheromones of the crab *Portunus sanguinolentus* and the crayfish *Procambarus clarkii* appear to be polar (Cristofferson, 1970, and Ameyaw-Akumfi and Hazlett, 1975, respectively). In addition, although Kittridge et al. (1971) hypothesized that crustecdysterone is the pheromone of most Crustacea, three species failed to respond to this substance or its metabolites: the lobster *Homarus americanus* (Atema and Gagosian, 1973; Gagosian and Atema, 1973), the crab *Carcinus maenas* (Seifert, 1982), and the crab *Callinectes sapidus* (Gleeson et al., 1984). The idea that the pheromone is an amino acid or derivative is not unreasonable, because some species respond to water-borne amino acids (summarized in Ache, 1982).

The data suggest that *Ulva lactuca* placed in the distal bottles with the females inhibits the males' responses to receptive female secretions. Males were attracted to receptive females' secretions significantly more often than to nonreceptive females' secretions when the substrate was sand, but not when the substrate was *U. lactuca*. In a previous study, under conditions similar to experiment I, males did respond to receptive female secretions (Borowsky, 1984). However, the previous tests were conducted for 5.5 h, as compared to the 1-h tests conducted for the present study. This suggests that the presence of *U. lactuca* lengthens the males' response time, but does not eliminate their ability to distinguish between receptive and nonreceptive females.

There are two ways to explain these observations. First, it is possible that females secrete more pheromone on a sand than on an *U. lactuca* substrate. Second, it is possible that *Ulva* effluents interfere with the males' perception of the pheromones (perhaps competing for receptor sites on sense organs), or even that the effluents repel males. Some support for the latter explanation comes from the recent observations of Johnson and Welsh (1985), who found that the by-products of the deterioration of *Ulva* were lethal to some crab larvae.

The present study suggests that this experimental approach can lead to the ultimate isolation and identification of the pheromone. For example, preliminary tests have been conducted in which anion exchange resins exposed to either receptive female water, nonreceptive female water, or waters with no animals were eluted with HCL, neutralized, then diluted in seawater. These diluted extracts were then tested on males in the Y-tube apparatus. The results demonstrated that males were not repelled by the extracts and, further, that they exhibited a positive response to extracts of receptive female but not to the other types of waters. These results were not statistically significant, but were sufficiently promising to warrant further investigations. These investigations are currently under way.

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ISOLATION OF 24-METHYLENECHOLESTEROL-DERIVED OXIDATION PRODUCTS FROM QUEEN HONEYBEE OVARIES (*Apis mellifica* L.)

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Abstract—The bioecological situation of 24-methylenecholesterol, the main sterol of many pollens and previously isolated from larvae, workers, and queens of the honeybee *Apis mellifica* L., is reviewed. A search for steroids in queen honeybee ovaries has led to the isolation of three compounds occurring in minute amounts: 24-methylenecholest-4-en-3-one (1), 24-oxocholesterol (2), and 24-oxocholest-4-en-3-one (3), which were identified by direct comparison with authentic samples prepared from 24-methylenecholesterol. These three substances have not been detected in lipids from other parts of queen or from worker honeybees, and their presence may reflect a particular oxidizing capability of the ovaries. The results so far observed are discussed in relationship with previous observations reported on honeybees.

Key Words—*Apis mellifica*, Hymenoptera, Apidae, honeybee, queen, ovaries, 24-methylenecholesterol, 24-methylenecholest-4-en-3-one, 24-oxocholesterol, 24-oxocholest-4-en-3-one.

INTRODUCTION

During the isolation of queen substance, the honeybee queen pheromone, 24-methylenecholesterol (Barbier et al., 1960b; Barbier and Lederer, 1960; Barbier, 1960, 1986), was obtained from the residual neutral fraction (Barbier and Schindler, 1959). This sterol was later found in many pollens, the main source of proteins for the honeybee (Barbier et al., 1960a; Hügel et al., 1964b; for a review see Barbier, 1966), mixed with the usual series of C_{27}, C_{28}, and C_{29} sterols (Standifer et al., 1968). 24-Methylenecholesterol was obtained in purer form from the queens than from worker honeybees or pollens, as if a kind of biological discrimination was accomplished between the food and tissue accu-

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mulation. This was emphasized by the isolation of nearly pure 24-methylenecholesterol from queen honeybee larvae collected at an early stage of development, that is, fed with royal jelly only (Barbier and Bogdanovsky, 1961). Svoboda et al. (1980, 1986) noticed a unique selective transfer mechanism of 24-methylenecholesterol from workers to developing brood, which is in agreement with this preliminary observation. During a systematic search for possible steroid hormones within the honeybee world, the existence of three 2,4-dinitrophenylhydrazine-reacting substances, present in trace amounts, was observed in the queen ovaries. At that time, due to the minute amounts existing in comparison with the mass of lipids and of sterols, only the main component was identified to 24-oxocholesterol, the two others being kept for further investigations. Later, while searching for queen substance, the queen honeybee pheromone (*E*)-9-oxo-2-decenoic acid, in the queen ovaries (Barbier and Pain, 1981), these experiments were developed to confirm the preceding result and to identify the as-yet-unidentified substances. These products were found to belong to the same biosynthetic series, 24-methylenecholest-4-en-3-one and 24-oxocholest-4-en-3-one, corresponding to successive oxidations of 24-methylenecholesterol. We subsequently completed this work by comparing the presence of these products in worker honeybees and in queens with ovaries removed, in which they have not been found within the limits of sensitivity of the methods. We now report these results.

The presence of 24-methylenecholesterol in the honeybee has been discussed (Svoboda et al., 1983, 1986; Svoboda and Lusby, 1986) in relationship to biochemical transformations and food requirements. 24-Methylenecholesterol is not transformed into C_{27} sterols, in agreement with reports about its accumulation in tissues. A discrepancy appears in the literature concerning the dealkylation of C_{29} sterols into cholesterol by honeybees (Allais et al., 1971; Svoboda et al., 1981). Allais observed an 18% transformation of labeled sitosterol into cholesterol, while 24-methylenecholesterol was recovered unlabeled after these experiments (Svoboda reported no dealkylation at position C-24 by using different conditions). Allais used the sitosterol propionate, and it may be that esterification of sterols is a prerequisite for further metabolism of the substance by honeybees following the usual dealkylation process (Allais and Barbier, 1971).

In pollen, the bioecological situation of 24-methylenecholesterol is related to an incomplete biosynthesis, a characteristic of these cells also noticed with pollinastanol (a 14-methyl-9,19-cyclostanol) in the rare pollen where cholesterol is the main sterol (Hügel et al., 1964a; Barbier, 1966; Devys et al., 1969). Corresponding 24-derivatives such as 24-methylene pollinastanol have also been isolated from pollen (Thompson et al., 1978).

Thus, the situation found in honeybees is rather particular concerning their sterols, as these insects are able to metabolize steroids (Veith et al., 1974) but

selectively accumulate 24-methylenecholesterol or, as shown here, oxidize it in their ovaries.

METHODS AND MATERIALS

Honeybee queen ovaries (800) were obtained through dissection from 400 queens (they are accessible even to nonspecialists by removing the sternites to uncover the ovaries as masses that occupy nearly all of the abdomen and are separated from the accessory glands. These ovaries were crushed in ethanol, filtered, and successively extracted by ethanol–ether 1 : 1 and ether. The collected extracts were dried in vacuo, the residue taken up by hot acetone, and the insoluble phospholipids discarded. The acetone-soluble lipids, after evaporation, were further saponified according to the usual procedure: 1 : 1 mixture of benzene with a 2 N methanolic KOH solution, 2 hr reflux under nitrogen, extraction by ether after addition of water, and crystallization from methanol. In these conditions, no noticeable oxidation of the 24-methylenecholesterol present in lipids from worker honeybees was observed by TLC of the mother liquors. Repeated crystallizations from MeOH led to 90% pure 24-methylenecholesterol (according to the mass spectrum of the acetate) and to a series of mother liquors which were collected. Substances 1, 2, and 3 (Figure 1) were isolated by preparative TLC of the residue from the mother liquors redissolved in ether for application to the SiO_2 layer (fluorescent films, Schleicher-Schüll, development with hexane–ethyl acetate 85 : 15 or 7 : 3, UV observation at 254 nm with a Desaga lamp for TLC, or 2,4-DNP spray, also comparing with standards on lateral position). All substances were extracted from the scraped SiO_2 by ether. The 2,4-DNP derivatives were obtained directly from the sprayed TLC plate and further submitted to control chromatography with a standard. The acetates were prepared for TLC and MS by action of acetic anhydride in pyridine (1 : 1), 20 hr at 20°C, and direct drying in vacuo previous to control TLC and developing with hexane–ethyl acetate 95 : 5. Observation of non-UV-absorbing, non-2,4-DNP-reacting sterols was by means of H_2SO_4 50%, 200°C by infrared lamp, red colorations. MS determinations were carried out on an AEI MS9 spectrometer, and UV spectra were obtained on an automatic Leres apparatus S-66 in ether.

RESULTS AND DISCUSSION

The lipids were extracted from the ovaries of 400 honeybee queens obtained through dissection, and the acetone-soluble fraction was saponified. The repeated crystallizations of the unsaponifiable fraction in methanol gave about 10 mg of a sterol fraction consisting mainly of 24-methylenecholesterol, R_f 0.45 (hexane–ethyl acetate 85 : 15), mp 139–142°C, acetate mp 132–134°C,

FIG. 1. A, 24-methylenecholest-4-en-3-one; B, 24-oxo-cholesterol; C, 24-oxocholest-4-en-3-one.

MS, *m/z* 380 (M-60), 296 (McLafferty rearrangement), 255, 213, (Barbier and Schindler, 1959; Barbier et al., 1960a; Hügel et al., 1964b). The mother liquors of the crystallizations were collected (46 mg) and studied through fluorescent SiO_2 TLC, showing the presence of two steroids in UV, reacting with 2,4-DNP. This reagent also gave a yellow color with a third component which could not be detected under the UV lamp in the reported conditions. After isolation, these three substances were identified by direct comparison with authentic samples prepared by oxidation on a standard of 24-methylenecholesterol. They all gave the McLafferty elimination of the side chain in MS because of the presence of a methylene group or an oxo function at position 24 (1, 3: 312, at 296 for 2).

Substance 1. UV λ_{max} 240 nm (ϵ 15,000 in ether), R_f 0.60 SiO_2 TLC (hexane–ethyl acetate 85:15), mp 77–84°C, 0.6 mg, MS *m/z* 396. This substance was identified as 24-methylenecholest-4-en-3-one by comparison with the authentic substance obtained from 24-methylenecholesterol through Oppenauer oxidation. The 2,4-DNP is orange, R_f 0.55 in the same developing solvent.

Substance 2. This product cannot be observed in UV on TLC, R_f 0.40 (hexane–ethyl acetate 85:15), reaction with 2,4-DNP or with H_2SO_4 200°C on a lateral deposit, mp of the acetate 122–127°C, MS 382 (M-60). Substance 2 was identified as 24-oxocholesterol by direct comparison with an authentic sample prepared from 24-methylenecholesterol acetate through ozonolysis. This substance is the major component in these oxo derivatives of 24-methylenecholesterol (0.8 mg). The 2,4-DNP is yellow, R_f 0.30 in the developing system mentioned.

Substance 3. R_f 0.55 (hexane–ethyl acetate 85:15), UV 240 nm (ϵ 15,000 in ether), mp 65–70°C, MS *m/z* 398. The compound was identified as 24-oxocholest-4-en-3-one by direct comparison with the authentic product prepared by Oppenauer oxidation of 24-oxocholesterol. This substance represents the minor component in the mixture of these oxo derivatives (0.4 mg). The corresponding 2,4-DNP (bis, according to the MS carried out on the standard) is orange and has an R_f of 0.45 in hexane–ethyl acetate (85:15). It has not been possible to carry out MS on the isolated 2,4-DNP because of the very small amount.

These substances (1, 2, and 3) occur in small amounts in queen honeybee ovaries, and it has not yet been possible to detect any of them in the lipids obtained from 320 carcasses or from 500 g of worker honeybees.

About the origin of these compounds, two hypotheses are possible: They may result from a systematic accumulation of traces present in food (pollen) or they may be produced in situ by the oxidizing capability of the ovaries. As far as we know, these substances have not been reported from pollen, and we failed to detect traces in the mother liquors of crystallization of the sterol fractions. A similar case has been found with makisterone A, a molting hormone detected in nanogram amounts in queen honeybee ovaries (Feldlaufer et al., 1986) which also probably results from the metabolism of 24-methylenecholesterol. Makisterone A is a 24-methyl derivative (Kaplanis et al., 1975) which could result from a reduction of the 24-methylene group. Highly active metabolites are often present in insect ovaries; for example, the ecdysteroid bombycosterol from *Bombyx mori* (Fujimoto et al., 1985). Organs often stock food products as demonstrated by the presence of a food contaminant, methyl *p*-hydroxybenzoate (Barbier and Schindler, 1959), introduced into the beehive with industrial molasses and accumulated in mandibulary glands of queens in high quantity. In the present case, the biological significance of the three oxo compounds 1, 2,

and 3 in the queen honeybee ovaries is not obvious. It could mean that these insects have adapted enzymatic systems, such as 3β-hydroxydehydrogenase, Δ_5-isomerase, Δ_4-reductase, that are able to transform a sterol into a steroid.

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IDENTIFICATION OF TRITERPENOID FEEDING DETERRENT OF RED PUMPKIN BEETLES (*Aulacophora foveicollis*) FROM *Momordica charantia*

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Abstract—The triterpenoids isolated from the leaves of *Momordica charantia* Linn (bitter gourd) were found to elicit feeding-deterrent activity against red pumpkin beetles (*Aulacophora foveicollis* Lucas). The most abundant triterpenoid which deterred feeding was identified as momordicine II, 23-*O*-β-glucopyranoside of 3,7,23-trihydroxycucurbita-5,24-dien-19-al. A concentration of 3200 μg/ml and above of the triterpenoids caused significant reduction of feeding by red pumpkin beetles in in vitro bioassay experiments, which compared favorably with the levels of triterpenoids in *M. charantia* leaves found in nature.

Key Words—Feeding deterrents, *Aulacophora foveicollis*, red pumpkin beetles, Coleoptera, Chrysomelidae, *Momordica charantia* (bitter gourd), cucurbitane triterpenes, momordicine, Cucurbitaceae.

INTRODUCTION

The chemical basis of insect feeding responses has been well established, and secondary plant substances are implicated as attractants, repellents, or deterrents (Schoonhoven, 1972, 1982). Cotyledons and leaves of *Momordica charantia* Linn (Cucurbitaceae) are unacceptable to red pumpkin beetles (*Aulacophra foveicollis* Lucas, coleoptera) which feed voraciously on the cotyledons and leaves of most of the other cucurbitaceous crops (Ramakrishna Ayyar, 1984). Efforts to establish the chemical basis for this phenomenon led to isolation of a feeding deterrent of red pumpkin beetles from leaves of *M. charantia* which was tentatively identified as a triterpene glucoside (Chandravadana and

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Pal, 1983). The chemical structure of the feeding deterrent and the optimum level at which it acts are reported in the present paper.

METHODS AND MATERIALS

Triterpenoids were isolated from leaves by the procedure reported earlier (Chandravadana and Pal, 1983). In this procedure dried and powdered leaf material was extracted with 90% methanol, concentrated under reduced pressure to $\frac{1}{10}$ the volume, extracted with petroleum ether (60–80°C) to remove the lipids, concentrated to dryness, suspended in water, and finally extracted with ethyl acetate. The concentrated ethyl acetate extract was passed through a column of alumina (neutral), washed thoroughly with ethyl acetate, and the triterpenoids were eluted in methanol. Thin-layer chromatography of this eluent on silica gel-G (0.25 mm) with the solvent system ethyl acetate–methanol–water (16 : 1 : 1) revealed eight distinct triterpenoid spots when sprayed with Liebermann-Burchard (LB) reagent (acetic anhydride–sulfuric acid, 19 : 1) followed by heating to 110°C for 10 min. Of the eight triterpenoids, the major triterpenoid alone was isolated in sufficient quantity for characterization. For its isolation, the crude mixture of triterpenoids (100 mg) was applied on a column of silica gel (60–100 mesh, 10 g) using ethyl acetate as an eluent. The fraction collected between 225 and 800 ml contained the major triterpenoid, and it was crystallized from the same solvent. Its [^{1}H]NMR and [^{13}C]NMR spectra were measured in pyridine-d_5 with tetramethylsilane as the internal standard on Hitachi-Perkin-Elmer (90 MHZ) and JEOL/FX-100 (25 MHZ) spectrometers, respectively. The infrared spectrum was recorded on a Perkin-Elmer IR spectrophotometer as a KBr pellet.

Bioassay. Adult red pumpkin beetles of mixed sex and age groups were collected from field-grown plants of bottle gourd (*Lagneria siceraria*) and pumpkin (*Cucurbita Maxima*). They were starved for 24 hr before using for bioassay. Excised, fully expanded cotyledons of pumpkin, which is an acceptable food for beetles, were used for bioassay.

Triterpenoid in 80% ethanol was applied uniformly on both surfaces of the cotyledon and the solvent was removed quickly by air drying. Cotyledons treated with 80% ethanol were used as control. Treated and control cotyledons were kept separately in Petri dishes, exposing their lower surface, for a no-choice test. Beetles were released at the rate of two per cotyledon. After 24 hr, the extent of feeding was assessed by measuring the area of cotyledon damaged, using a graph sheet. The number of fecal pellets was also counted. Evidence for feeding deterrence was construed based on reduction in the extent of feeding damage and number of fecal pellets in the treated one as compared to control. Bioassays were separately conducted for major triterpenoid, minor triterpenoids, and the crude mixture of triterpenoids extracted from leaves.

Concentrations. Five concentrations of the major triterpenoid (300, 800, 1600, 3200, and 6400 μg/ml) were prepared in 80% ethanol. On each cotyledon, with a mean area of 4.8 ± 0.50 cm^2 and a mean weight of 300 ± 28 mg, 0.3 ml of one of the above solutions was applied. This corresponds to an average triterpenoid concentration of 19, 50, 100, 200, and 400 μg/cm^2 or 0.03, 0.08, 0.16, 0.32, and 0.64% w/w of fresh cotyledon. There were five replicates for each treatment, and the statistical significance of the data was assessed using analysis of variance according to Snedecor and Cochran (1967).

The minor triterpenoids were isolated by resolving 20 mg of the crude mixture on a thin-layer chromatogram. After development with the solvent system ethyl acetate–methanol–water (16:1:1), a portion of the plate was sprayed with LB reagent for visualization of triterpenoids, and the corresponding bands from the unsprayed portion of the plate were eluted in ethanol and evaporated to dryness. The quantity of each triterpenoid so obtained was bioassayed in duplicate.

The crude mixture of leaf triterpenoids which eluted together from the column of alumina was bioassayed at 300, 800, 1600, 3200, and 6400 μg/ml levels.

Triterpenoid Estimation in Vivo in M. charantia Leaves. Leaves collected from 10 plants at flowering and fruiting stages were pooled and dried. Triterpenoids were extracted by the procedure described earlier. The extract from 1 g of dried leaves was passed through a column of alumina (2 g) and washed with ethyl acetate (50 ml). The triterpenoids were eluted in methanol (100 ml), concentrated, and dissolved in methanol (1 ml). An aliquot of 0.25 ml was evaporated to dryness, mixed with 4 ml of LB reagent, and the optical density was measured immediately at 545 nm. The standard curve was prepared using the major triterpenoid. The concentration was computed based on estimations of 10 pooled samples.

RESULTS AND DISCUSSION

The [^{1}H]NMR, [^{13}C]NMR, and IR spectral data of the major triterpenoid feeding deterrent isolated from *M. charantia* has been obtained. [^{1}H]NMR revealed methyl group signals at 0.90, 0.92, 1.18, 1.51, 1.70, and 1.78 ppm, olefinic proton signals at 5.6 and 6.28 ppm, formyl proton at 10.6 ppm; and protons on carbon-bearing hydroxyl groups between 3.8 and 5.1 ppm. [^{13}C]NMR revealed hydroxymethine carbons at 104.1, 78.82, 78.21, 75.53, 75.29, 71.76, 65.66, and 62.92 ppm; formyl carbon at 207.8 ppm; carbons for two trisubstituted double bonds at 124.20, 129.01, 132.18, and 145.64 ppm; and quaternary carbons at 41.7, 45.9, and 48.2 ppm. During the course of the present study, Yasuda et al. (1984) reported the identification of the major bitter principle in the leaves of *M. charantia* as momordicine II, 23-*O*-β-glucopyr-

FIG. 1. Momordicine II.

anoside of 3,7,23-trihydroxy-cucurbita-5,24-dien-19-al ($C_{36}H_{58}O_9$, Figure 1). A comparative study of the spectral data revealed a close correspondence of the [^{1}H]NMR and [^{13}C]NMR peaks of momordicine II and the deterrent we have isolated. Furthermore, the IR spectrum of the feeding deterrent was superimposable on that of standard momordicine II. The present study therefore establishes that the major feeding deterrent of red pumpkin beetles is momordicine II, which was reported as the bitter principle by Yasuda et al. (1984). The method used for isolation of the bitter principle by Yasuda et al. is, however, different from that employed here.

The in vitro bioassay carried out in the present study with starved beetles was aimed at determining the effective levels of the major leaf triterpenoid (identified as momordicine II) as a feeding deterrent. Among the five concentrations tested (Table 1), 3200 μg/ml (0.32% w/w) and 6400 μg/ml (0.64% w/w) levels caused significant feeding inhibition (81% and 94%, respectively), in terms of the reduction in area damaged by beetles. Reduction to the extent of 80% and 94% in the number of fecal pellets produced by beetles at these two concentrations also corroborate the above observation. At lower concentrations of the triterpenoid (300–1600 μg/ml), the observed reduction in feeding damage was not statistically significant.

It was considered desirable to check the feeding deterrency of the other minor triterpenoids, as they might elicit additive or synergistic effects in vivo. Among the eight fractions distinguished on the basis of R_f values (Table 2), five of them (including momordicine II) exhibited feeding deterrent activity. All the above five fractions probably elicit additive effects in vivo, since a bioassay involving the naturally occurring mixture of triterpenoids recorded significant feeding-deterrent activity at 3200 μg/ml (0.32% w/w) and above, levels at which the major triterpenoid alone deterred feeding. It is interesting that the levels of feeding-deterrent activity of the naturally occurring mixture of triterpenoids were

TABLE 1. FEEDING DETERRENCY OF MAJOR LEAF TRITERPENOIDS AT DIFFERENT LEVELS IN NO-CHOICE EXPERIMENT

Concentration applied			Area damaged by beetles mean	Extent of defecation	inhibition
μg/ml	%/w/w	μg/cm²	(cm²)	(mean)	(%)[a]
Control	nil	nil	0.95	54	—
300	0.03	19	0.68	36	—
800	0.08	50	0.62	30	—
1600	0.16	100	0.52	27	—
3200	0.32	200	0.18*[b]	11*	81.1
6400	0.64	400	0.06*	3*	93.7
F test			*	*	
SEM			0.16	8	
Critical difference at 1%			0.63	32	

[a]Inhibition (%) = 100 × (area damaged on control − area damaged on treated)/area damaged on control.
[b]Asterisk indicates significant at 1% level.

at par with momordicine II alone, even though the mixture included triterpenoid fractions 4, 5, and 6 with no feeding-deterrent activity (Table 2). It will be worthwhile to identify the minor triterpenoids and ascertain their threshold concentrations for feeding-deterrent effects.

The in vivo concentration of triterpenoids in leaves of *M. charantia* was estimated, and it ranged from 0.31% to 0.36% w/w on a fresh weight basis.

TABLE 2. FEEDING DETERRENCY OF LEAF TRITERPENOID COMPONENTS

Component number[a]	R_f^b	Feeding inhibition (%)[c]
1	0.24	94.2
2 (major triterpenoid)	0.34	96.4
3	0.40	90.0
4	0.47	12.1
5	0.54	0.7
6	0.64	00.0
7	0.80	92.1
8	0.90	94.2

[a]The concentration of the components in 0.3 ml of 80% ethanol, used for bioassay, corresponds to the quantity of each obtained by resolving 20 mg of the crude mixture of triterpenoids on a thin-layer chromatogram and their subsequent elution.
[b]R_f: relative mobilities with respect to solvent on silica gel-G (0.25 mm) in solvent system ethyl acetate–methanol–water (16 : 1 : 1).
[c]Refer table 1.

This concentration compared favorably with the effective levels established by in vitro bioassay, which suggests that the natural levels of triterpenoids in leaves of *M. charantia* are adequate to cause inhibition to feeding by red pumpkin beetles.

The above results provide evidence for resistance imparted by triterpenoids in leaves of *M. charantia* against red pumpkin beetles. The levels at which the feeding-deterrent activity of the triterpenoids was discerned may not truly reflect the situation in vivo where it may be effective even at a lower concentration. This is primarily because, unlike in *M. charantia*, cotyledons of *C. maxima* used in bioassay studies contain cucurbitacins B and E (Enslin et al., 1957) which are known to stimulate feeding by red pumpkin beetles (Sinha and Krishna, 1970; Vashista and Choudury, 1971). Momordicine and cucurbitacins, both of which are cucurbitane triterpenes, seem to cause a contrasting feeding response by red pumpkin beetles which might be due to difference in their structure. Momordicine II is unique in having an aldehyde group at C-19 and a 23-*O*-β-glucopyranosyl unit and in not having a carbonyl function at C-11. The effect of momordicine on feeding behavior of other insect pests of cucurbits merits further study.

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FEMALE SEX PHEROMONE OF THE COMMON FURNITURE BEETLE *Anobium punctatum* (COLEOPTERA: ANOBIIDAE): Extraction, Identification, and Bioassays

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Abstract—Observations and reports on the common furniture beetle *Anobium punctatum* suggested that, on emergence, females use a sex pheromone to attract males. GLC analysis of ovipositor extracts showed the presence of a single component, which was found to be active by EAG and coupled GLC-EAG techniques, and to attract males in both walking and flying assays. The pheromone was identified by GC-MS as 2,3-dihydro-2,3,5-trimethyl-6-(1-methyl-2-oxobutyl)-4H-pyran-4-one (stegobinone), which is the sex pheromone of another anobiid, the drugstore beetle, *Stegobium paniceum*. Male *A. punctatum* responded equally to ovipositor extracts of either species, at both the sensory (EAG) and behavioral levels, which poses the question as to how species specificity in mate attraction is achieved.

Key Words—Common furniture beetle, *Anobium punctatum*, drugstore beetle, *Stegobium paniceum*, Coleoptera, Anobiidae, sex pheromone, stegobinone, 2,3,dihydro-2,3,5-trimethyl-6-(1-methyl-2-oxobutyl)-4H-pyran-4-one, behavior, electroantennogram, species specificity.

INTRODUCTION

Anobium punctatum de Geer, the common furniture beetle, is a widespread and destructive pest of seasoned wood. Indigenous to northern Europe and Asia, it has been distributed by man on a worldwide scale and is now found in Australasia, South Africa, and the eastern coast of North America (Hickin, 1963).

The damage is done by the larvae—known as woodworm—which can feed on soft and hardwoods, pulpboards, plywood, and even cardboard, and as a result, both structural timbers and furniture are subject to attack. The female

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lays her eggs in cracks in the surface, and the larvae tunnel into the wood. After pupation, the adults cut their way out, leaving a characteristic flight hole (Hickin, 1963).

Surprisingly, despite the economic importance of *A. punctatum*, little attention has been paid to its reproductive behavior or that of anobiid beetles in general. A recent review (Tumlinson, 1985) listed only two identified pheromones from this family: the drugstore beetle, *Stegobium paniceum* L. (Kuwahara et al., 1975, 1978) and the cigarette beetle, *Lasioderma serricorne* F. (Chuman et al., 1979).

Several observations, however, have suggested the presence of a female sex pheromone in *A. punctatum*. Kelsey et al. (1945) reported "swarming," and on examining a swarm found it to contain 167 males clustered around three females! R.W. Berry reported that males, which emerge first, locate females as they emerge from their flight holes. Our own observations have noted female "calling" behavior, which has previously been reported in *A. punctatum* (Cymorek, 1964) and in another anobiid beetle—*Ptilinus pectinicornis* L. (Cymorek, 1960): The female stands with the abdomen raised and periodically protrudes the ovipositor.

This paper describes the extraction, identification, and bioassay of the sex pheromone of *A. punctatum* and provides comparisons with other anobiid pheromones.

METHODS AND MATERIALS

Insect Material. Cultures of hazel twigs infested with *A. punctatum* larvae and pupae were obtained from the Building Research Establishment, Princes Risborough, Buckinghamshire, U.K., and were kept at 22°C in constant darkness. Emerging adults were removed daily and sexed using the presence or absence of a depression on the ventral surface of the male's terminal abdominal segment (Kelsey et al., 1945). The sexes were kept separately in glass tubes on moist filter paper until use. *S. paniceum* was kept in a mixed-sex culture on rabbit pellets at 22°C.

Extraction Procedure. Ten newly emerged female *A. punctatum* were dissected and the ovipositors removed and extracted in 0.5 ml of pentane for 24 hr at 4°C. Three replicate extractions were made. Ovipositors of *S. paniceum* were extracted in a similar manner.

Gas Chromatography. Both extracts were analyzed using a Varian 3700 GLC (Walton-on-Thames, Surrey, U.K.) fitted with a 50-m × 0.32-mm ID fused silica column coated with chemically bonded OV-1 (Phase Separation Ltd. Queensferry, Clwyd, U.K.). Samples were injected in splitless mode with the oven at 50° C for 1 min, then programmed at 5°C/min to 250°C, using helium carrier gas at 1.5 kg/cm^2. Quantitative analyses used naphthalene as a standard.

Gas Chromatography-Mass Spectrometry (GC-MS). GLC of the *A. punctatum* and *S. paniceum* extracts each showed the presence of a single major component which was identified using a Finnigan 1020 GC-MS.

Electroantennography (EAG). Initial EAG recordings showed that male *A. punctatum* antennae gave a strong EAG response to the conspecific ovipositor extract. An EAG experiment was therefore conducted to determine whether males and females are equally responsive to female *A. punctatum* extract, and whether they respond differently, at the EAG level, to extracts of *A. punctatum* and *S. paniceum.* EAG responses were recorded from isolated furniture beetle heads mounted on a cork stage using double-sided adhesive tape. The indifferent glass microelectrode was inserted into the base of the head, and the opening sealed with petroleum jelly. The recording microelectrode was inserted into a small hole punched in the terminal antennal segment using a finely etched tungsten needle. Electrodes were filled with saline and connected, using chloridized silver wires, to a Grass P-16 DC (Quincy, Massachusetts) preamplifier and a Tektronix 5223 oscilloscope.

The odor delivery system was similar to that described by Payne and Dickens (1976), using a two-way solenoid valve to divert the filtered (activated charcoal) and humidified airflow via a test cartridge into a glass Y delivery tube. The airflow was maintained at 1000 ml/min with a pulse length of 1 sec. Cartridges consisted of a strip of filter paper impregnated with 10 μl of the test extract, inside a Pasteur pipet. Parallel series of dilutions of female *A. punctatum* and *S. paniceum* extracts were made, giving matched pairs containing equal concentrations of the major component. The two series were checked for concentration by GLC and stored at $-80°C$ before use. Test cartridges were presented pairwise in ascending order of concentration. Control presentations of solvent were interspersed between test presentations, and the response subtracted from the test response before analysis of the results.

Six individuals of each sex of *A. punctatum* were tested in this way. Results were analyzed using a split-plot analysis of variance to identify differences between the sexes and between the responses to the two extracts.

Coupled Gas Chromatography-Electroantennography (GC-EAG). This was carried out to confirm that the EAG responses recorded to the ovipositor extracts were due to the single major component rather than to any trace components. The GC-EAG system was essentially similar to that described for GC-single cell recordings by Wadhams (1984). The GLC and OV-1 column were as described above, using a temperature program of 150–230°C at 5°C/min. The column effluent was split 1 : 1 between two lengths of an OV-1-coated capillary column using a glass lined T piece. One arm led to the FID detector while the other ran via a heated jacket (maintained at 220°C) to the airflow impinging on the antenna.

Behavioral Assays. Bioassays were conducted to determine the response of male and female *A. punctatum* to conspecific ovipositor extract and to test

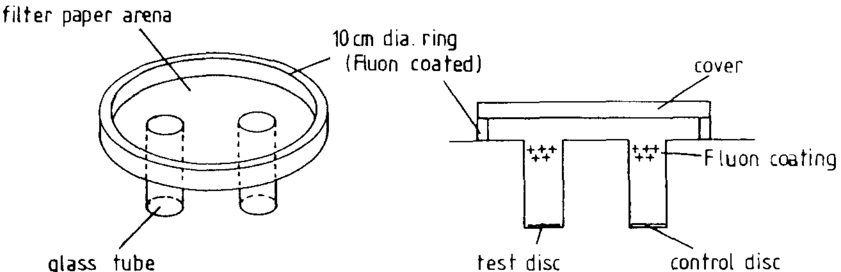

FIG. 1. Two-choice pitfall bioassay apparatus.

whether the response of *A. punctatum* to this extract differed from that to *S. paniceum* ovipositor extract.

A two-choice pitfall assay was used to compare the response of the sexes (Figure 1). Two 5 × 2.5-cm glass tubes with necks coated in Fluon were glued below holes cut in an 11-cm-diameter filter paper disc, which was supported on a metal baseplate. A 10-cm-diameter Perspex ring coated with Fluon was placed on the filter paper and covered with a glass plate.

The insects (3–15 per test) were introduced into the arena and allowed 10 min to settle. Test and control stimuli were then introduced, consisting of 10 μl of female extract (0.2 female equivalents), and 10 μl of pentane, respectively, applied to a 1-cm filter-paper square. The assay was left for 1 hr at 25°C, and then the number of beetles in each tube was counted. Five replicates were conducted for each sex, using each individual once only. Results were combined to give totals of each sex trapped in the test or control tubes. Differences between test and controls were tested for significance using a chi-square test.

A preliminary assay was also conducted in a wind tunnel 160 cm long × 75 cm high × 70 cm wide, using a wind speed of ca. 0.2 m/sec. The test stimulus (0.4 female equivalents of ovipositor extract) was placed on a filter paper strip at the upwind end of the tunnel, and either males or females placed on a platform 1.5 m downwind of the source, and their behavior observed.

An open arena assay was employed to compare the behavioral response of male *A punctatum* to the two extracts. The arena again consisted of a 10-cm-diameter ring coated in Fluon placed on an 11-cm-diameter filter paper and covered by a glass plate. Males were released singly into the arena and allowed 10 min to settle. The test extract was then applied to a 1-cm filter paper square as described above, the paper placed in the center of the arena, and the arena covered. Each individual was observed for up to 5 min, and the time taken for it to reach the center of the arena (defined as crossing a 2-cm-diameter circle drawn around the test paper) recorded. Beetles not responding in this period were given the default time of 5 min. Three concentrations plus a control were

tested for each of the extracts, with 10 replicates of each, in a randomized design. Results were analyzed using analysis of variance to determine whether the response time differed between the two extracts.

RESULTS

GLC. Analysis of the *A. punctatum* ovipositor extract showed the existence of a single major peak with a retention time of 27.5 min using the OV-1 column and the conditions described (Figure 2). Quantitative GLC showed that the mean (N = 10) amount of this material per female *A. punctatum* was 240 ng. Analysis of the *S. paniceum* extract also showed a single major peak with an identical retention time, which was confirmed by coinjection.

GC-MS. The electron impact spectra for the major components of *A. punctatum* and *S. paniceum* ovipositor extracts were found to be identical (Figure 3). They suggested a compound of molecular weight 224, this being the highest ion of significance in both spectra. The spectra were also identical to that published for stegobinone, the sex pheromone of *S. paniceum* (Kuwahara et al., 1975, 1979). Therefore, on the basis of identical spectra and retention times, the major component of the *A. punctatum* ovipositor extract was identified as stegobinone, or 2,3-dihydro-2,3,5-trimethyl-6-(1-methyl-2-oxobutyl)-4H-pyran-4-one (Figure 4).

EAG. Results of the EAG experiment (Figure 5) showed that the EAG response of males was significantly greater than that of females to both *A. punctatum* and *S. paniceum* extracts ($P < 0.001$). However, within each sex, there

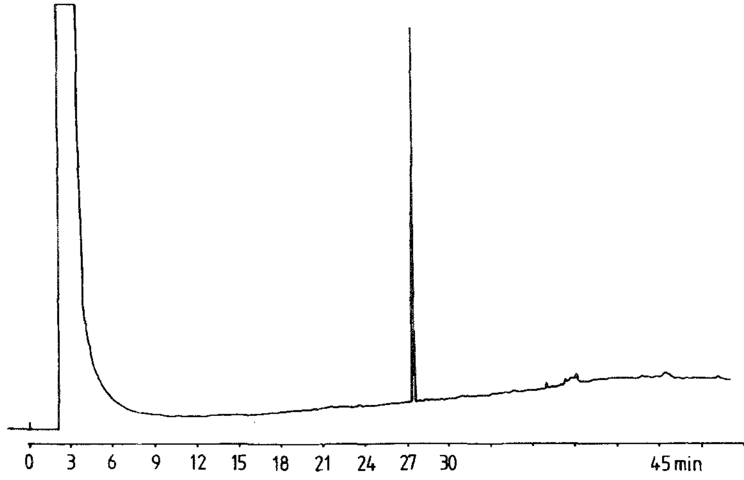

FIG. 2. Gas chromatogram of *A. punctatum* ovipositor extract, using 50-m OV-1 column. Oven initially at 50°C for 1 min, then programmed at 5°C/min to 250°C.

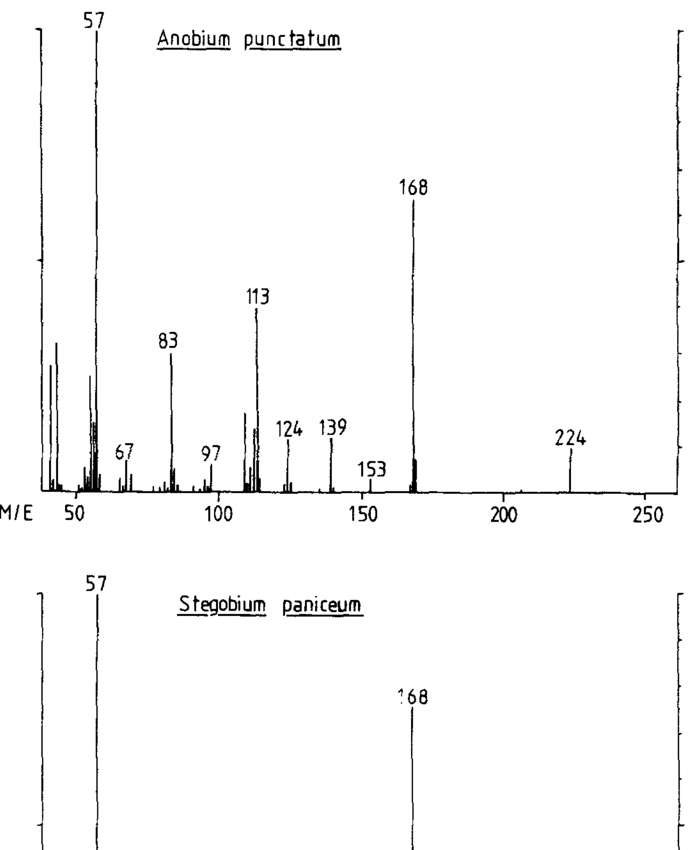

FIG. 3. Electron impact mass spectra of major components of *A. punctatum* and *S. paniceum* ovipositor extracts.

FIG. 4. Structure of 2,3-dihydro-2,3,5-trimethyl-6-(1-methyl-2-oxobutyl)-4H-pyran-4-one (stegobinone).

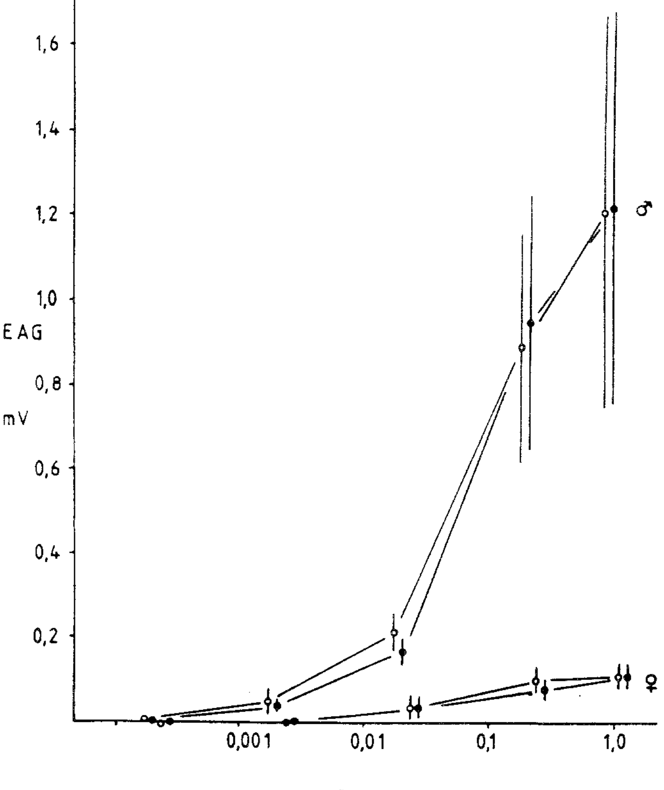

FIG. 5. Electroantennogram (EAG) response of *A. punctatum* to ovipositor extracts of *A. punctatum* (open circles) and *S. paniceum* (closed circles). ($\pm$ standard error, N = 6). Using analysis of variance, difference between male and female response was F = 13.5, $P < 0.001$; difference between extracts $F = 0.0$, NS.

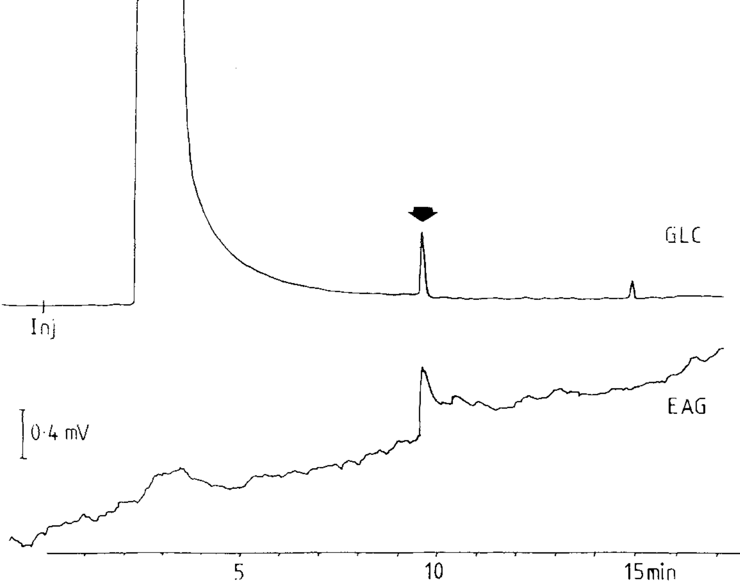

FIG. 6. Coupled GC-EAG response of male *A. punctatum* to conspecific ovipositor extract. GC oven intially at 150°C for 1 min, then programmed at 5°C/min to 230°C.

was no significant difference between the responses to the two extracts. Coupled GC-EAG traces for male *A. punctatum* (Figure 6) showed a single EAG response that was synchronous with elution of the single major component of the conspecific ovipositor extract. The EAG response to the female extract was therefore due to a response to the major component and not to any trace components.

Behavioral Assays. Results of the pitfall assays are presented in Table 1. Significantly more male *A. punctatum* were trapped by the conspecific female extract than the control at the concentration tested ($P < 0.001$), but there was no evidence of such a bias in females.

TABLE 1. ATTRACTION OF *A. punctatum* TO CONSPECIFIC OVIPOSITOR EXTRACT IN TWO CHOICE PITFALL ASSAY.[a]

	Test (0.2 FE)	Control (pentane)	No response	Total
Male	30	0	13	43
Females	0	2	29	31

[a] Difference between test and control: males $\chi^2_{(1)} = 30.0$, $P < 0.001$; females, NS.

A similar result was found in the wind-tunnel trials (data to be presented elsewhere). At least 20 of the 40 males tested responded by walking to the upwind edge of the platform, taking off and flying upwind, and landing on or within 2 cm of the source. Males flew to both *A. punctatum* and *S. paniceum* extracts. By comparison, no females flew in the wind-tunnel trials.

The response of males in the open arena assays (Figure 7) showed a significant effect of concentration ($P < 0.001$) with the time taken to reach the source decreasing with increasing dosage. Males responded to the odor by raising their antennae and then orienting in a series of zigzags to the source. Once at the source, males performed tight turning movements on the filter paper square. In contrast, when exposed to the solvent controls, males either remained stationary at the edge of the arena, or walked slowly around the perimeter. There was no difference, however, between the response of males to the two extracts. Female responses to the ovipositor extracts were not significantly different from responses to the solvent, even at the highest concentration tested (Figure 7).

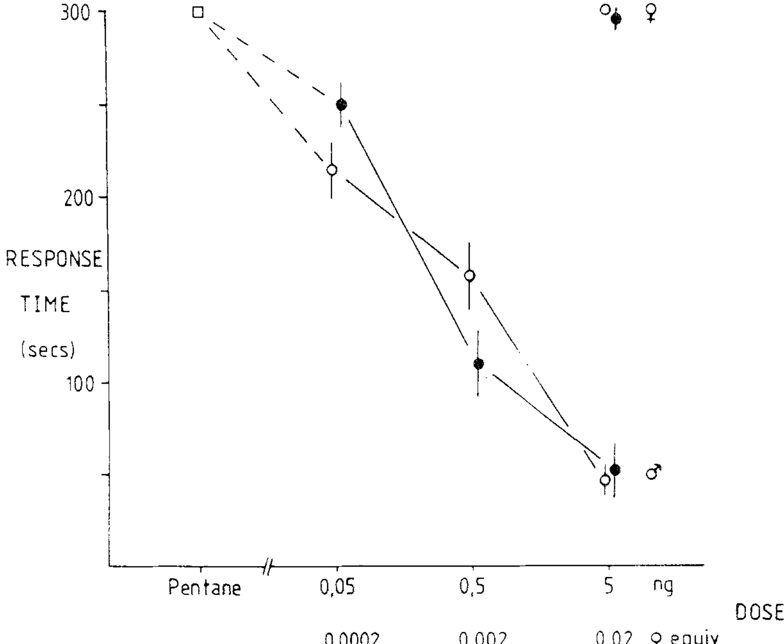

FIG. 7. Response of *A. punctatum* males to ovipositor extracts of *A. punctatum* (open circles) and *S. paniceum* (closed circles) in open arena behavioral assay. Response time gives mean time (± standard error, $N = 10$) taken to reach center of arena. Using analysis of variance, effect of dose $F = 40.6$, $P < 0.001$; difference between extracts $F = 0.1$, NS.

DISCUSSION

The female common furniture beetle, *A. punctatum*, was shown to release from the ovipositor a sex pheromone which attracts males. The GC-EAG results suggest that only one component from the ovipositor produces the response, and on the basis of mass spectra and retention times this was identified as stegobinone [2,3-dihydro-2,3,5-trimethyl-6-(1-methyl-2-oxobutyl)-4H-pyran-4-one]. This molecule possesses three chiral centers, giving eight possible stereoisomers. So far we have not been able to determine directly the configuration of the stegobinone extracted from *A. punctatum*, although the configuration of the sex pheromone of *S. paniceum* has been established by X-ray analysis as (2*S*,3*R*,7*R*)-stegobinone (Hoffmann et al., 1981). Since male furniture beetles responded identically to the extracts from *A. punctatum* and *S. paniceum* in both behavioral and electrophysiological assays, the sex pheromone of *A. punctatum* appears to have the same configuration. However, further chemical analysis is required to confirm this.

Males have a low threshold for this material, responding in the behavioral assay at doses below 0.05 ng (2×10^{-4} female equivalents) and in the EAG assay at 0.25 ng (10^{-3} FE), and attraction of males was further demonstrated in a flight tunnel. To our knowledge, this is the first instance where attraction of flying beetles to a pheromone source has been demonstrated in the laboratory. Choudhury and Kennedy (1980) reported this response in flying *Scolytus multistriatus*, but in uniform concentrations of pheromone.

Female common furniture beetles produce large amounts of the sex pheromone. The mean titer per female was 240 ng, and since female size varied widely (probably reflecting differences in the duration of the larval stage), some females are likely to contain considerably more than this. Kuwahara et al. (1975) found similar levels of pheromone in *S. paniceum* (50–200 ng per female). Furthermore, pheromone titer was found to rise following emergence in both *S. paniceum* (Kuwahara et al., 1975) and *Lasioderma serricorne* (Coffelt and Burkholder, 1972), reaching a plateau after five days. In this study female furniture beetles were extracted at 1 to 3 days following emergence, so they may not have reached a peak of pheromone content. This high pheromone content may result from strong selection acting on females to ensure that they are located by males, since the adult life-span is only about two weeks after a larval development that may last four years.

The results suggest that both *A. punctatum* and *S. paniceum* use the same sex pheromone. Although both species are placed in the subfamily Anobiinae, they are not generally positioned immediately adjacent to each other (White, 1971; Toskina, 1974). Crowson suggests (personal communication) that the two genera separated 40–50 million years ago in the Palaeocene, yet they appear to have retained the same sex pheromone. We intend to study other anobiid beetles to see if similar compounds are found.

Since male common furniture beetles respond equally well to the two extracts, it appears that they will be equally attracted to calling female drugstore beetles. We have yet to investigate the response of male drugstore beetles to determine whether they are able to discriminate between the two extracts, but at least in the case of *A. punctatum* there is the possibility of confusion if the species coexist. While other barriers to interspecific mating are likely to exist, selection should act to promote discrimination between species at the earliest possible stage, i.e., during location of the female, to avoid wasting time and energy following false trails (Linn et al., 1986; Cardé and Baker, 1984).

The two species appear to have been allopatric prior to association with human habitation; *A. punctatum* from the forests of northern Europe and Asia (Hickin, 1963), and *S. paniceum* from the Mediterranean or Middle East (Crowson, personal communication). They were further separated ecologically, since the former develops in wood, while the latter is found in bird nests (Woodroffe, 1953) and stores of organic material. However, *S. paniceum* has become a cosmopolitan pest of warehouses, stores, and houses (Mallis, 1960) since it can develop on almost any dried organic material (Pant and Fraenkel, 1954) and is now sympatric with *A. punctatum* over much of its range. Therefore, at least in houses, stores, and animal buildings, the two species may coexist. It will be interesting to determine whether confusion by males between the calling females does occur under such conditions, and if so, whether discriminating mechanisms develop.

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DEFENSIVE CHEMISTRY OF THE FLOUR BEETLE *Tribolium brevicornis* (LeC.): Presence of Known and Potential Prostaglandin Synthetase Inhibitors

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Abstract—The defensive secretion of *Tribolium brevicornis* contains 12 organic components, including quinones, hydroquinones, hydrocarbons, aromatic ketones, and aromatic esters. The two ketones, 2′-hydroxy-4′-methoxyacetophenone and 2′-hydroxy-4′-methoxypropiophenone, and the two esters, methyl 2,5-dihydroxy-6-methylbenzoate and methyl 2,5-dihydroxy-6-ethylbenzoate, represent ca. 0.25% of the biomass of the beetles. Mass spectral and NMR analyses were used to elucidate the structures of all components. The ketones are potent prostaglandin synthetase inhibitors (PSI), and the esters are suspected to be PSI.

Key Words—Biosynthesis, Coleoptera, Tenebrionidae, *Tribolium brevicornis*, prostaglandin, allomones, synthetase inhibitor, defensive secretion, 2′-hydroxy-4′-methoxyacetophenone, 2′-hydroxy-4′-methoxypropiophenone, methyl 2,5-dihydroxy-6-methylbenzoate, methyl 2,5-dihydroxy-6-ethylbenzoate.

INTRODUCTION

The defensive secretions of tenebrionid beetles often contain a diversity of natural products. The best known of these products are the ubiquitious quinones and olefins (Tschinkel, 1975a), but in addition compounds such as isocoumarins (Lloyd et al., 1978), terpenes (Gnanasunderam et al., 1981), aliphatic carbonyl

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compounds (Tschinkel, 1975b; Gnanasunderam et al., 1982), salicylate esters (Gnanasunderam et al., 1984), and β-hydroxy aromatic ketones (Suzuki et al., 1975; Howard et al., 1986) have also been found. The physiological significance of most of the nonquinone and hydrocarbon components is unknown. We recently reported, however, that the red flour beetle, *Tribolium castaneum* (Herbst), contains microgram per beetle levels of 2′-hydroxy-4′-methoxyacetophenone (**1**) and 2′-hydroxy-4′-methoxypropiophenone (**2**) (Howard et al., 1986) and that these aromatic ketones are potent inhibitors of both insect and mammalian prostaglandin synthetases. We have subsequently found that similar aromatic prostaglandin synthetase inhibitors (PSI) are present in a diversity of other insect exocrine secretions (Jurenka et al., 1986).

As part of our continuing efforts to identify insect-derived PSI, we have reexamined (Markarian et al., 1978; Wirtz et al., 1978) the defensive secretion of the North American flour beetle *Tribolium brevicornis* LeC. We have found that this insect contains substantial quantities of ketones **1** and **2** as well as two previously undescribed β-hydroxy aromatic esters. We report here the structure determination of these compounds and discuss their possible physiological significance.

METHODS AND MATERIALS

Insects. Cultures of *T. brevicornis* were obtained from Dr. R. Strong, Department of Entomology, University of California, Riverside, California, and from Dr. W. Burkholder, USDA-ARS, Department of Entomology, Madison, Wisconsin. They have gone through approximately five generations in our laboratory and have been reared on whole wheat flour mixed with 3% brewer's yeast in constant darkness at 30 ± 1 °C, except for brief periods of examination in room light.

Isolation Methods. Beetles from each source population were chilled in an ice bath for ca. 10 min, causing many of the beetles to release their yellowish defensive secretion, which then solidified onto the cuticle. The solidified secretions were transferred with an insect pin to a 1-ml Reactivial,[3] diluted with diethyl ether, and examined by capillary gas chromatography–mass spectrometry (GC-MS). Other beetles were killed by freezing at −30°C, the entire insect extracted with diethyl ether, and then the extract was examined by GC-MS. Both methods with either population of beetles gave the same mixture of low-molecular-weight components in the same relative proportions. Therefore, all subsequent analyses utilized the whole-body extraction procedure.

Preparative Methods. Approximately 900 adult *T. brevicornis* (10.23 g)

[3]Mention of a proprietary product in this paper does not imply its approval by USDA to the exclusion of other products that may also be suitable.

were killed by freezing and then extracted 3× with 25-ml portions of diethyl ether. The combined extracts were concentrated in vacuo to yield 170 mg of a dark reddish yellow oil. This oil was chromatographed through a 2 × 20-cm column of BioSil A with 60-ml fractions of hexane, 1:9, 2:8, 4:6, 6:4, 8:2 ether–hexane, and 100% ether. Fractions were collected (7 ml) and monitored by GC-MS. Fractions containing aromatic components (10% ether and 20% ether) were concentrated in vacuo and further purified by thin-layer chromatography. Unactivated Kieselgel 60 F254 plates were predeveloped in $CHCl_3$, then streaked with CH_2Cl_2 solutions of fractions from the preparative column chromatography and redeveloped in $CHCl_3$, air dried, and examined under long-wavelength UV light. The β-hydroxy ketones, quinones, and esters all appeared as separate light-blue fluorescent bands. These bands were scraped from the plates and thoroughly extracted with CH_2Cl_2. The resulting CH_2Cl_2 solutions were concentrated in vacuo to yield the pure defensive secretion components. Individual components were stored in CH_2Cl_2 until used.

Quantitative Analysis of Individual Insects. Individual live beetles were placed in microvials constructed from Pasteur pipets and 50 μl of CH_3CN containing 0.5 μg vanillin/μl (as an internal standard) was then added. After 20 min, 1-μl aliquots of the CH_3CN solution were analyzed by capillary GC using a flame ionization detector. A comparison of peak areas of the internal standard to those of the quinones, ketones, and esters was used to calculate the abundance of these chemicals in individual beetles. Some of the beetles were subsequently crushed in the microvial and reanalyzed to assess how much of the defensive secretions was not recovered by the 20-min soaking.

Derivatization. A 10-μl portion of the ether solution from our preliminary isolation was treated with 100 μl Trisil® (Pierce Chemical Company, Rockford, Illinois), held at room temperature for 30 min, and then examined by GC-MS.

Instrumentation. GC-MS analyses were conducted on a Hewlett Packard model 5790A capillary GC (Hewlett Packard, Inc., Palo Alto, California) equipped with a 30-m × 0.2-mm DB-1 capillary column (J & W Scientific, Inc., Rancho Cordova, California), interfaced to a Hewlett Packard model 5970 mass selective detector operated at 70 eV, and a Hewlett Packard model 5730A gas chromatograph containing a 12.5-m × 0.2-mm HP cross-linked methyl silicone capillary column with a flame ionization detector. Nitrogen was the carrier gas, and injections utilized the splitless mode. Signals from the GC were stored and analyzed using a Hewlett Packard model 3380 Integrator. [^{1}H]NMR spectra were acquired on a Bruker WM-400 spectrometer (USA Bruker Instruments, Inc., Billerica, Massachusetts) using a 5 mm C/H probe, 32 K data points over a 6024-Hz spectral width, a 2.0-sec delay and, unless otherwise indicated, the spectra were processed with a 0.1-Hz line broadening. The samples were dissolved in "100%" $CDCl_3$ (Aldrich Chemical Co., Milwaukee, Wisconsin), and the chemical shifts were referenced to the protiochloroform peak taken as 7.26 ppm relative to tetramethylsilane (0 ppm). Specific reso-

nance homonuclear decouplings were done with 10L to 20L (low) settings using a 5-sec total delay time. All of the specific compound resonances reported integrated to the correct number of protons within ±10% when $C(O)CH_3$ or OCH_3 were taken as 3.0, except for the methyl protons of the 6-methyl compound **3**, which indicated approximately four hydrogens due to an overlapping signal from an unidentified impurity.

Sources of Chemicals. Reference compounds were either purchased from Aldrich Chemical Company, Milwaukee, Wisconsin, or were synthesized by R. Howard using standard synthetic procedures. Dr. Ronald Bentley, University of Pittsburgh, Pittsburgh, Pennsylvania, generously provided a series of aromatic esters as model compounds for our mass spectral studies.

RESULTS

Individual beetles contain substantial quantities of quinones, ketones, and esters in their defensive secretions (Table 1). A comparison of the amount extracted by simply soaking the intact beetle to that obtained by crushing the insect in the solvent indicates that soaking removes only about 20% of the actual amount present (Table 1). Since individual beetles weigh ca. 10 mg, their quinones and aromatic defensive secretion components together comprise ca. 2% of their biomass, with the ketones and esters representing ca. 0.25% of the insects biomass.

The acetophenone **1** and propiophenone **2**, which we isolated from *T. brevicornis*, were identical in all respects with the compounds previously isolated from *T. castaneum* (Howard et al., 1986). Our previous structure proofs relied on mass spectral and infrared spectral data coupled with total synthesis. In this paper we report 400-MHz [^{1}H]NMR data which further confirm the two reported structures (Figure 1A and 1B; Table 2). Compound **1** showed two methyl peaks, three aromatic protons, and a singlet far downfield at 12.716 ppm. The

TABLE 1. QUANTITIES OF QUINONES, AROMATIC KETONES, AND AROMATIC ESTERS EXTRACTED FROM WHOLE AND CRUSHED *Tribolium brevicornis* LeC.

Compound		Amount (μg per beetle, $\bar{X}$ ± SEM)	
		Whole (N = 18)	Crushed (N = 5)
2-Methylbenzoquinone		13.1 ± 3.1	51.9 ± 9.0
2-Ethylbenzoquinone		32.1 ± 7.2	122.9 ± 24.7
2′-Hydroxy-4′-methoxyacetophenone	**1**	0.4 ± 0.1	1.3 ± 0.2
2′-Hydroxy-4′-methoxypropiophenone	**2**	3.7 ± 0.8	20.6 ± 5.3
Methyl 2,5-dihydroxy-6-methylbenzoate	**3**	0.4 ± 0.1	3.8 ± 0.6
Methyl 2,5-dihydroxy-6-ethylbenzoate	**4**	0.4 ± 0.1	2.8 ± 0.4

most upfield of the aromatic resonances (Figure 1A) at 6.360 ppm was a doublet with a 2.4-Hz coupling constant, whereas the signal just downfield from it (6.390 ppm) was a doublet of doublets with 8.8- and 2.5-Hz coupling constants. The most downfield of the aromatic peaks (7.575 ppm) showed only the 8.8-Hz coupling. The observed coupling pattern strongly suggests ortho coupling between the 7.575 and 6.390 ppm resonances, combined with meta coupling between the 6.390 and 6.360 ppm protons, as expected for the trisubstituted aromatic ring of **1.** Table 2 compares the observed chemical shifts to those previously reported for **1** (Suzuki et al., 1975).

A confirmatory NMR analysis of the propiophenone **2** proved to be less straightforward. Its spectrum (Figure 1B) showed a singlet for the methoxy methyl resonance at 3.837 ppm, an upfield triplet and quartet indicative of an ethyl group, a complex set of peaks in the aromatic region, and a hydroxy proton signal at 12.845 ppm. Homonuclear decoupling of the 6.439 ppm resonance essentially collapsed the 7.664 ppm multiplet, indicating that the apparent complexity was due to second-order effects. Chemical shifts and coupling constants for the three aromatic protons were therefore obtained by simulation techniques using the Bruker PANIC program. The observed and calculated aromatic region spectra are compared in Figure 1B and C, and the calculated shifts and coupling constants for all protons are given in Table 2. These results confirm the 1,2,4-trisubstitution pattern of **2.**

The structures of the two esters **3** and **4** were established using mass spectral and NMR analyses in conjunction with selected derivatizations. The mass spectrum of **3** (Figure 2) showed a prominent molecular ion at *m/z* 182 which

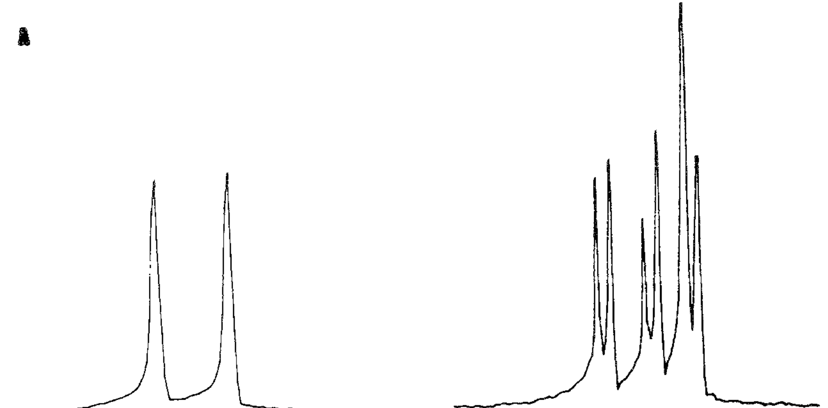

FIG. 1A. [^{1}H]NMR spectra at 400 MHz of the aromatic region of the ketones of *T. brevicornis* in $CDCl_3$. The spectra in A and B were processed with −0.25 Hz line broadening. (A) Compound **1,** 2′-hydroxy-4′-methoxyacetophenone.

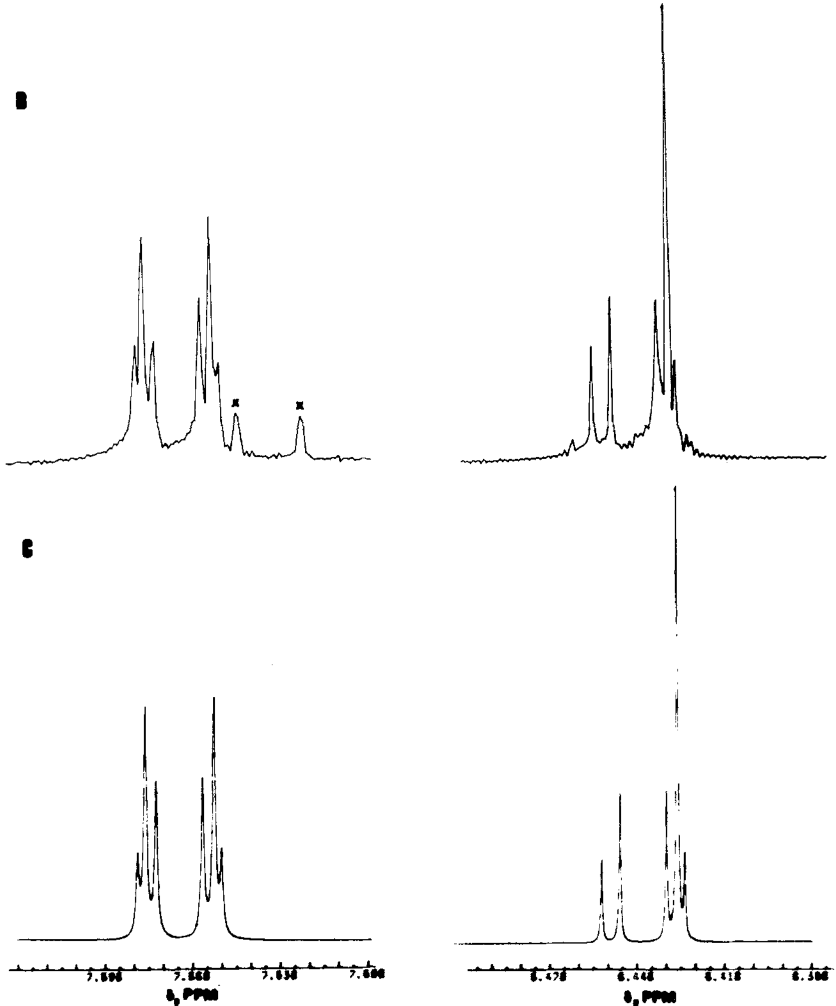

FIG. 1B, C. [^{1}H]NMR spectra at 400 MHz of the aromatic region of the ketones of *T. brevicornis* in $CDCl_3$. (B) compound **2**, 2′-hydroxy-4′-methoxypropiophenone; (C) simulated aromatic region spectrum of **2** using the Bruker PANIC program. In this spectrum the downfield set of peaks are displayed with a line width of 0.3 Hz and the upfield sets of peaks with a 0.5 Hz line width. The peaks marked × are from an unidentified contaminant.

fits a molecular formula of $C_9H_{10}O_4$. The base peak at *m/z* 150 (M-32) and the prominent ion at *m/z* 122 (M-32-28) strongly suggested that compound **3** was a substituted salicylate (Budzikiewicz et al., 1967). The two subsequent losses of mass 28 to give the ions at *m/z* 94 and *m/z* 66 indicated an aromatic molecule

TABLE 2. ^1H CHEMICAL SHIFTS AND COUPLING CONSTANTS OF 2′-HYDROXY-4′-METHOXYACETOPHENONE **1** AND 2′-HYDROXY-4′-METHOXYPROPIOPHENONE **2**

Compound	[^{1}H]NMR parameter[a]	H atom							
		3-H	5-H	6-H	C(O)CH_3	C(O)$\underline{CH_2}CH_3$	C(O)$CH_2\underline{CH_3}$	OCH_3	2-OH
1	δ	6.360(d)	6.390(dd)	7.575(d)	2.504(s)			3.787(s)	12.716(s)
	δ^b	6.33	6.40	7.58	2.51			3.80	12.72
	J		8.8	8.8					
		2.4	2.5						
2	δ^c	6.428(dd)	6.439(dd)	7.664(dd)		2.955(q)	1.230(t)	3.837(s)	12.845(s)
	J^c		8.79	8.79		7.33	7.34		
		2.50	2.50						
		0.51		0.51					

[a] δ is in ppm downfield from tetramethylsilane (0 ppm) as referenced to the $CHCl_3$ solvent peak taken as 7.26 ppm. J values are in Hz.
[b] Suzuki et al. (1975).
[c] The aromatic proton δ and J values were obtained from simulated spectra (see Results).

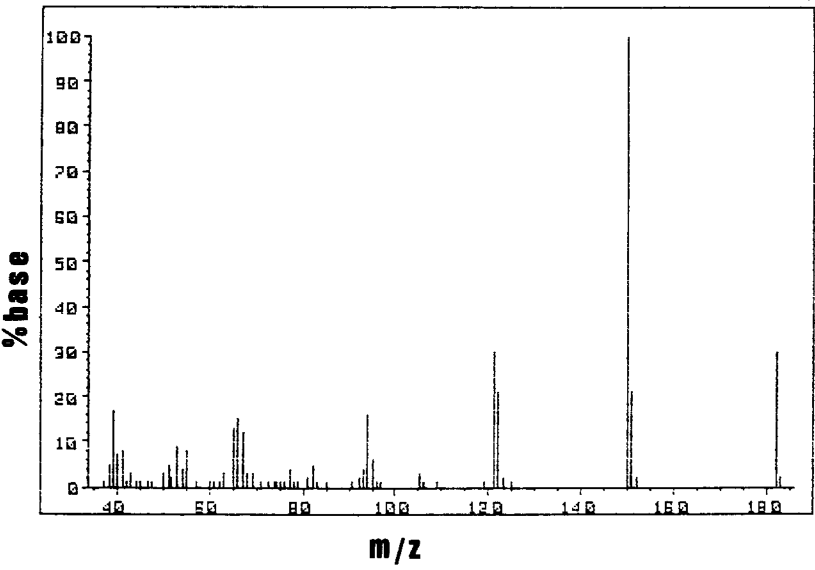

FIG. 2. EI mass spectrum of methyl 2,5-dihydroxy-6-methylbenzoate **3**.

with two ring hydroxyls, and by subtraction the remaining substituent on the ring had to be a methyl group. Derivatization of **3** with TMS yielded a product with a molecular ion (base peak) at *m/z* 268, corresponding to the addition of one methyl group and one trimethylsilyl residue to the starting ester. Other major fragment ions from this derivative occurred at *m/z* 253 (loss of methyl), *m/z* 237 (loss of -OCH_3), and *m/z* 73 (trimethylsilyl). These data thus confirmed our conclusion regarding the presence of two hydroxyl groups on the aromatic ring.

To this point the data argued for a trisubstituted methyl benzoate with one of the substituents being a hydroxy ortho to the carboxylate function. The placement of the methyl group could not be unequivocally determined from mass spectral data alone, although we suspected it to be at the other ortho position to the carboxylate functionality, from biosynthetic considerations and by analogy to the esters reported by Gnanasunderam et al. (1984). Similarly, the precise location of the second hydroxyl could not be determined solely from mass spectral evidence. It was possible, however, to exclude the second hydroxyl being at C-3 of the aromatic ring from the following considerations. Hydroquinones are known to undergo facile oxidation to the corresponding quinones in the hot injection ports of gas chromatographs, and indeed the production of such quinones was always observed in our analyses. Orthoquinones have very prominent $M + 2$ ions in their mass spectra which frequently are as intense as the M^+ ions (Zeller, 1974). The quinone derived from **3** has a prominent M^+

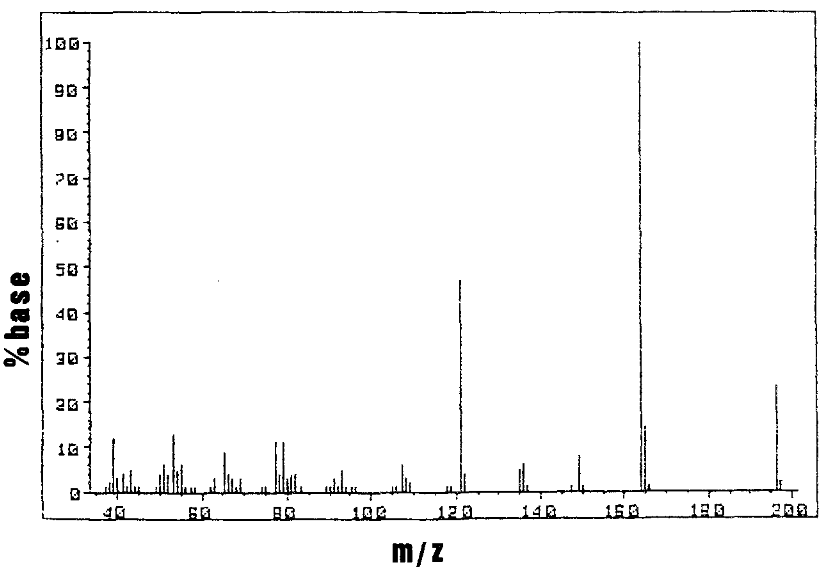

FIG. 3. EI mass spectrum of methyl 2,5-dihydroxy-6-ethylbenzoate **4**.

ion at *m/z* 180 and an M + 2^+ ion abundance of ca. 3% which clearly does not match known *o*-quinone behavior.

Inspection of the mass spectrum of compound **4** (Figure 3) suggested that it was probably an alkyl homolog of **3**. A prominent molecular ion at *m/z* 194 suggested a molecular formula of $C_{10}H_{12}O_4$, and the base peak again arose by a loss of methanol. The three subsequent successive losses of mass 28 producing ions at *m/z* 136, 108, and 80 again indicated a dihydroxy methyl benzoate with one of the hydroxyls ortho to the carboxylate group. The prominent ion at *m/z* 121 at first did not seem consistent with **4** being the ethyl homolog of methyl substituted **3**, and we considered the possibility that **4**, like **1** and **2**, contained a methoxy group. This possibility was tested by subjecting **4** to derivatization with the TMS reagent and examining the mass spectrum of the resulting derivative. That product had an M^+ at *m/z* 282 corresponding, as in **3**, to the addition of both CH_3 and trimethylsilyl to the starting ester, thus demanding that both ring oxygen functionalities be hydroxyls. As with ester **3**, the second hydroxyl was not at carbon 3 because of the low abundance of the M + 2 ion of the corresponding quinone. Therefore, other possible mechanisms that might lead to the ion at *m/z* 121 from the base peak at *m/z* 164 were considered. If the *m/z* 164 ion had a 6-ethyl substituent, it rationally could produce the *m/z* 121 ion by a loss of the methyl group from the ethyl side chain in conjunction with a ring expansion and 1,2-H shift to produce a keto tropylium ion, which in turn could lose carbon monoxide to produce a resonance stabilized

p-hydroxybenzene acylium ion of mass 121. Placement of an ethyl group elsewhere on the ring would not be expected to lead to a prominent *m/z* 121 ion nor would any dimethyl structure that we considered (Budzikiewicz et al., 1967). Our analysis to this point for compounds **3** and **4** strongly suggested that we were dealing with methyl 2,X-dihydroxy-6-alkylbenzoates.

From biosynthetic considerations it seemed likely that the second hydroxyl was at C-5. We accordingly synthesized methyl 2,5-dihydroxybenzoate **5** from the corresponding acid and used it as a standard for NMR comparisons. In Figure 4A, the [^{1}H]NMR spectrum of **5** is compared with that of ester **3** from *T. brevicornis* (Figure 4B). The natural product showed an additional 3H singlet resonance at 2.432 ppm due to the ring methyl. The chemical shift for the 6-methyl protons was in good agreement with the 2.48 ppm chemical shift reported for that group in methyl 2-hydroxy-6-methylbenzoate (Chan and Brownbridge, 1980). The 2.432 ppm singlet clearly is not a methoxy methyl since those chemical shifts are at 3.8 ppm or higher (Table 2). In addition, Chan and Brownbridge (1980) report the chemical shift of the C-4 methyl in methyl 2-hydroxy-4,6-dimethylbenzoate to be at 2.25 ppm.

In the aromatic region the alkylated benzoate **3** has only two resonances, 6.750 (d) and 6.933 ppm (d) compared to three for compound **5:** 6.872 (d), 7.010 (dd), and 7.273 ppm (d) (Figure 4A and B). That the missing proton at 7.273 ppm corresponded to the 6-position followed directly from the assignments of the ring hydrogens of **5.** Since the 7.010 ppm signal of **5** was a doublet of doublets with coupling constants of 8.9 and 3.1 Hz, that resonance corresponded to H-4, because 3.1 Hz would be unrealistically large for para coupling between H-3 and H-6 and therefore must have arisen from four-bond coupling between H-4 and H-6. The same coupling of 3.1 Hz was observed only at the 7.273 ppm resonance which identifies it as H-6.

Examination of the [^{1}H]NMR spectrum of ester **4** indicated it to be very similar to that of compound **3.** Differences include the absence of the 3H singlet at 2.432 ppm and the presence of a triplet at 1.191 ppm and a quartet at 2.917 ppm with identical coupling constants (J = 7.4 Hz), thus confirming the presence of an ethyl group in this compound. The shift of 2.917 ppm for the methylene protons excludes the possibility of an ethoxy derivative since in that case the shift would have been 4.0 ppm or greater (Jackman and Sternhell, 1969). The chemical shifts and coupling constants for compounds **3, 4,** and **5,** as well as literature (Gnanasunderam et al., 1984) values for methyl 6-methyl- and methyl 6-ethylsalicylate (**6** and **7,** respectively) are listed in Table 3.

The chemical shift values for the hydroxyl protons reported in Table 3 varied somewhat from sample to sample, apparently depending on the states of dryness of the sample. This variability is a well-known phenomenon for phenols, as evidenced by the range of chemical shift values reported for salicylates in the Sadtler Standard NMR Spectra (1970) (5 to ca. 13 ppm in $CDCl_3$). For compounds **3** and **4,** two OH peaks were observed near 12.75 and 10.5 ppm.

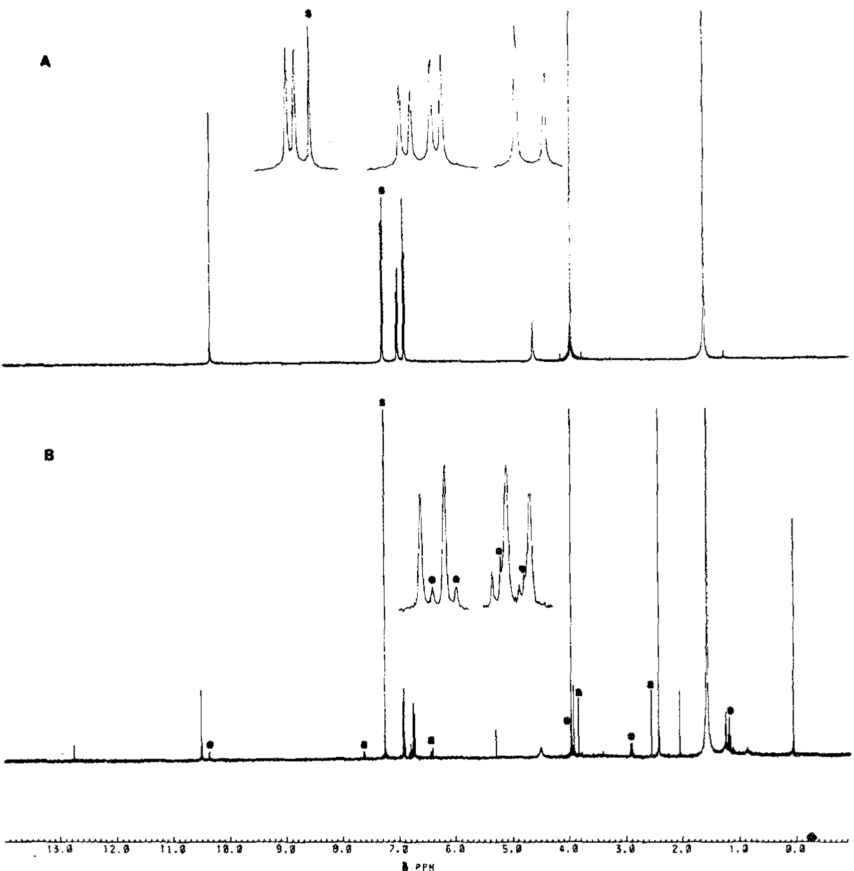

FIG. 4. [^{1}H]NMR spectra of the methyl benzoates at 400 MHz in $CDCl_3$. (A) Authentic methyl 2,5-dihydroxybenzoate. The expanded peaks are those between 6.8 and 7.3 ppm of the full spectrum with "s" denoting the protiochloroform peak. The large peak at ca. 1.5 ppm is an impurity from the isolation procedures, probably hexane. (B) Methyl 2,5-dihydroxy-6-methylbenzoate from *T. brevicornis*. The expanded peaks are those between 6.7 and 7.0 ppm of the full spectrum. The letter "e" above a peak denotes that it is from the corresponding 6-ethylbenzoate **4** and "a" indicates the acetophenone **1**. The other smaller peaks between 5.5 and 1.0 ppm, as well as the larger peaks at ca. 1.5 and 0 ppm, are contaminants from the extraction procedures.

Unfortunately, the spectrum of compound **5** (Figure 4A) gave only one signal in this region (10.4 ppm), but showed a broad feature in the water region at 5.5 ppm. The 2-O<u>H</u> chemical shifts in the β-hydroxy ketones **1** and **2** (Table 2) came at ca. 12.8 ppm, and the corresponding hydroxy signals for methyl 2-hydroxy-6-methyl benzoate **6** and methyl 2-hydroxy-4,6-dimethylbenzoate **8** are reported to occur at 11.03 and 11.00 ppm, respectively (Chan and Brownbridge,

TABLE 3. ^{1}H CHEMICAL SHIFTS AND COUPLING CONSTANTS OF METHYL 6-ALKYL-2,5-DIHYDROXYBENZOATES (**3**, **4**), METHYL 2,5-DIHYDROXYBENZOATE (**5**), AND METHYL 6-ALKYL-2-HYDROXYBENZOATES (**6**, **7**)

Compound	[^{1}H]NMR parameter[a]	H atom									
		3-H	4-H	5-H	6-H	CO_2CH_3	6-CH_3	6-$\underline{CH_2}CH_3$	6-$CH_2\underline{CH_3}$	2-OH	5-OH
3	δ	6.750(d)	6.933(d)			3.970(s)	2.432(s)			12.754(s)	10.501(s)
	J	8.8	8.9								
	δ(5-3)	0.122	0.077			−0.049					−0.113
	δ(6-3)	−0.02	0.34			0.01	<0.1			−1.47	
4	δ	6.758(d)	6.923(d)			3.975(s)		2.917(q)	1.191(t)	12.755(s)	10.369(s)
	J	8.8	8.8					7.4	7.4		
	δ(5-3)	0.114	0.087			−0.054					0.019
	δ(7-4)	0.00	0.35			−0.255[b]		0.02	0.01	−1.48	
5[c]	δ	6.872(d)	7.010(dd)		7.273(d)	3.921(s)				ca 5.5(s, br)	10.38(s)
	J	8.9	8.9								
			3.1		3.1						
6[d]	δ	6.73	7.27(dd)	6.85(d)		3.98(s)	2.5(s)			11.28(s)	
	J	7.4	7.4	8.2							
			8.2								
7[d]	δ	6.76(d)	(7.27)[e]	6.86(d)		3.72(s)		2.94(q)	1.2(t)	11.3(s)	
	J	7.3		8.0				7.4	7.5		

[a] The chemical shifts, δ, are in ppm downfield from tetramethylsilane (0 ppm) as referenced to the solvent $CHCl_3$ peak taken as 7.26 ppm. The coupling constants, J, are in Hz.
[b] This difference is probably too large because the CO_2CH_3 shift of 3.72 ppm reported for 7 seems to be in error.
[c] Authentic methyl 2,5-dihydroxybenzoate (see Methods and Materials).
[d] Gnanasunderam et al. (1984), who used δ = 7.27 ppm for the $CHCl_3$ reference. Assignments of 3-H and 5-H for **6** and **7** are based on the current results for **3** and **4**.
[e] Peak obscured by $CHCl_3$.

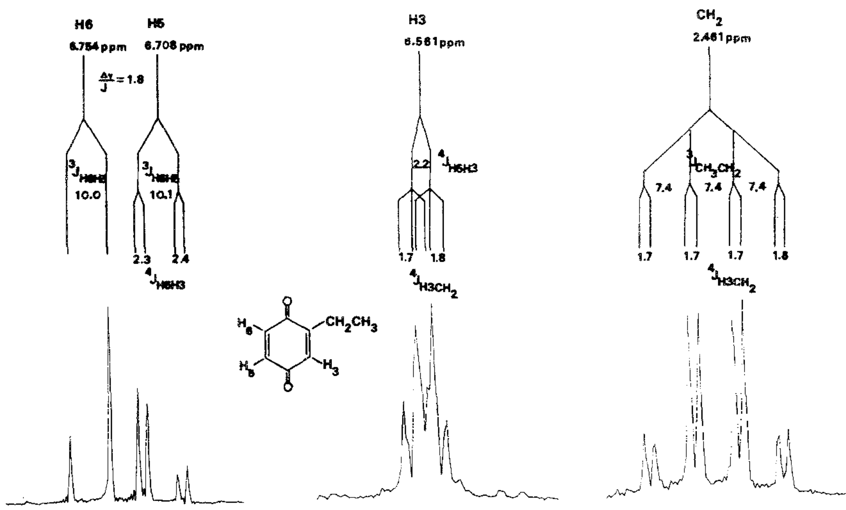

FIG. 5. [^{1}H]NMR multiplets of 2-ethylbenzoquinone from *T. brevicornis* at 400 MHz in $CDCl_3$. The triplet at 1.136 ppm for CH_2CH_3 did not show any long-range coupling and is not shown in this figure. The spectrum was processed with a −0.25 Hz line broadening. The coupling constants values shown are those obtained directly from the spectrum at each multiplet.

1980). Although we cannot unequivocally assign chemical shifts to the 2-O<u>H</u> and 5-O<u>H</u> resonances of **3** and **4**, we are tentatively assigning the most downfield resonance to the 2-O<u>H</u> hydrogen since it is involved in internal hydrogen bonding in $CDCl_3$ and matches most closely to the observed chemical shift for the β-hydroxy ketones of **1** and **2**. Thus considering all of the data, the structures of the *T. brevicornis* esters can be assigned as methyl 2,5-dihydroxy-6-methylbenzoate for **3** and methyl 2,5-dihydroxy-6-ethylbenzoate for **4**.

Although 2-methyl- and 2-ethylbenzoquinone are well-known defensive secretion products, we obtained a NMR spectrum of the ethyl derivative (Figure 5) in preparation for biosynthetic studies. Observation at 400 MHz of the long-range coupling between H-3 and the methylene protons of the 3-ethyl group rendered the proton assignments straightforward (Table 4). We note that even at 400 mHz, the aromatic protons still show slight second order effects.

DISCUSSION

The defensive secretion of *T. brevicornis* was previously reported by Markarian et al. (1978) and Wirtz et al. (1978) to contain 2-methylbenzoquinone, 2-ethylbenzoquinone, the corresponding hydroquinones, and 1-pentadecene. These are indeed the major components of this beetle's defensive secretion, but

TABLE 4. ^1H CHEMICAL SHIFTS AND COUPLING CONSTANTS OF 2-ETHYLBENZOQUINONE

[^{1}H]NMR parameter[a]	H atom				
	3-H	5-H	6-H	$\underline{CH_2}CH_3$	$CH_2\underline{CH_3}$
δ	6.561(dt)	6.708(dd)	6.754(d)	2.46(qd)	1.136(t)
J		10.1	10.0	7.4	7.4
	2.2, 1.8	2.4		1.7	

[a] δ is in ppm downfield from tetramethylsilane (0 ppm) as referenced to $CHCl_3$ of the solvent (δ = 7.26 ppm).

we have also found substantial quantities of at least 10 other components, including six additional olefins (Howard, 1987), the two ketones, and the two esters which are the subject of this paper. Complex blends such as these are typical of insect defensive secretions. However, the functions of all components are not always so clear. Components such as the quinones and hydroquinones are presumably cytotoxicants or irritants, whereas the olefins most likely are serving as "biosolvents" to aid in the cuticular penetration of the toxicants (Blum, 1981) and the prostaglandin synthetase inhibitors.

Prostaglandins are local cell hormones which have been isolated from nearly every type of mammalian tissue (Bergstrom et al., 1968), from several nonmammalian vertebrates and marine invertebrates (Bundy, 1984), and from insects (Brady, 1983; Lange, 1984; Murtaugh and Denlinger, 1982; Wakayama et al., 1986). In vertebrates, prostaglandins have been shown to modulate adenylate cyclase activity and facilitate ion transport, nerve transmission, platelet aggregation, and gastrointestinal function (Samuelsson et al., 1978, 1980). In invertebrates, prostaglandins have been shown to mediate ion regulation in mollusks (Graves and Dietz, 1979; Freas and Grollman, 1980), to produce fevers in crayfish (Casterlin and Reynolds, 1978) and scorpions (Cabanac and Guelte, 1980), to regulate oviposition behavior in two species of crickets (Destephano et al., 1982; Loher et al., 1981) and a silkmoth (Yamaja Setty and Ramaiah 1980), and to be required for the normal emergence and flight capabilities of a mosquito (Dadd and Kleinjan, 1984). Future studies will undoubtedly show that prostaglandins play as important a role in invertebrate physiology as they do in mammals.

Given that prostaglandins do play such a vital role in the normal physiology of animals, it is not surprising that insects have evolved chemicals that would inhibit the biosynthesis of prostaglandins in their competitors, parasites, or predators. At least three orders of insects (Coleoptera, Hemiptera, and Hymenoptera) have been reported to possess exocrine secretions that contain aromatic compounds analogous to those that we have found in *T. brevicornis*

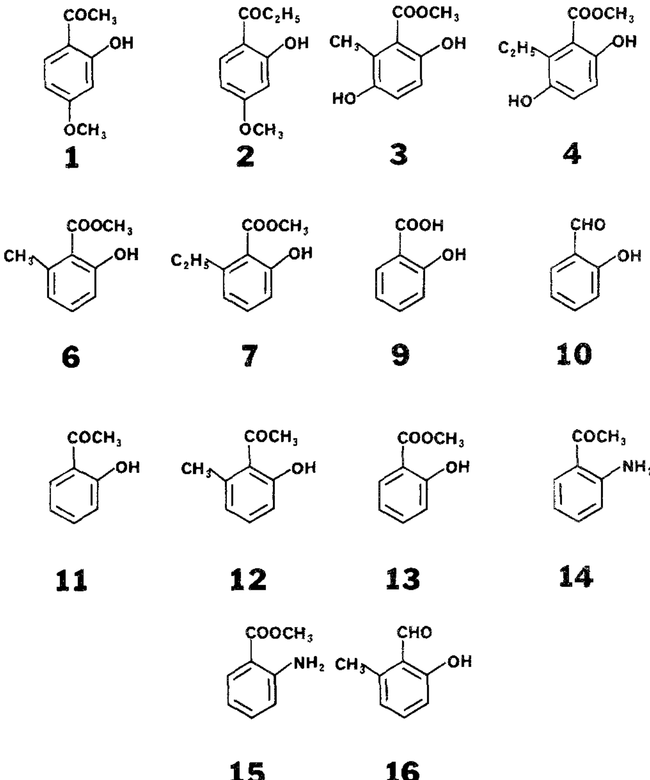

FIG. 6. Insect exocrine chemicals with known or suspected prostaglandin synthetase activity.

(Blum, 1981) (Figure 6). The common feature of all these compounds is the presence of the β-hydroxy or β-amino carbonyl moiety. Compounds **1, 2, 10, 13, 14,** and **15** (Figure 6) have been shown to be excellent prostaglandin synthetase inhibitors in both mammalian and insect systems (Howard et al., 1986; Jurenka et al., 1986). Although salicylic acid (**9,** Figure 6) has been reported to be only minimally active as a PSI, it has been shown to be converted in vivo to 2,5-dihydroxybenzoic acid, which is a very active PSI compound (Robinson and Vane, 1974). Such evolutionary "backfiring" of an organism's detoxification mechanism is of course well known to insecticide toxicologists (Matsumura, 1975). We consider it highly likely that the compounds in Figure 6, including the *T. brevicornis* esters **3** and **4,** which have not yet been tested for their PSI activity, will also prove to be excellent prostaglandin synthetase inhibitors, as will other compounds of a similar structure from other insects. Studies are now underway in our laboratories to identify such chemicals, to elucidate their ecological roles, and to further clarify the physiological roles of prostaglandins in insects.

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EXTREME SENSITIVITY OF GRANDISAL TO ACIDS

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Abstract—Attempted purification of synthetic racemic grandisal **1** by silica gel chromatography resulted in severe decomposition. The nature of this reaction was studied on silica gel and in an ether solution of *p*-toluenesulfonic acid. The same products resulted from both reaction systems, although in different ratios. Five racemic, rearrangement products were isolated by preparative GC and identified as follows: (1*RS*,3*SR*,6*RS*)-1-methyl-5-methylenebicyclo[4.2.0]octan-3-ol (**4**); (1*RS*,3*RS*,6*RS*)-1-methyl-5-methylenebicyclo[4.2.0]octan-3-ol (**5**); (1*RS*,3*RS*,6*RS*)-1,5-dimethylbicyclo[4.2.0]oct-4-en-3-ol (**6**); (1*RS*,3*SR*,6*RS*)-1,5-dimethylbicyclo[4.2.0]oct-4-en-3-ol (**7**); and 3-methyl-7-methylenecyclooct-3-en-1-ol (**8**). The stereochemical assignments are based on our proposed mechanism, which also accounts for all products observed. The racemic bicyclic enone (**3**) was a by-product of grandisol (**2**) oxidation.

Key Words—Grandisal, rearrangement, isomerization, acid catalysis, silica gel catalysis, *Pissodes* spp., Coleoptera, Curculionidae.

INTRODUCTION

Grandisal (*cis*-2-isopropenyl-1-methylcyclobutaneethanal) (**1**)[1] has been found in incubations of macerated boll weevil abdomens, *Anthonomus grandis* Boheman, but the compound was inactive (Hedin, 1977). This aldehyde, along with the corresponding alcohol, grandisol **2**, has been identified in the males of *Pissodes approximatus* and *Pissodes strobi* (Booth et al., 1983). These compounds, as racemates, are effective aggregation pheromone components for *P.*

[1] Structures are drawn as single enantiomers to represent relative stereochemistry; all compounds are racemic.

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approximatus (Booth and Lanier, 1974; Booth, 1978), but their function in *P. strobi* is not clear.

During preparation of racemic grandisal **1** from grandisol **2** by pyridinium chlorochromate oxidation, we attempted purification by silica gel chromatography, in the course of which the sample decomposed. This extreme sensitivity led us to investigate the decomposition both on silica gel and in ether containing a catalytic amount of *p*-toluenesulfonic acid and to identify the products. We call attention to particular hazards of silica gel chromatography.

METHODS AND MATERIALS

GC isolations were carried out on a Varian-Aerograph model 1700 equipped with an effluent splitter and a thermal gradient collector (Brownlee and Silverstein, 1968). The following packed columns were used: column A was a 2.4-m × 7-mm-ID glass column packed with 10% Carbowax 20 M on Chromosorb W 60–80 mesh; column B was a 6.1-m × 4-mm-ID glass column packed with 4% Apiezon-L on Chromosorb G, 60–80 mesh. Column B was used isothermally to calculate Kovats' retention indices (Kovats, 1958). GC-MS was performed on a Finnigan GLC 9500 fitted with a 30-m × 0.2-mm-ID DB-1 capillary column (column C) interfacing a Finnigan 4000 mass spectrometer (ionizing voltage 70 eV). High-resolution mass spectra were obtained on an AEI-MS-902 instrument with 10,000 resolution. Infrared spectra were obtained on a Perkin-Elmer 1310; samples were run as neat films between NaCl plates. [^{1}H]NMR spectra were obtained on a Varian XL-100 (100 MHz) and a Bruker Widebore WM-360 (360 MHz) instrument. [^{13}C]NMR spectra were recorded on the Varian XL-100 instrument at 25.2 MHz. All NMR samples were run in $CDCl_3$ and are recorded as ppm from Me_4Si as internal reference. Elemental analyses were performed at Micro-Analysis, Inc., Wilmington, Delaware. Synthetic grandisol was obtained from Chemical Samples Company, Columbus, Ohio, and was judged to be about 92% pure by NMR and GC analysis.

cis-*2-Isopropenyl-1-methylcyclobutaneethanal (Racemic Grandisal) (1).* The procedure is essentially the one used by Corey and Suggs (1975). In a 100 ml, round-bottom flask fitted with a reflux condenser was suspended a mixture of 2.10 g (9.75 mmol) of pyridinium chlorochromate and 0.16 g (1.95 mmol) of sodium acetate in 10 ml of anhydrous methylene chloride. Racemic grandisol (**2**) (1.00 g, 6.5 mmol) in 5 ml of anhydrous methylene chloride was added in one portion to the magnetically stirred solution at room temperature. After 2 hr, the oxidation, monitored by GC, was judged complete. The black reaction mixture was diluted with five volumes of anhydrous ether and decanted from the black gum. The black solids were washed thoroughly with 3 × 10 ml of ether. The combined solution was passed through a short pad of Florisil. Pure grandisal was obtained in 68% yield by chromatography on column A (125°C

for 12 min, programmed to 150°C at 4°/min, N_2 60 ml/min): [^{1}H]NMR (Figure 1); MS *m/e* 53(13%), 55(15%), 56(4%), 57(8%), 67(60%), 68(100%), 69(18%), 79(4%), 81(8%), 83(5%), 93(7%), 95(4%), 96(5%), 97(5%), 107(2%), 108(37%), 109(22%), 123(1%); Kovats' index 1122.

(1RS,6RS)-*1,5-dimethylbicyclo*[*4.2.0*]*oct-4-en-3-one* (*3*).[2] Bicyclic enone **3** was the major by-product (5%) of grandisol (**2**) oxidation. After the initial fractionation on column A above, final purification was done with column B (isothermal at 150°C, N_2 60 ml/min): [^{1}H]NMR (Figure 2); [^{13}C]NMR δ21.7, 23.8, 27.7, 30.6, 37.2, 44.5, 46.6, 125.7, 162.0, 199.8; IR 1655, 1620, 1281, and 825 cm^{-1}; MS *m/e* 39(30%), 40(5%), 41(22%), 51(8%), 52(4%), 53(10%), 56(4%), 65(12%), 67(42%), 77(20%), 79(60%), 80(8%), 91(13%), 93(15%), 94(28%), 95(100%), 96(6%), 108(22%), 121(4%), 122(64%), 135(6%), 150(13%); Kovats' index 1242. Anal. calcd for $C_{10}H_{14}O$: C, 79.96; H, 9.39. Found: C, 80.03; H, 9.25.

Silica Gel-Catalyzed Rearrangement of Pure Grandisal (1). The silica gel (grade 923, 100–200 mesh, Aldrich) was activated at 130–140°C for 2 hr and cooled to room temperature under N_2. Two grams of this material was packed into a 20-cm × 1-cm-OD glass column as a pentane slurry under N_2. Grandisal (10 mg, 0.066 mmol) in 0.5 ml of pentane was added to the top of the column. The stopcock was opened, and the grandisal was allowed to drain into the gel bed. The column was closed at both ends and allowed to stand at room temperature for 14 hr. The column was eluted with diethyl ether. Removal of the solvent and short path distillation at 75°C (0.5 mm Hg) of the residual oil gave 8.5 mg of volatile components.

p-*Toluenesulfonic Acid-Catalyzed Rearrangement of Pure Grandisal (1).* To 10 mg (0.066 mmol) grandisal and 0.76 ml anhydrous ether in a 20-ml vial was added 0.24 ml (0.007 mmol) of a 5 mg/ml solution of *p*-toluenesulfonic acid monohydrate in anhydrous ether. The solution was held at room temperature for 12 hr. The reaction solution was shaken with dry sodium carbonate powder and then passed through 0.3 g of Florisil in a disposable pipet (17 cm × 7 mm OD). The solvent was removed, and vacuum distillation at 75°C (0.5 mm Hg) gave 8.7 mg of volatile material.

*Isolation of (1*RS,*3*SR,*6*RS*)-1-Methyl-5-methylenebicyclo*[*4.2.0*]*octan-3-ol (4j), (1*RS,*3*RS,*6*RS*)-1-Methyl-5-methylenebicyclo*[*4.2.0*]*octan-3-ol (5), (1-*RS,*3*RS,*6*RS*)-1,5-Dimethylbicyclo*[*4.2.0*]*oct-4-en-3-ol (6), (1*RS,*3*SR,*6*RS*)-1,5-Dimethylbicyclo*[*4.2.0*]*oct-4-en-3-ol (7), and 3-Methyl-7-methylenecyclooct-3-en-1-ol (8).* Since both rearrangement reactions gave the same products (although in different ratios, Table 1), the same procedure was used to isolate products from either system. Initial fractionation on column A (130°C for 12 min, programmed to 150°C at 4°/min, N_2 80 ml/min) gave four fractions, which

[2]In these racemic compounds, the relative configuration is shown by designating the first chiral center as *RS* and the others relative to it.

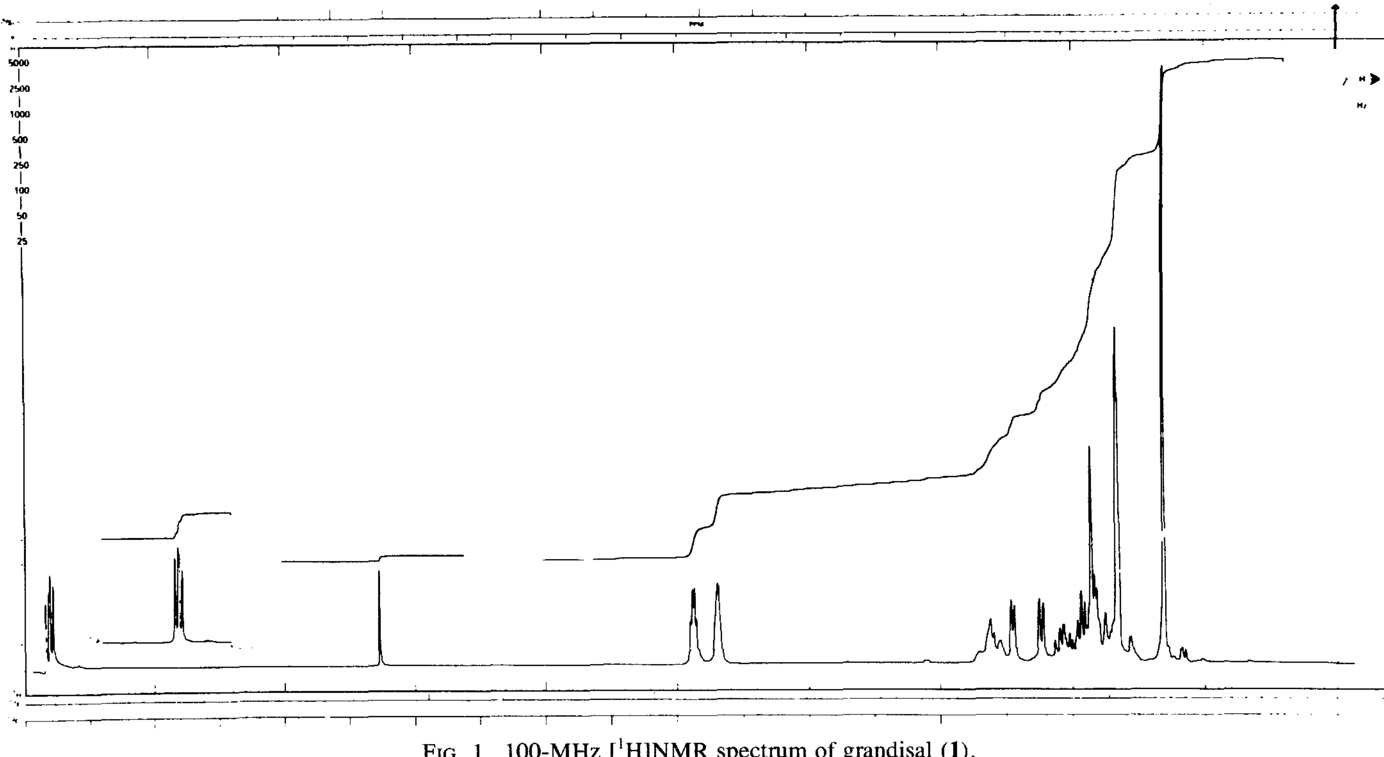

FIG. 1. 100-MHz [^{1}H]NMR spectrum of grandisal (1).

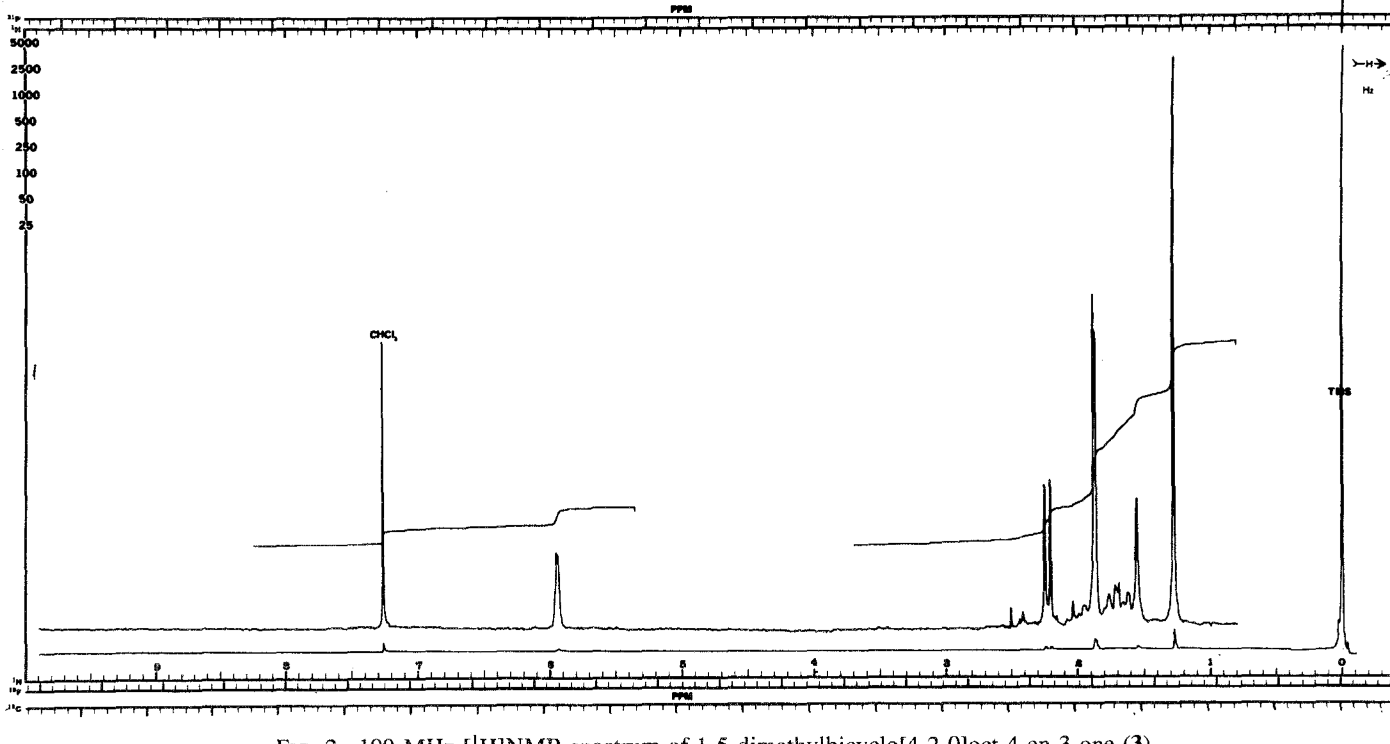

FIG. 2. 100-MHz [^{1}H]NMR spectrum of 1,5-dimethylbicyclo[4.2.0]oct-4-en-3-one (3).

TABLE 1. RELATIVE RATIO OF ISOMERS FROM GRANDISAL REARRANGEMENT

	Isomer (%)	
Compound	From silica gel rearrangement	From *p*-TSA rearrangement
4	51	27
5	12	8
6	5	12
7	10	31
8	22	22

were collected separately. The first fraction contained a small amount of unreacted grandisal (**1**). The second fraction from column A was further fractionated on column B (150°C, N_2 60 ml/min) to give a pure compound identified as **4** with the stereochemistry tentatively assigned (see Discussion): [^{1}H]NMR (Figure 3); [^{13}C]NMR δ22.5, 30.2, 39.4, 40.2, 42.9, 46.5, 68.3, 111.8, 146.5; IR 3345, 1638, 1035, 880 cm^{-1}; MS *m/e* 39(58%), 40(12%), 41(100%), 43(28%), 44(6%), 51(12%), 52(6%), 53(29%), 55(30%), 56(15%), 57(20%), 65(15%), 67(49%), 68(71%), 69(51%), 77(27%), 79(47%), 81(52%), 82(8%), 83(9%), 84(12%), 85(7%), 91(49%), 92(12%), 93(36%), 95(36%), 96(31%), 97(38%), 105(14%), 106(50%), 108(97%), 109(41%), 110(7%), 119(23%), 123(8%), 124(26%), 134(12%), 137(21%), 152(4%); Kovats' index 1178. Anal. calcd for $C_{10}H_{16}O$: C, 78.88; H, 10.59. Found: C, 78.78; H, 10.57.

The third fraction from column A was fractionated on column B (150°C, N_2 60 ml/min). Two fractions were obtained. One fraction was shown to be a pure compound and has been identified as **5** with the stereochemistry tentatively assigned: [^{1}H]NMR (Figure 4); IR 3300, 1638, 1030, 880 cm^{-1}; MS *m/e* 39(53%), 40(13%), 41(100%), 43(28%), 44(67%), 51(10%), 53(31%), 55(46%), 56(14%), 57(21%), 65(15%), 67(52%), 68(68%), 69(49%), 77(26%), 79(42%), 81(41%), 82(6%), 83(77%), 84(8%), 85(77%), 91(40%), 92(11%), 93(32%), 95(28%), 96(30%), 97(26%), 105(14%), 106(40%), 108(79%), 119(22%), 121(5%), 123(7%), 124(20%), 134(7%), 137(15%), 152(4%); Kovats' index 1151. High-resolution mass spectrum; calculated for $C_{10}H_{16}O$, 152.1201; found, 152.1198.

The other fraction from column B was shown to be a mixture of two compounds on column C (80°C, programmed after 1 min to 130°C, 4°/min, 16 psi He). These compounds were identified as **6** and **7** (with the stereochemistry tentatively assigned) by comparing them to an authentic mixture of **6** and **7** produced by the reduction of **3**. Capillary GC-MS analysis of **6** gave *m/e* 39(20%), 41(25%), 43(14%), 51(6%), 53(10%), 55(11%), 65(7%), 67(9%), 69(10%), 77(16%), 79(19%), 81(11%), 91(27%), 93(22%), 95(16%), 97(63%), 105(6%), 106(12%), 107(12%), 108(28%), 109(100%), 110(10%),

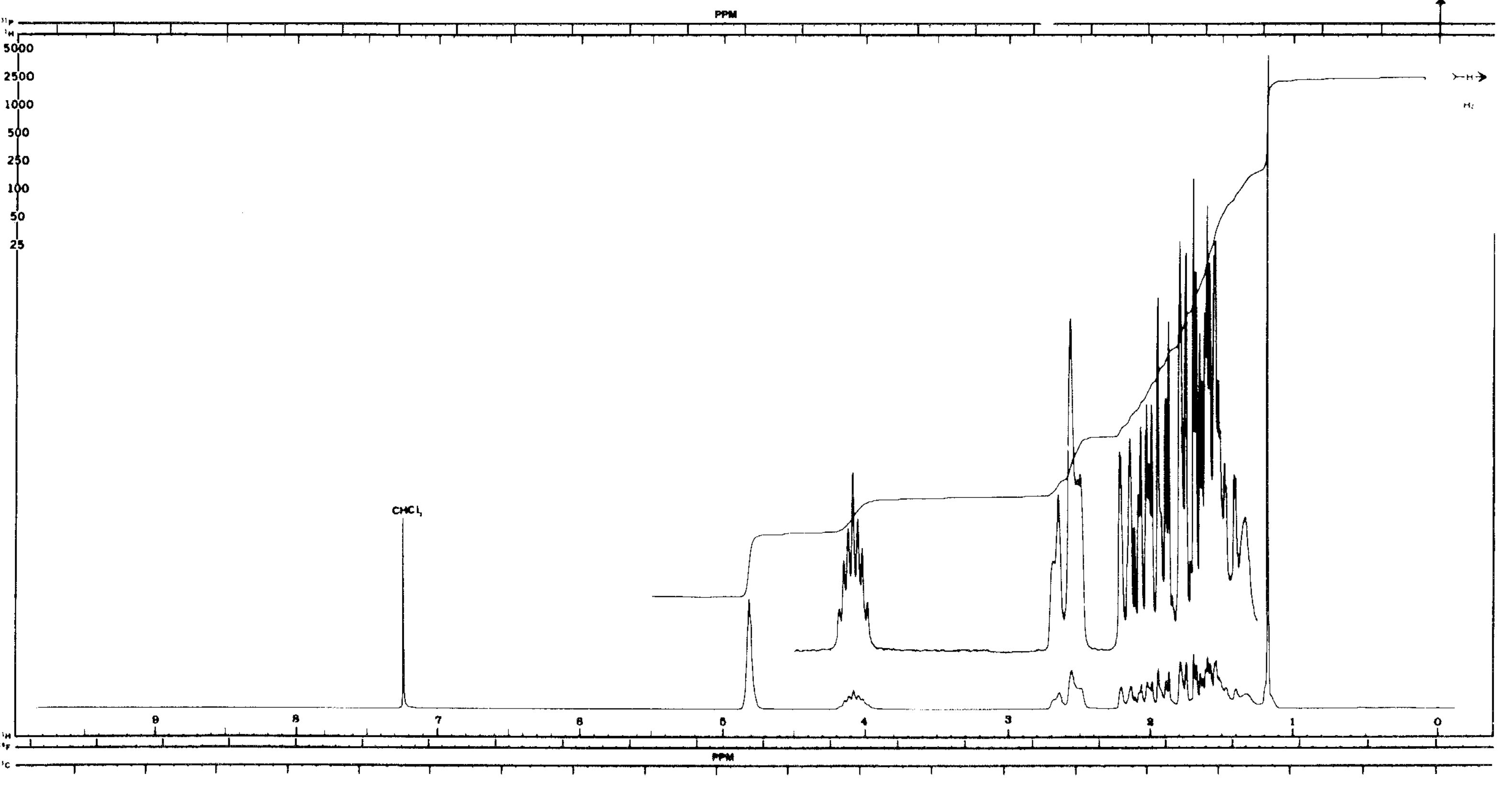

FIG. 3. 100-MHz [^{1}H]NMR spectrum of *endo*-1-methyl-5-methylenebicyclo[4.2.0]-octan-3-ol (**4**).

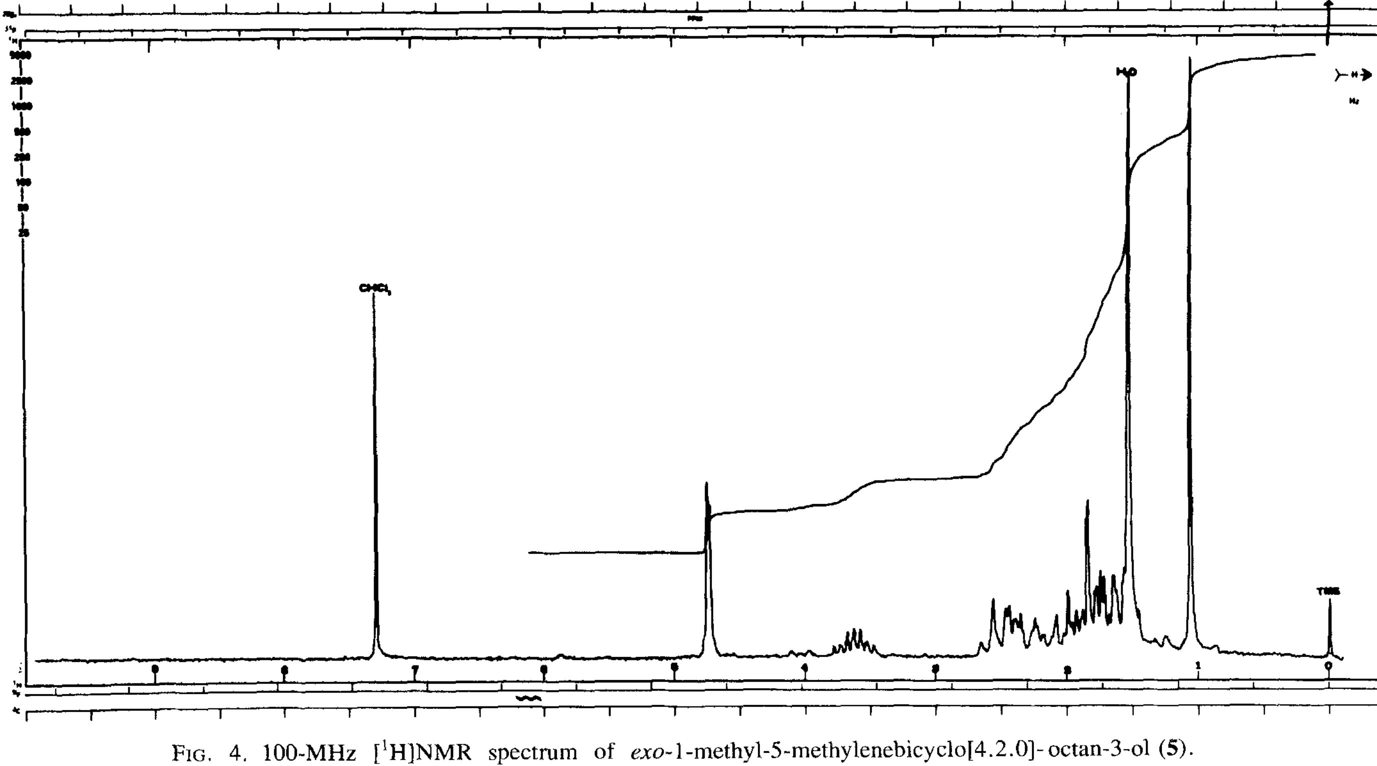

FIG. 4. 100-MHz [^{1}H]NMR spectrum of *exo*-1-methyl-5-methylenebicyclo[4.2.0]-octan-3-ol (**5**).

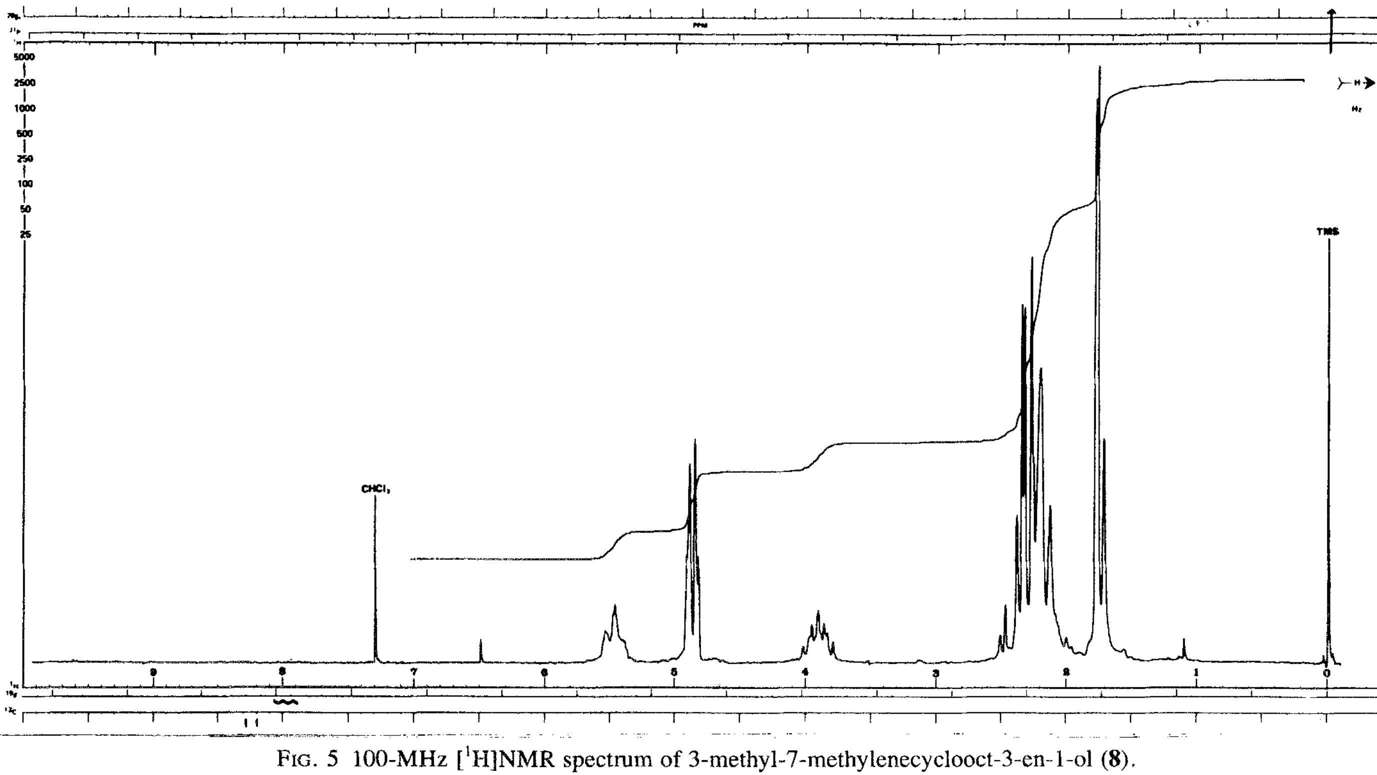

FIG. 5 100-MHz [^{1}H]NMR spectrum of 3-methyl-7-methylenecyclooct-3-en-1-ol (**8**).

119(5%), 123(20%), 124(7%), 137(24%), 152(6%); Kovats' index 1185. Mass spectral analysis of **7** gave *m/e* 39(22%), 41(26%), 43(15%), 51(5%), 53(11%), 55(11%), 65(6%), 67(10%), 69(10%), 77(13%), 79(18%), 81(12%), 91(26%), 93(16%), 95(19%), 97(48%), 105(6%), 106(11%), 107(11%), 108(22%), 109(100%), 110(9%), 123(23%), 137(18%), 152(6%); Kovats' index 1181.

The fourth fraction (essentially one compound) from column A was further purified on column B (150°C, N_2 60 ml/min). This compound was identified as **8**: [^{1}H]NMR (Figure 5); [^{13}C]NMR δ25.3, 26.6, 39.3, 39.5, 41.8, 70.8, 115.6, 125.2, 135.1, 146.1; IR 3350, 1636, 1032, 887, 823 cm^{-1}; MS *m/e* 39(49%), 40(129%), 41(67%), 43(19%), 44(7%), 51(7%), 53(32%), 55(33%), 56(16%), 65(8%), 67(42%), 68(31%), 69(72%), 77(16%), 79(32%), 81(31%), 83(14%), 84(21%), 91(26%), 93(62%), 95(18%), 97(19%), 105(9%), 106(7%), 108(100%), 109(28%), 119(16%), 124(5%), 134(8%), 137(5%), 152(3%); Kovats' index 1229.

Reduction of **3** *to a mixture of* **6** *and* **7**. A 1-ml tapered vial was flame dried and covered with a rubber septum. A solution of 2.7 mg (0.018 mmol) of **7** in 20 μl anhydrous THF was injected into the vial by a syringe. The vial was cooled to 0°C, and 44 μl (0.022 mmol) of a 0.5 M solution of 9-BBN (9-borabicyclo[3.3.1]nonane) in THF was injected into the reaction vial. The mixture was held at 0°C for 3 hr and shaken for another hour at 25°C. Methanol (1 μl) was added followed by 8.8 μl (0.026 mmol) of 3 N NaOH and 7.2 μl (0.06 mmol) of 30% H_2O_2 (exothermic reaction). This mixture was shaken for 0.5 hr at 60°C. The aqueous phase was saturated with anhydrous potassium carbonate and the organic phase was removed. The aqueous phase was extracted with two 50-μl portions of ether. The combined organic extract was washed twice with a 50-μl portion of water and dried ($MgSO_4$), and two compounds were collected from column A (150°C, N_2 80 ml/min). The identity of compounds **6** and **7** from treatment of grandisal and those from reduction of **3** was demonstrated by coinjection on column C and by capillary GC-MS.

H CH3 H O
1

H HO CH3
2

H O
3

H HO
4

H HO
5

RESULTS AND DISCUSSION

Identification of Compounds **4** *and* **5**. The mass spectra of these two compounds are nearly identical (M^+ 152). Combustion analyses and high-resolution mass spectrometry furnish the molecular formula $C_{10}H_{16}O$. Both compounds contain a hydroxyl group as evidenced by a strong broad peak at 3345 cm^{-1} for **4** and at 3300 cm^{-1} for **5** in their IR spectra (which are also nearly identical). The other prominent functional group in **4** and **5** is the vinylidene group (CH_2=). Absorptions from this group are prominent in both IR spectra (strong absorption at 880 cm^{-1}, medium at 1638 cm^{-1}). NMR (^1H and ^{13}C) analysis confirmed the presence of the vinylidene group. The two-proton absorption at δ4.83 for **4** and at δ4.73 for **5** is typical of a strongly coupled AB absorption. The [^{13}C]NMR of **4** (there was an insufficient amount of **5**) contains a peak at δ111.8 (typical for $=\underline{C}H_2$) and at δ148.5 (typical for $=\underline{C}R_2$). The methyl singlets in the [^1H]NMR spectra (δ1.19 for **4** and δ1.03 for **5**) and the lack of other double bonds (shown by the lack of other olefinic carbon absorptions in the [^{13}C]NMR) are strong evidence that the cyclobutane ring remained intact.

Since the molecular formula indicates three degrees of unsaturation, and since only two have been accounted for (one for the vinylidene group and one for the cyclobutane), the compound must contain another ring. That the rings are fused and four- and six-membered can be inferred from the starting structure and the proposed mechanism. Thus we have two diastereomers of 1-methyl-5-methylenebicyclo[4.2.0]octan-3-ol (**4** and **5**). The stereochemical assignments are discussed below.

Identification of **3**. Although enone **3** is not a direct product of grandisal rearrangement, its identification is included here for two reasons: first, it is the main by-product of grandisal oxidation with pyridinium chlorochromate, and second, because the identification of **6** and **7** hinges on structure **3**. Combustion analysis and a molecular ion peak at *m/e* 150 indicated a molecular formula of $C_{10}H_{14}O$. Evidence for a conjugated ketone group was obtained from the IR spectrum (strong absorption at 1655 cm^{-1} and medium absorption at 1281 cm^{-1}) and from the [^{13}C]NMR spectrum (a C=O peak at δ199.8). One double bond is present since two olefinic carbon peaks are found in the [^{13}C]NMR spectrum (δ125.7 and δ162.0). Since only a single olefinic proton can be found in the [^1H]NMR spectrum (δ5.95), the double bond must be trisubstituted. A three-proton singlet at δ1.28 and a three-proton doublet at δ1.87 ($J < 2$ Hz) represent methyl groups. Decoupling showed that the methyl at δ1.87 is allylic because

irradiation at δ5.95 collapsed the doublet to a sharp singlet. A two-proton apparent doublet at δ2.23 is actually a tightly coupled AB system representing a methylene group adjacent to the carbonyl. By the same reasoning as above for the number and sizes of the rings present, compound **3** is 1,5-dimethylbicyclo[4.2.0]oct-4-en-3-one.

Identification of 6 and 7. Compounds **6** and **7** could not be separated on the packed columns used, but separation on the capillary GC-MS system (column C) gave a mass spectrum for each compound that shows M^+ at *m/e* 152. Since the mass spectra of **6** and **7** are nearly identical to those of **3** and **4**, respectively, it is probable that **6** and **7** were merely endocyclic double bond isomers of **4** and **5**. Thus, enone **3**, obtained from grandisol oxidation, on reduction with 9-BBN (9-borabicyclo[3.3.1]nonane) (Krishnamurthy and Brown, 1977), gave a mixture of diastereomers, namely *endo-* and *exo-*1,5-dimethylbicyclo[4.2.0]oct-4-en-3-ol (**6** and **7**). Chromatographic and mass spectral analysis unequivocally showed that compounds **6** and **7** from isomerization of grandisal are identical to the reduction products. The stereochemistry is discussed below.

Identification of 8. High-resolution mass spectral analysis gave a molecular formula of $C_{10}H_{16}O$ (again, isomeric with grandisal). The IR spectrum suggested the presence of a hydroxyl group (strong absorption at 3350 cm^{-1} and a medium absorption at 1032 cm^{-1}). The [^{13}C]NMR clearly indicated the presence of two double bonds because there are four olefinic peaks (δ115.6, 125.9, 135.1, and 146.1). The upfield and downfield peaks (δ115.6 and 146.1, respectively) suggest a vinylidene group; the [^{1}H]NMR spectrum clearly supports this idea with a two-proton absorption at δ4.82. Since there is only one other olefinic proton in the [^{1}H]NMR, the other double bond must be trisubstituted. The three-proton doublet ($J < 2$ Hz) at δ1.76 represents a methyl group. The multiplet at δ2.19–2.37 consists of four methylene groups (eight protons). Decoupling provided the following information: irradiation at δ5.44 (one proton) collapsed the methyl doublet (allylic coupling) at δ1.76 to a sharp singlet, and it affected one of the methylene groups between δ2.19 and 2.33. Thus, the trisubstituted double bond is substituted by the methyl group, and the olefinic proton is adjacent to a methylene group. Irradiation at δ3.94 (the proton on the hydroxyl-bearing carbon) affected only the methylene groups (multiplet at δ2.19–2.33); thus, the hydroxyl carbon is attached only to methylene groups. Finally, since the compound must contain a ring (one degree of unsaturation remaining), the substitution pattern only allows for an eight-membered ring. Thus, component **8** is 3-methyl-7-methylenecyclooct-3-en-1-ol.

We have tentatively assigned the stereochemistry for compounds **4, 5, 6,** and **7,** on the basis of the following arguments. Figure 6 outlines a proposed mechanism (shown for a single enantiomer) to account for the products found in the grandisal rearrangement. We believe that the reaction is a concerted "ene"

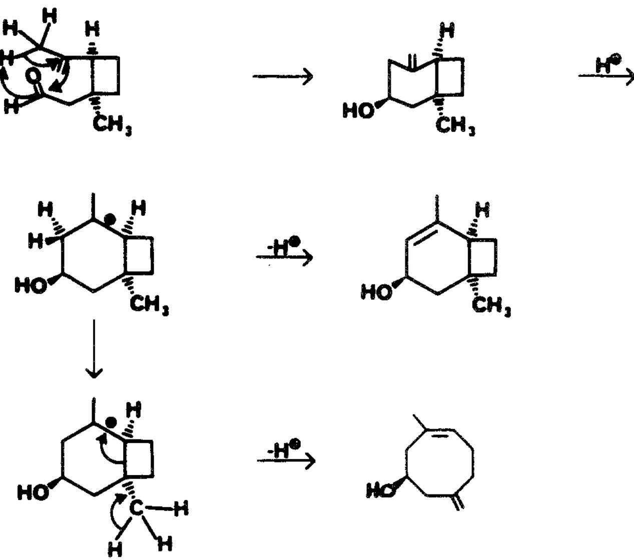

FIG. 6. Proposed mechanism to account for products and stereochemistry of grandisal rearrangement.

reaction (or nearly concerted) to account for the predominance of compounds **4** and **5** bearing the exocyclic double bond (Table 1). In this mechanism, attack of the aldehyde carbon (which probably has considerable cationic character due to the acid-catalyzed nature of the reaction) can occur from either side, but the backside (Figure 6) is relatively more accessible; attack from this side should occur more readily. Since attack from the front is not prohibited, both diastereomers should result. Acid-catalyzed isomerization of the double bond probably occurs at nearly the same rate with each diastereomer; thus, the ratio of **4** to **5** produced initially should be reflected in the ratio of **6** and **7**. Also, since *p*-tosic acid is a better protic acid than silica gel, the double-bond isomerization would be expected to proceed faster in the *p*-tosic acid reaction. The ratios recorded in Table 1 support these arguments. An external check on the stereochemistry can be made from the results of the reduction of enone **3**. Reduction of **3** would be expected to occur from the more accessible side of **3**, thus producing the *endo* alcohol. In other words, the ratio of **6** to **7** is expected to be similar in both cases, and this indeed was found to be the case.

Although the extensive isomerization of grandisal on silica gel, which is very weakly acidic, was quite unexpected, similar results under the weakly acidic conditions of pyridinium chlorochromate oxidation have been demonstrated (Corey and Boger, 1978). Grandisal contains the optimum features described by Corey for acid-catalyzed intramolecular attack by a double bond on

a carbonyl group: formation of a six-membered ring forming a fused-ring system.

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ALLELOPATHIC EFFECT OF PARTHENIUM (*Parthenium hysterophorus* L.) EXTRACT AND RESIDUE ON SOME AGRONOMIC CROPS AND WEEDS[1]

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Abstract—Allelopathic effects of entire shoot extract, plant part extracts, and shoot residue of parthenium (*Parthenium hysterophorus* L.) on corn (*Zea mays* L.), ryegrass (*Lolium multiflorum* Lam.), wheat (*Triticum aestivum* L.), velvetleaf (*Abutilon theophrasti* Medik.), and soybean [*Glycine max* (L) Merr.] growth were examined. Parthenium shoot contained water-soluble materials that were toxic to root growth of velvetleaf and wheat. At 4% (w/v) concentration, root growth of velvetleaf and wheat were reduced by 60 and 75%, respectively. The order of increasing sensitivity to parthenium was ryegrass, corn, wheat, and velvetleaf. There was a strong correlation between extract concentration and increased toxicity to test species. The toxicity of plant part extracts was also concentration dependent. At 1 and 2% (w/v), the inflorescence and leaves caused more root inhibition than stem extract. Parthenium shoot incorporated in soil at 1% (w/w) caused significantly more root inhibition of wheat than soybean, corn, and ryegrass. At 4% (w/w), root growth of all the test species was inhibited compared to the control. Toxicity of parthenium residue to wheat diminished with increasing periods of decomposition. Residue decomposed for four weeks was less toxic than the undecomposed residue.

Key Words—Allelopathy, parthenium, *Parthenium hysterophorus*, Compositae, corn, *Zea mays*, ryegrass, *Lolium multiflorum*, wheat, *Triticum aestivum*, velvetleaf, *Abutilon theophrasti*, soybean, *Glycine max*, inhibition.

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INTRODUCTION

Parthenium is an annual, Compositae, weed native to the Americas. It is an aggressive weed of disturbed sites and commonly found in cultivated fields. The weed commonly forms huge pure stands and in such areas the vegetation seldom contains other plant species. Such a situation suggests competitive ability and a possible allelopathic mechanism being operative. Weeds like *Sorghum halepense, Salvia leucophylla*, and *Artemisia californica* form pure dense stands and are reported to exert allelopathic influence (Abdul-Wahab and Rice, 1967; Muller et al., 1964).

Discharge of allelochemicals into the environment occurs by exudation of volatile chemicals from living plant parts (Muller, 1965), by leaching of water-soluble toxins from above-ground parts in response to the action of rain (Grummer and Beyer, 1960; Tukey, 1966), by exudation of water-soluble toxins from below-ground parts (Fay and Duke, 1977; Guenzi and McCalla, 1962; Kozel and Tukey, 1968; Woods, 1960), by release of toxins from nonliving plant parts through leaching of toxins from litter decomposition (Guenzi et al., 1967; Patrick, 1971) or microbial by-products resulting from litter decomposition (Kaminsky, 1981; Kimber, 1973; Linderman, 1970).

Allelopathic plants may be used to control weeds or their inhibitory effect on crops could be avoided if several preconditions are met. Their selective toxicity to different plant species should be known. The plant parts with the most inhibitor should be identified and the relationship between inhibition and concentration must be understood. Despite the numerous reports on the allelopathic potentials of parthenium (Kanchan and Jayachandra, 1979a,b; Picman and Picman, 1984; Sarma et al., 1976), the above information is not documented for this important weed. This study was undertaken to fill the information gap.

The objectives of this investigation were to (1) determine the relative toxicity of parthenium water extracts and shoot residue to corn, ryegrass, wheat, and velvetleaf; (2) compare the toxicity of parthenium plant part extracts on root growth of wheat; and (3) evaluate the persistence of parthenium toxicity to wheat.

METHODS AND MATERIALS

Collection and Preparation of Parthenium Residues. Parthenium shoots with seeds were collected in November 1985 from fields near the Tropical Research and Education Center, Homestead, Florida. Plant samples were divided into two portions: plants sectioned into stems, leaves, and inflorescence, and whole shoots. Both plant portions were cut into 1- to 2-cm sections and oven dried at 40°C for a week. All experiments were repeated and the combined data were analyzed.

Plant Species Response to Parthenium Water Extract. Whole parthenium shoot sections were ground in a Wiley mill to pass a 1.27-mm^2 screen and stored in sample bottles at room temperature. Rates of 0, 0.5, 1.0, 2.0, and 4.0% (w/v) were prepared by mixing ground residues in distilled water at room temperature for 5 min with a magnetic stirrer in 250-ml Erlenmyer flasks. Extracts were filtered by vacuum filtration on a Buchner funnel with No. 2 Whatman filter paper. Osmotic potential of the different filtrates was determined by 5100 B Osmometer (Wescor Inc., Logan, Utah). Ten seeds of corn, wheat, ryegrass, and velvetleaf were placed in separate Petri dishes (10 cm diameter) with two No. 2 Whatman filter papers and 10 ml of each extract were added. The Petri dishes were kept in a dark incubator at 25°C and radicle lengths were measured after five days. Each treatment (extract) was replicated four times and the average root length of germinated seedlings per Petri dish was used for statistical analysis. Treatments were arranged in a randomized complete block design. The relationship between root growth and extract concentration for all test species was analyzed by linear regression.

Phytotoxicity of Parthenium Parts to Wheat. Dried sections of stem, leaves, and inflorescence (with seeds) were separately ground and stored in sample bottles at room temperature. Water extracts of each plant part were prepared at 0, 0.5, 1.0, 2.0, and 4.0% (w/v) as above. Ten seeds of wheat were placed in Petri dishes (10-cm diameter) with filter paper, and 10 ml of extract from each plant part were added. Wheat seedlings were grown as described above. Each treatment was replicated four times in a randomized complete block design with 3 (plant parts) × 5 (concentrations) factorial arrangement.

Rate Study with Parthenium Residues. Dried parthenium shoot sections were mixed at 0, 1, 2, and 4 g/100 g of sand and placed in plastic cups (16 oz) without any provision for drainage. Similar concentrations of sphagnum moss were added to controls to provide the same amount of soil organic matter (AlSaadawi and Rice, 1982; Schon and Einhellig, 1982). Ten seeds of wheat, corn, soybean, and ryegrass were planted in each cup at 0.5 cm below the soil surface. Cups were watered as necessary and 20 ml of 1% (w/v) of nutrileaf fertilizer (20-20-20 N,P,K) was added once every week. This fertilizer rate was found adequate to overcome the nutrient immobilization by microbes using sphagnum peat (Achhireddy and Singh, 1984). After 10 days, seedlings were thinned (uprooted) to three plants per cup. Four weeks after planting, plants were harvested and shoot and root lengths were measured. There were four cups per treatment, and cups were arranged in a randomized complete block design. The data were subjected to analysis of variance and least-significant differences of treatment means were determined.

Persistence of Inhibitors from Parthenium Residue. Oven-dried parthenium shoot sections were incorporated into sand at 0, 1, 2, and 4 g/100 g of sand and placed into 15-cm-diameter pots without any provision for drainage. Similar concentrations of sphagnum moss were also added to the sand and placed

in pots. This incorporation was done three times with two-week intervals to give 4, 2, and 0 weeks of decomposition before planting. The growing conditions were as described for the rate study. Each treatment was replicated four times in a randomized complete block design with 4 (residue rate) × 3 (decomposition period) factorial arrangement. After analysis, means for each decomposition time were calculated by averaging across residue rates.

RESULTS AND DISCUSSION

Plant Species Response to Parthenium Water Extracts. Extraction of parthenium shoot residue with water attempts to simulate the natural release of compounds that might be caused by rain acting on the standing or fallen plant material. The osmotic potential of the water extract was −1.8 bar for the highest concentration (4%) and pH was 6.5. Species differed in their response to parthenium water extracts (Figure 1). Ryegrass was the most tolerant while velvetleaf was the most sensitive. Corn and wheat were intermediate in their response. Root growth of the tolerant ryegrass was inhibited by 15%, whereas root length of velvetleaf was reduced by about 75% at the 4% concentration level. This demonstrates species-specific allelopathy. Overland (1966) found that the inhibitory action of barley (*Hordeum vulgare* L.) varied with test species. In general, chickweed [*Stellaria media* (L.) Cyp.] growth was strongly inhibited, shepherdspurse [*Capsella bursa-pastoris* (L.) Medik] and tobacco (*Nicotiana tabacum* L.) moderately inhibited, and wheat was not affected in the presence of living plants and dead roots of barley. The data (Figure 1) also showed that the allelopathic response of the two sensitive species, wheat and velvetleaf, was concentration dependent. Root inhibition increased with an in-

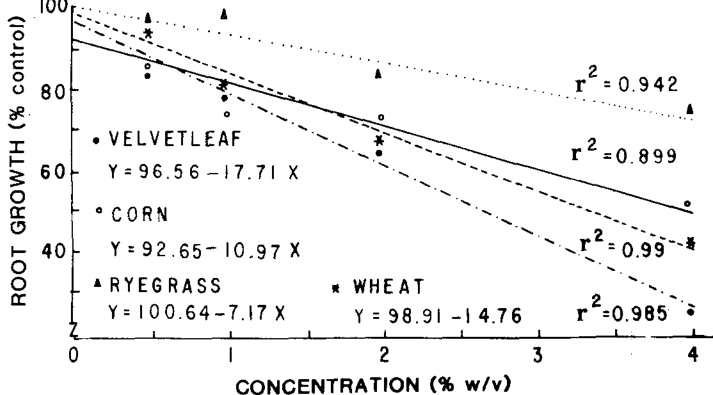

FIG. 1. The effect of various concentrations of parthenium extracts on root growth of corn, ryegrass, velvetleaf, and wheat.

crease in the concentration of the water extract. The relationship between root inhibition and extract concentration was similar for velvetleaf and wheat. However, the rate of root inhibition for the tolerant ryegrass was significantly lower ($P = 0.05$) than for the other species. Results are in agreement with previous investigations (Caussanel, 1979; Bhowmik and Doll, 1982) in that the activity of water extracts from allelopathic weeds was directly related to the extract concentrations. The results of these experiments demonstrated that water extracts of parthenium residue produced allelopathic inhibition, and the effects were dependent on extract concentration and test species.

Phytotoxicity of Parthenium Plant Part Extracts to Wheat. The effects of parthenium plant parts to wheat were also concentration dependent (Figure 2). At the lowest concentration (0.5%), the inflorescence extract significantly reduced wheat root growth compared to the leaf and stem extract. However, at 1 and 2%, the wheat root inhibition was similar for the inflorescence and leaves. At the highest extract concentration (4%), there was no significant difference among the plant parts. The differences observed at the lowest concentration of the inflorescence extract and the other plant parts, which were obtained from the same amounts of material, suggest that the inflorescence contains proportionally higher amounts of the allelopathic compounds than other plant parts. Kanchan and Jayachandra (1979a) also reported that soil from which parthenium plants were removed at the rosette and flowering stages caused greater inhibition of bean (*Phaseolus vulgaris* L.) nodulation and root and shoot growth than soil from which the weed was removed at the four-leaf stage.

Rate Study with Parthenium Residue. The incorporation of peat at all rates did not affect the shoot or root growth of corn, wheat, and soybean (Table 1).

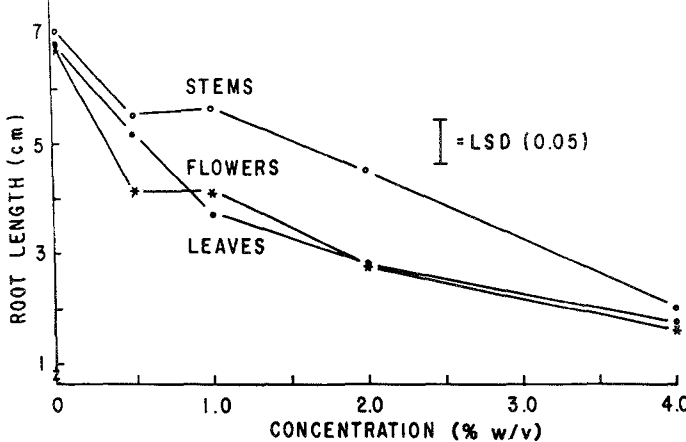

FIG. 2. Effects of water extracts of parthenium plant parts on the root lengths of wheat.

TABLE 1. EFFECT OF PARTHENIUM SHOOT RESIDUE ON ROOT AND SHOOT LENGTHS OF CORN, WHEAT, SOYBEAN, AND RYEGRASS

Species	Residue rate (%, w/w)	Root length (cm)		Shoot length (cm)	
		Peat	Parthenium	Peat	Parthenium
Corn	0	25.6 ± 1.3[a]	25.6 ± 1.3	42.8 ± 3.5	42.8 ± 3.5
	1	27.0 ± 1.9	24.9 ± 1.4	41.5 ± 2.4	44.2 ± 1.0
	2	26.5 ± 2.4	25.6 ± 1.0	39.3 ± 1.3	37.9 ± 1.4
	4	30.3 ± 1.9	18.8 ± 1.3	40.5 ± 2.0	29.8 ± 1.4
LSD (0.05)		NS	3.3	NS	6.4
Wheat	0	11.3 ± 1.7	11.3 ± 1.7	15.8 ± 1.0	15.8 ± 1.0
	1	13.8 ± 1.3	7.3 ± 0.4	15.8 ± 0.4	13.0 ± 0.9
	2	13.5 ± 1.5	5.3 ± 0.5	16.0 ± 0.8	10.7 ± 0.5
	4	11.5 ± 1.7	2.4 ± 0.4	14.4 ± 0.5	5.5 ± 0.2
LSD (0.05)		NS	3.4	NS	2.5
Soybean	0	13.8 ± 0.2	13.8 ± 0.2	16.0 ± 1.7	16.0 ± 1.7
	1	11.3 ± 0.9	13.5 ± 1.7	15.2 ± 0.3	17.7 ± 0.8
	2	11.0 ± 0.9	10.3 ± 1.3	13.4 ± 0.8	15.7 ± 0.8
	4	14.2 ± 0.9	6.6 ± 0.6	13.1 ± 0.9	8.1 ± 1.1
LSD (0.05)		NS	3.3	NS	3.0
Ryegrass	0	15.7 ± 0.8	15.7 ± 0.8	19.7 ± 0.7	19.7 ± 0.7
	1	20.6 ± 1.3	21.9 ± 0.5	24.6 ± 1.6	19.5 ± 1.0
	2	20.4 ± 1.0	19.4 ± 2.0	25.8 ± 1.3	16.8 ± 1.5
	4	20.2 ± 0.8	3.8 ± 0.7	28.8 ± 1.1	7.5 ± 1.5
LSD (0.05)		3.4	3.6	3.1	3.4

[a]Means ± SE.

In ryegrass, growth was significantly stimulated by peat compared to sand. This lack of root growth inhibition by peat even at 4% shows that any growth effect by parthenium shoot is not due to mere plant residue presence in the sand. Rather, it indicates a possible allelopathic effect by parthenium shoot on the test species.

The plant species differed in their response to the rate of parthenium residue in the growing medium. The data indicated that the root growth of corn and soybean was affected only by the highest rate (4%), whereas wheat was significantly inhibited by the lowest rate of parthenium. Root length of ryegrass in sand was lower than in all rates of peat as well as parthenium. However, root length of ryegrass in the highest rate of parthenium (4%) was significantly lower than the two lower rates (1 and 2%). The sensitivity of wheat observed in this experiment was in agreement with the results of the extract experiment (Figure 1). In both studies, wheat was more sensitive to parthenium than corn or ryegrass.

Allelochemicals from parthenium could be released through two possible mechanisms: either allelochemicals were produced by microorganisms during residue decomposition or they were leached directly from the residue. Residue extracts were toxic as evidenced in the laboratory experiments (Figure 1).

The allelochemicals released by the parthenium residue could have inhibited root growth by at least two mechanisms. The first possible target is cell division in roots (Bhowmik and Doll, 1982). Phenolics represent one of the largest groups of allelochemicals, and Avers and Goodwin (1956) have shown that various phenolic compounds inhibited cell division. It is also possible that cell elongation was affected by extracts of weed residues. Tomaszewski and Thimann (1966) found many allelochemicals inhibited gibberellin, and indoleacetic acid induced growth. These phenomena may account for the root inhibition of test species by the highest rate of parthenium residue.

Persistence of Inhibitors from Decaying Parthenium Residue. Wheat planted in the undecomposed parthenium had shorter root length than wheat in the residues decomposed for two and four weeks (Table 2). The means were averaged across four residue rates for each decomposition period. The statistical analyses also showed a significant interaction between decomposition period and root growth. Patrick et al. (1963) also reported decreased toxicity of barley, rye, and wheat residues to lettuce (*Lactuca sativa* L.) and spinich (*Spinacia oleracea* L.) seedlings with increasing periods of decomposition. Such diminished phytotoxicity with increasing decomposition period could be due to breakdown of inhibitors by microorganisms. The undecomposed parthenium residue also reduced shoot length of wheat more than residues decomposed for two and four weeks before planting (Table 2). However, shoot length means for two and four weeks were not significantly different as in the case of root length. This was similar to results obtained for residue rate study (Table 1) where roots were found to be more sensitive than shoots. In field studies, Patrick et al. (1963) found allelopathic injury to roots of lettuce and spinach seedlings was confined mainly to those parts in direct contact with or in the immediate vicinity of decomposing plant fragments in the soil.

TABLE 2. EFFECTS OF DIFFERENT LENGTHS OF DECOMPOSITION OF SOIL-INCORPORATED PARTHENIUM SHOOT ON ROOT AND SHOOT GROWTH OF WHEAT[a]

Decomposition time (weeks)	Root length (cm)	Shoot length (cm)
0	9.3c	20.4b
2	15.2b	31.8a
4	18.1a	32.7a

[a] Means within a column followed by the same letter are not significantly different at the 5% level according to Duncan's multiple-range test.

The results of this investigation demonstrated that the water extracts of parthenium shoot produced allelopathic inhibitors, and the effects were species-specific and concentration dependent. Furthermore, the study showed that the inflorescence contained proportionally higher amounts of inhibitors than the other above-ground parts. Toxicity from parthenium residue diminished with increased time of decomposition, indicating a breakdown of inhibitors within four weeks.

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FLIGHT AND COPULATION OF FEMALE SPRUCE BUDWORM IN PHEROMONE-PERMEATED AIR

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Abstract—High concentrations of synthetic sex pheromone caused increased flight activity among mated female spruce budworm of all ages, a fact that may indicate an increase in dispersal. Flight activity also increased among older virgin females, but not among females one day old, the age at which they usually mate. Receptivity of young virgin females to courting males was not affected by pheromone-permeated air. Synthetic sex pheromone is therefore not likely to influence mating frequency through its effects on female activity.

Key Words—Spruce budworm *Choristoneura fumiferana*, Lepidoptera, Tortricidae, sex pheromone, female response, flight, copulation, dispersal.

INTRODUCTION

In the majority of Lepidoptera, male moths are "attracted" to the female moth by female-emitted sex pheromones. In a few species it has been shown that the female moths themselves are able to perceive the sex pheromone of their own species (Grant, 1970; Mitchell et al., 1972; Birch, 1977; Palaniswamy and Seabrook, 1978; Light and Birch, 1979).

The pheromone receptors of female spruce budworm [*Choristoneura fumiferana* (Clem.)] are on the antennae (Ross et al., 1979). Palaniswamy and Seabrook (1978) found that 3-day-old virgin female spruce budworm moths reacted to the synthetic pheromone of their own species by increased walking, increased flexing of the abdomen, and extension of the ovipositor. They also found that the number of moths responding was related to the dosage of the synthetic pheromone. Later Palaniswamy and Seabrook (1985) showed that a

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higher proportion of females ''call'' (extrude the pheromone gland) in the presence of pheromone and that they start calling at least 2 hr earlier than in pheromone-free air. The implications of this behavior for population dynamics and control of spruce budworm could be profound. First, since moving female budworm do not call, high concentrations of pheromone that cause an increase in activity could result in a reduction in mating and hence a reduction in population density. Second, it could influence the dispersal of mated female moths, an important factor in the population dynamics of spruce budworm (Greenbank et al., 1980). The outcome of computer simulations of population dynamics is profoundly affected by whether or not dispersal is related to population density (Clark, 1979). The available evidence suggests that dispersal does not occur at low population densities (Greenbank et al., 1980). If this is so, then the question arises as to which factors trigger dispersal. One possibility is the high concentration of pheromone associated with high population density (Palaniswamy and Seabrook, 1978; Sanders, 1979a).

The experiments described here were carried out to determine if flight activity of female spruce budworm moths is increased, and if female spruce budworm are less receptive to the advances of male moths, in the presence of synthetic pheromone.

METHODS AND MATERIALS

All insects were from laboratory-reared stock, maintained at the Forest Pest Management Institute and the Great Lakes Forestry Centre, of the Canadian Forestry Service, Sault Ste. Marie, Ontario. The larvae were reared on synthetic diet (Grisdale 1970) and the insects were sexed as pupae, which were placed in separate cages for emergence. Conditions were maintained at 20°C and 70% relative humidity throughout.

Female Flight Activity. Flight activity was recorded by the technique described previously (Edwards, 1962; Sanders and Lucuik, 1975) of measuring the electrostatic disturbance caused by a moth flying inside a Faraday cage, 30 × 30 × 30 cm. There were four treatments: mated or virgin females with or without pheromone.

Freshly emerged female moths were collected between 0900 and 1200 hr the day before each experiment began. When mated females were required, the freshly picked-off females were placed in a screen cage with 1-day-old male moths. Mated pairs were removed from this cage every hour, placed in a separate cage, and left overnight. At 1200 hr the following day, 10 females were placed in the Faraday cage. Shoots of balsam fir, about 15 cm long, were placed in each of the four corners of the cage as resting and oviposition sites, and these were replaced with fresh shoots each morning. Electrostatic charges caused by moths flying in the cage were amplified and relayed to an event recorder. Each experiment was run continuously for three days.

For the experiments involving the pheromone, 14 pheromone dispensers were pinned to the outside of the cage, one at each of the eight corners, and one in the center of each side and the floor and roof. The dispensers were polyvinyl chloride pellets (Sanders, 1981) measuring 10 mm long × 4 mm diameter, containing 0.3% w/w of a 95 : 5 blend of (*E* : *Z*)-11-tetradecenal, the major components of the sex pheromone of the spruce budworm (Sanders and Weatherston, 1976; Silk et al., 1980). The pellets were aged 7–10 days before use, at which time the release rate of pheromone in moving air is estimated to be between 100 and 200 ng/hr (Sanders, 1981; Meighen et al., 1983).

The Faraday cage was housed inside a 0.12-m^3 (4-ft^3) controlled environment cabinet, maintained at 20°C with a light–dark cycle of 17 : 7. The air in the cabinet was recirculated and would therefore have been heavily laden with pheromone, although the concentration was not measured. In order to minimize the possibility of contamination by pheromone, treatments were run in the following sequence. Two treatments each of virgin and mated females in clean air were followed by one treatment each of virgin and mated females in pheromone-permeated air. An ultraviolet lamp was then placed in the environment cabinet for 10 days to hasten the degradation of the pheromone. The Faraday cage and the inside of the cabinets were then washed with soap and water and left for 60 days before the above sequence of treatments was repeated.

Female Copulation. The objective of this experiment was to determine the reaction of calling (i.e., sexually responsive) female moths to courting males in pheromone-laden air. The experiments were carried out in a glass wind tunnel 90 × 90 cm in cross-section, and 2 m long. A nylon screen was placed across the tunnel about 50 cm in from the downwind end. Attached to the screen were 16 circular "fences" made of aluminum screening 2 cm high and 12 cm in diameter. These were arranged at uniform spacing, and their purpose was to provide "roosting" niches for the female moths, to encourage them to remain on the screen and not to walk on the sides or roof of the tunnel.

Two hours before "lights-off," 1-day-old virgin female moths were placed on the screen and allowed to move around freely. After 1 hr the assays began. Two-day-old male moths in small wire cages were held, one at a time, downwind from each female moth that was on the screen. When a male responded by wing-fanning, the cover of the wire cage was removed and the male was allowed to fly upwind towards the female. As soon as the male touched the female, a stop watch was started. The reaction of the female on being touched by the male was recorded. If she moved more than 2.5 cm, she was recorded as having moved. The time was recorded until the pair copulated or until they lost contact. Assays were recorded only for those females that were on the screen, at least 2.5 cm from the edge, where males could approach and land on the screen without landing on the sides of the tunnel.

To minimize the risk of contamination, a new screen was used after each treatment, and a sequence of experiments began with a clean air treatment,

followed by treatments of successively more concentrated pheromone. After a full sequence of treatments, the tunnel was left for one week with a UV lamp inside it. It was then washed with soap and water. The complete sequence was repeated six times. The air in the tunnel was permeated with pheromone by pinning pieces of polyvinyl chloride impregnated with pheromone to a screen at the upwind end of the tunnel as previously described (Sanders, 1982). The screen was intersected by a 9 × 9 grid of 2.5-cm-wide masking tape, and a piece of PVC, 4 mm diam × 10 cm long, was pinned at each of the 81 intersecting points. The tape created turbulence which thoroughly mixed the air, as could be demonstrated by visualization of the airflow with smoke. At a point 30 cm downwind from the first screen, the air passed through a second screen which smoothed out the airflow as it passed into the observation area of the tunnel. PVC pellets containing three different concentrations of the pheromone were used: 0.0003, 0.003, and 0.03% synthetic pheromone w/w. On the basis of release-rate measurements (Sanders, 1981, Sanders and Meighen, in preparation) and an airflow of 25 cm/sec, concentrations of pheromone in the tunnel were estimated to be 10, 100, and 1000 p/m^3, respectively (Sanders, 1982.). The numbers of female moths used in a treatment varied, depending upon the supply; for any one treatment the maximum was 40, the minimum 10.

Numbers of moths responding in the different treatments were analyzed for differences by the G test (Sokal and Rohlf, 1981). The times from contact to copulation were transformed to logarithms and then subjected to analysis of variance.

RESULTS

Female Flight Activity. The flight activity of virgin female moths in clean air and pheromone-laden air is shown in Figure 1A, that of mated females in Figure 1B. For both virgin and mated females, the circadian pattern of activity was similar; it began early in the afternoon, peaked between 1600 and 2000 hr, and decreased to virtually zero by midnight. Flight activity was higher, by a factor of 10, among the mated females than the virgin females. Both virgin and mated females showed higher levels of activity in the presence of the pheromone. Among the virgin females, there was very little flight activity on the first day following emergence, in either clean or pheromone-laden air. On the second and third days, activity was still very low in clean air, reaching a peak of only 4 flights/hr. In the pheromone-laden air, activity increased to 17 flights/hr on the second day and to over 30 on the third day. Among the mated females, differences were most pronounced on the first two days, increasing from peaks of 10 in clean air to over 100 flights/hr in the pheromone-permeated air on the first day, from 46 to over 200 on the second day, but from 130 to only 195 on the third day.

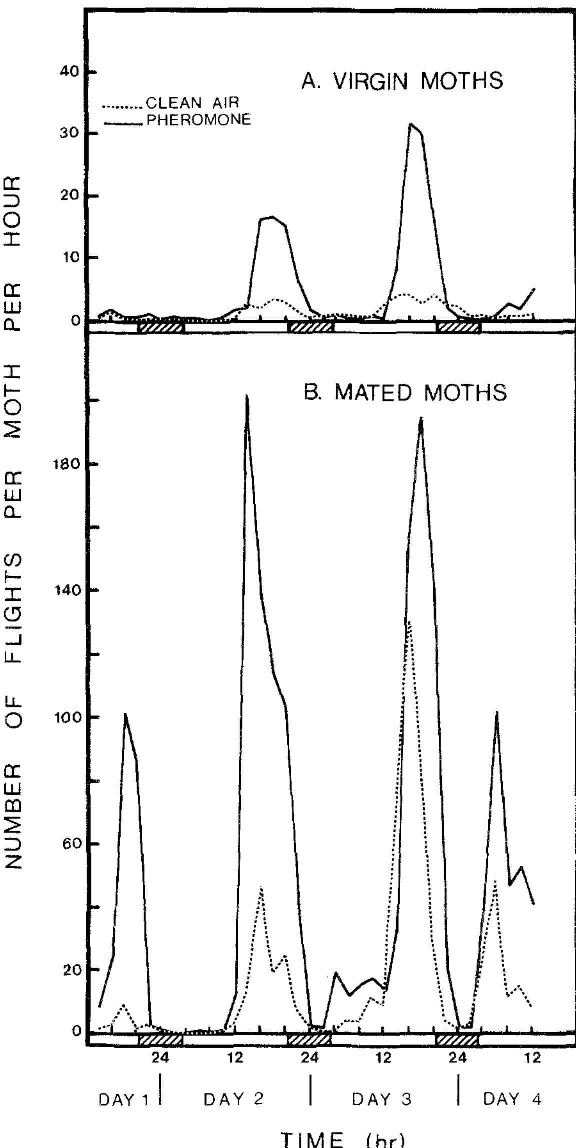

FIG. 1. Number of flights per hour per female spruce budworm moth recorded in an activity chamber in pheromone-permeated air (solid lines) and clean air (dotted lines). (A) Virgin female moths; (B) mated female moths. The day of emergence and mating is counted as day 0; recordings started when females were 24 hr old. In both A and B, the pheromone treatment = 2 replicates of 10 females each, the clean air = 5 replicates of 10 females each.

Female Copulation. No significant differences were recorded in the numbers of females that settled on the screen in the wind tunnel in the four different treatments. In clean air, 84 out of a total of 195 (43%) females settled on the screen; in the pheromone-laden air, in order of increasing concentrations, the figures were 70/179 (39%), 76/181 (42%) and 50/145 (34%).

In the clean air and in the lowest concentration of pheromone, males responded to the females by wing-fanning and flying upwind from a distance of 50 cm. However, at the two higher pheromone concentrations, males started wing-fanning as soon as they encountered the air flow in the tunnel; in order to have them "lock-on" to the pheromone plume from a female, they had to be held 10–20 cm from the females. Once the males were locked onto a female pheromone plume, their behavior was similar in all four treatments; they landed close to the female and ran around, fanning their wings until they contacted her. The first contact of a female by a male was usually with its antennae (confirmed in many instances by video recordings). After antennal contact, males began courting the female as described in Sanders (1979b). Occasionally the approaching male landed on top of the female, causing the female to fly immediately or drop from the screen. However, since this occurred in only 3% of the contacts, the effect on the overall results was negligible. On being contacted by the male, females were classified as remaining stationary or moving. To qualify for the latter category they had to move at least 2.5 cm before stopping.

The reactions of the females to the males in the four treatments, and the times between contact and copulation, are shown in Table 1. There were no significant differences among the four treatments. Fewer male–female contacts were obtained at the highest pheromone concentration. This was because the males had difficulty locating the females against the high background of pheromone, and not because of changes in female behavior. In 137 (62%) of the total of 222 contacts, the females remained stationary. None of these 137 females showed any visible reaction to the male, and in 121 (88%) of the encounters copulation followed in an average time of 3.3 sec from time of first contact. In the other 16 (12%) encounters, either the males lost their footing and departed before successfully copulating, or the females moved off after the males had made repeated but unsuccessful copulatory attempts.

In the remaining 79 encounters, the female moth walked away when contacted, and in 24 instances (28% of all those in which the female moved or flew), the male pursued and copulated with the female. This, of course, took longer than when the female remained stationary, the average duration from contact to copulation being 11.4 sec.

DISCUSSION

In the absence of pheromone, the levels of flight activity and the circadian patterns of flight activity of both virgin and mated females are very similar to

TABLE 1. RESPONSES OF VIRGIN FEMALE SPRUCE BUDWORM MOTHS AFTER BEING CONTACTED BY ATTRACTED MALE IN WIND TUNNEL IN CLEAN AIR AND PHEROMONE-PERMEATED AIR

		Response of female after contact by male					
		Females remaining quiet			Females moving		
Concentration of pheromone (pg/m³)	Number of moths assayed (n_1)	n_2a (% of n_1)	% of n_2[b] copulating	Time to copulation[d] (sec ± 1 SE)	n_3 (% of n_1)	% of n_3[c] copulating	Time to copulation[e] (sec ± 1 SE)
0	66	53	97	3.45 ± 0.07	47	23	12.75 ± 0.08
10	57	63	89	2.60 ± 0.07	37	38	30.62 ± 0.08
100	65	72	83	3.68 ± 0.07	28	33	8.90 ± 0.11
1000	34	56	84	3.51 ± 0.07	44	20	17.08 ± 0.09
Pooled	222	62	88	3.27 ± 0.06	38	28	11.38 ± 0.07

[a] $G = 5.82$, N.S.
[b] $G = 5.11$, N.S.
[c] $G = 2.22$, NS ($df = 3$).
[d] Analysis of variance, $F = 1.78$, $df = 3/119$.
[e] Analysis of variance, $F = 0.17$, $df = 3/20$.

those reported in previous experiments (Sanders and Lucuik, 1975). As in the previous experiment, mated moths were more active than virgin moths, particularly on days 2 and 3 (counting the day of emergence and mating as day 0. This is when dispersal probably begins, at least at higher population densities (Sanders and Lucuik, 1975; Greenbank et al., 1980). Flight activity peaked on day 3, the day on which female antennae show maximum response to pheromone (Ross et al., 1979).

The presence of the pheromone clearly increased flight activity levels of both virgin and mated females, although the virgin females remained inactive for a full 48 hr after emergence, even in the presence of the pheromone. On day 3, virgin females showed a marked increase in activity in the presence of the pheromone. This corresponds with observations of increased walking and oviposition in 3-day-old virgin spruce budworm in the presence of pheromone reported by Palaniswamy and Seabrook (1978). The same authors pointed out that mobile moths do not copulate, a factor that could contribute to the disruptive effect of pheromone on mating. However, the effects of pheromone on the activity of virgin females may not be of great significance in the field, since most females are mated in the first or second day after emergence when activity levels are low. Therefore, the evidence here suggests that the incidence of mating is not likely to be decreased because of increased activity of the virgin females in the presence of the pheromone

Among mated females, the largest increase in flight activity associated with pheromone occurred on day 2, the day of maximum oviposition and maximum dispersal activity (Sanders and Lucuik, 1975; Greenbank et al., 1980). However, levels of flight activity as measured in the activity cage must be interpreted with caution (Sanders et al., 1978). There is no way of differentiating between local flights associated with oviposition, long-range dispersal flight, or flight in response to the pheromone. The fact that peak activity in the cage corresponds to the time of peak dispersal suggests that much of the increased activity of mated females in the presence of pheromone is related to dispersal. This suggests that the incidence of dispersal is increased in the presence of high concentrations of pheromone. However, in field trials designed to determine the effects of synthetic pheromone on mating and dispersal behavior, no increase in dispersal activity has been observed from scaffold towers above the forest canopy (Sanders, 1979c; Sanders and Silk, 1982).

Results from the activity cage (Figure 1) indicate that female moths do not become habituated to the pheromone. Even though they were subjected to continuous high concentrations of pheromone for several days, increased flight activity occurred each day and persisted over several hours each day. Such behavior would be necessary for the pheromone to act as a dispersal cue.

The wind-tunnel bioassays (Table 1) indicate that the incidence of mating is not affected by changes in the receptivity of the female moth to courting males

in the presence of pheromone-laden air: there were no significant differences in any of the criteria of Table 1 attributable to the pheromone. It is possible that the tests were biased, because the bioassays were restricted to those females that had settled on the screen; any females that did not settle were removed from the sample. Palaniswamy and Seabrook (1985) have shown that a greater proportion of virgin female spruce budworm moths call and that they start calling earlier in the presence of pheromone; this would result in a higher proportion of receptive females and more settling on the screen in the presence of the pheromone in the present experiment. However, the fact that the proportions of females settling on the screen were not significantly different among the four treatments suggests that there was no bias.

Therefore, it is concluded that pheromone-permeated air does not affect the incidence of mating of spruce budworm moths by changing the activity levels of the virgin female moths, or by altering the receptivity of virgin females to courting males. This suggests that disruption of spruce budworm mating by synthetic sex pheromone is caused solely by the effects of the pheromone on the male moths. High concentrations of pheromone may result in more dispersal of mated female moths, since the activity level of mated females is markedly increased in pheromone-permeated air, but it is questionable if this would occur at the pheromone concentrations attained in field disruption trials.

These experiments were carried out with the two major components of the spruce budworm pheromone. It is possible that minor pheromone components (Silk et al., 1980; Sanders, 1984) could significantly affect female moth behavior.

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DIFFERENTIAL INHIBITION BY CASTANOSPERMINE OF VARIOUS INSECT DISACCHARIDASES

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Abstract—The indolizidine alkaloid, castanospermine (1,6,7,8-tetrahydroxy-octahydroindolizidine—a stereochemical mimic of glucose found in the Australian legume *Castanospermum australe*), differentially inhibited cellobiose, lactose, maltose, sucrose, and trehalose hydrolyzing enzymes from a broad taxonomic spectrum of insects (19 species from 12 different families). It was a potent inhibitor of cellobiase activity of all insects tested (50% inhibition at $\leq 3.2 \times 10^{-5}$ M castanospermine). With one exception, it also inhibited lactase activity of all insects examined. Only in the sap-feeding Homoptera did castanospermine inhibit all disaccharidase activities assayed. Trehalase activity of the Lepidoptera and Diptera was generally inhibited by castanospermine, whereas inhibition of trehalase activity of the Coleoptera by castanospermine was exiguous or not detectable. Castanospermine was a significant feeding deterrent towards pea aphids, *Acyrthosiphon pisum*, with an ED_{50} of 1×10^{-4} M in artificial diets. Two compounds stereochemically related to castanospermine, deoxynojirimycin and 6-epicastanospermine, were each slightly active at deterring the feeding of green peach aphids, *Myzus persicae*, ($ED_{50} = 2.5 \times 10^{-3}$ M) and greenbugs, *Schizaphis graminum* ($ED_{50} = 5 \times 10^{-3}$ M), respectively. Among the insects studied there was no distinct relationship between enzyme inhibition and adaptation to host plants containing castanospermine or other toxic alkaloids.

Key Words—Castanospermine, 6-epicastanospermine, deoxynojirimycin, cellobiase, lactase, maltase, sucrase, trehalase, endosymbiote, insect-plant interactions, allelochemicals, Homoptera, Coleoptera, Lepidoptera, Diptera.

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INTRODUCTION

The indolizidine alkaloid, castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizidine) (Figure 1A), occurs in the leguminous tree *Castanospermum australe* A. Cunn., the Moreton Bay chestnut or black bean, native to Queensland, Australia (Hohenschutz et al., 1981). The seeds of *Castanospermum* contain 0.3% castanospermine and are toxic to mammals (Elbein and Molyneux, 1986). A number of recent studies have shown that this alkaloid is either toxic or a feeding deterrent to insects. Studies by Nash et al. (1985) showed that larval development of the cow pea weevil, *Callosobruchus maculatus* F., was totally inhibited by 0.03% castanospermine incorporated into seed meal from the cow pea, *Vigna unguiculata* (L.) Walp. Fecundity of the confused flour beetle, *Tribolium confusum* du Val was reduced by 50% and 95% when castanospermine was incorporated into the diet at 0.01% and 0.1%, respectively. These same researchers found that castanospermine differentially inhibited feeding by two locusts. Whereas nymphs of *Locusta migratoria* (Reiche & Fairmaire) (the migratory locust) were deterred from feeding on sucrose by 0.02, 0.1, and 0.5% incorporation of castanospermine, nymphs of *Schistocerca gregaria* (Forskal) (the desert locust) were unaffected by the same concentrations. Dreyer et al. (1985) found that castanospermine was the most potent deterrent to feeding by the pea aphid, *Acyrthosiphon pisum* (Harris) of 11 pyrrolizidine, indolizidine, and quinolizidine alkaloids tested [50% of aphids were deterred from feeding on an artificial diet by only 0.002% (1×10^{-4} M) castanospermine].

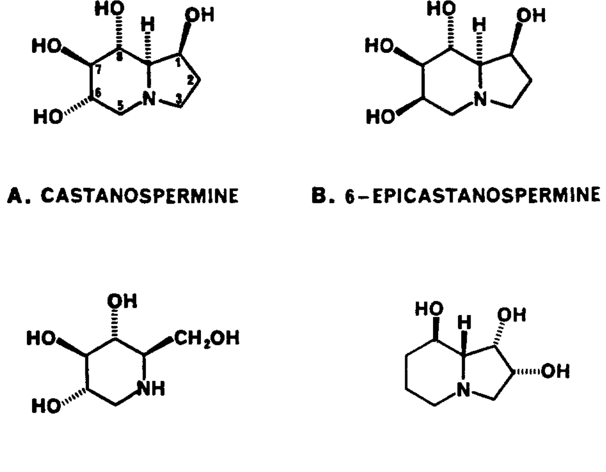

FIG. 1. Chemical structures of some polyhydroxy alkaloids known to inhibit the activity of certain glycosidases.

Castanospermine is a structural and stereochemical mimic of glucose and has been shown to be a potent inhibitor of various glycosidases from a number of sources. Scofield et al. (1986) found that castanospermine inhibited all mouse intestinal mucosal disaccharidase activities tested, including a number of α- and β-glucosidases and β-galactosidases. Castanospermine concentrations that resulted in 50% inhibition ranged from 1.7×10^{-5} M for β-glucosidase to 4.2×10^{-8} M for sucrase. However, castanospermine had differential inhibitory activity when screened against a variety of commercially available glycosidases (Saul et al., 1983). Castanospermine was most inhibitory to β-glucosidase of almond emulsin (50% inhibition at 5×10^{-5} M) and least inhibitory to β-xylosidase from *Aspergillus* (50% inhibition at 5×10^{-4} M). Additionally, castanospermine was a potent inhibitor (50% inhibition at $\leq 5 \times 10^{-5}$ M) of fibroblast β-glucocerebrosidase and lysosomal α-glucosidase. However, it was not inhibitory to a number of glycosidases from various sources including certain α-glucosidases, α- and β-galactosidases, α- and β-mannosidases, α- and β-fucosidases, β-glucuronidases, and β-*N*-acetylhexosaminidases. The β-glucosidase inhibition was not time-dependent and was seemingly of the mixed type.

Trugnan et al. (1986) found that castanospermine was a potent inhibitor of sucrase from a cell culture line of human colon cancer cells (Caco-2). They also determined that this inhibition was noncompetitive (interacting with the enzyme), and time- and dose-dependent. A preincubation period of 1 hr before assay resulted in castanospermine inhibiting sucrase activity by 50% at as low a concentration as 4×10^{-9} M, the most potent inhibition of sucrase known to date.

In view of the variable activity of castanospermine on inhibiting glycosidases from the above sources, we felt it would be of interest to examine the inhibitory activity of castanospermine on five disaccharidases known to occur in the midguts of a number of insect species (Treherne, 1967). We were particularly interested in the prospect of castanospermine as a selective inhibitor of insect trehalase. Also, we investigated the effect of castanospermine and two other structurally related polyhydroxy-imino alkaloids, 6-epicastanospermine (Figure 1B) and desoxynojirimycin (Figure 1C), on the feeding behavior of a legume-feeding aphid and two other species of aphids which do not naturally feed on legumes. In view of the stereochemical similarities of these alkaloids to hexose substrates, we hoped to determine if there was a connection between disaccharidase inhibition and effect on feeding behavior that would relate to insect–plant interactions. Next, we examined if a relationship occurred between disaccharidase inhibition by castanospermine and the taxonomic relatedness of the insects assayed. Finally, we investigated whether castanospermine might facilitate the biochemical characterization of certain insect glycosidases which are difficult to separate by traditional chromatographic or electrophoretic techniques.

METHODS AND MATERIALS

Castanospermine. Castanospermine was extracted from seeds of *C. australe* and purified according to procedures previously described (Hohenschutz et al., 1981).

Insects. All homogenates were made using live insects from colonies kept for ongoing research or collected from host plants in the field. Larvae of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), the pink bollworm, *Pectinophora gossypiella* (Saunders), the western spruce budworm, *Choristineura occidentalis* (Freemann), and the codling moth, *Cydia pomonella* L., were maintained from egg-hatch on standard artificial diets. Adults of the maize weevil, *Sitophilus zeamais* Motchulsky, and the rice weevil, *S. oryzae* (L.), and larvae of the Angoumois grain moth, *Sitotroga cerealella* (Olivier), were from colonies maintained on wheat grain (*Triticum aestivum* L. var. Scout 66). Adults of the Egyptian alfalfa weevil, *Hypera brunneipennis* (Boheman), were reared on alfalfa (*Medicago sativa* L. var. CUF-101). Spotted alfalfa aphids, *Therioaphis maculata* (Buckton), and pea aphids, *Acyrthosiphon pisum* (Harris), were from colonies maintained on alfalfa variety Caliverde. Larvae of the tobacco hornworm, *Manduca sexta* (Johannson), were reared on tomato plants (*Lycopersicon esculentum* Mill. var. Rutgers). Aphids of the greenbug, *Schizaphis graminum* (Rondani), were from a colony maintained on sorghum [*Sorghum bicolor* (L.) Moench. var. BOK-8]. Mealybugs, *Pseudococcus longispinus* (Targioni-Tozzetti), were from a colony maintained on Moreton Bay chestnut plants. Aphids of the green peach aphid, *Myzus persicae* (Sulzer), were from a colony maintained on mustard seedlings (*Brassica alba* Rabenh. var. Tendergreen). Larvae of the navel orangeworm, *Amyelois transitella* (Walker), were from a colony maintained on commercially purchased walnuts. All of the above insects are moderate to serious agricultural pests.

Seed beetles, *Acanthoscelides aureolus* (Horn), were collected in the field from locoweed, *Astragalus oxyphysus* Gray. Larvae of a tephritid were collected from pods of a wild lupin, *Lupinus* sp. These insects were included in the study because of their adaptation to poisonous range plants which contain quinolizidine or indolizidine alkaloids, some of which are known glycosidase inhibitors (Elbein and Molyneux, 1986). Lastly, the only predacious insect studied was the convergent ladybird beetle, *Hippodamia convergens* Guérin-Méneville, adults of which were purchased from Natural Pest Controls, Sacramento, California.

Enzyme Preparation and Assay. Insects were homogenized at 2% live wt/*v* in 0.2 M acetate buffer (pH 5.1), 0.01 M dithiothreitol (DTT), with 1.0% polyvinylpyrrolidone, using a glass tissue grinder (Ten Broeck). The homogenates were centrifuged at 15,000 rpm for 10 min, the supernatant recentrifuged and then "desalted" on Poly-Prep columns (Bio Rad, Richmond, California) packed with Bio Gel P-6, using the 0.2 M acetate −0.01 M DTT buffer as the

eluting solvent. The protein-containing fractions were recentrifuged at 15,000 rpm for 10 min and the supernatants used for the enzyme assays. All enzyme preparative procedures were performed at 4°C. The following disaccharides served as substrates: cellobiose (4-*O*-β-D-glucopyranosyl-D-glucose), lactose (4-*O*-β-D-galactopyranosyl-D-glucose), maltose (4-*O*-α-D-glucopyranosyl-D-glucose), sucrose (β-D-fructofuranosyl-α-D-glucopyranoside), and trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) (Sigma Chemical Co., St. Louis, Missouri). Assays were performed by first adding 100 μl aliquots of 0.04 M solutions of substrates in 0.2 M acetate buffer (pH 5.1), containing diminishing concentrations of castanospermine (range = 5×10^{-4} M to 3×10^{-8} M castanospermine), to individual wells of microtiter plates (NUNC, Denmark). Next, 100 μl of enzyme preparation were added to the substrate making the final concentration of substrate 0.02 M. Each substrate–castanospermine concentration was replicated three times. The enzyme–substrate–castanospermine mixtures were incubated at 27°C on a shaker for 0.5 hr.

The reaction was terminated by addition of 100 μl of a 2.5% solution of Flozyme [a commercially prepared reagent for quantitation of glucose using a coupled-enzyme system of glucose oxidase and peroxidase as modified by Trinder (1969); Worthington Diagnostic Systems Inc., Freehold, New Jersey] with an additional 0.5 hr of incubation for chromophore development. Liberated glucose was quantitated by measuring the oxidized quinoneimine chromophore spectrophotometrically at 500 nm. The Flozyme reagent was not affected by even the highest concentration (2.5×10^{-4} M) of castanospermine. Trace quantities of free glucose in insect–enzyme preparations were considered in calculating background enzyme activity. No attempt was made to purify or separate any of the enzymes, and activities simply reflect the rate of hydrolysis of the substrates. Specific activities of the disaccharidases are expressed in katals per milligram protein; where 1 katal equals one mole of substrate hydrolyzed/sec. Quantitation of protein content in insect enzyme preparations was determined using the Bio-Rad Protein Assay (Bio-Rad, Richmond, California) as originally developed by Bradford (1976). Inhibition of enzyme activity is based upon the concentration of castanospermine that inhibited substrate hydrolysis by 50%. This concentration was determined using a computer-generated polynomial regression (Draper and Smith, 1966) of percent enzyme-activity inhibition (in comparison to controls) versus $\log_{10}$ concentration castanospermine. All polynomial regressions for calculating the 50% inhibition concentrations of all enzymes assayed had an $R^2 \geq 0.97$. Castanospermine was considered to be inactive for a given enzyme if concentrations of $>2.5 \times 10^{-4}$ M were required to achieve 50% inhibition. Because the enzymes were not purified, K_m and K_i values and type of enzyme inhibition could not be reliably determined.

Aphid Feeding Bioassays. Bioassays to test the feeding deterrency towards aphids of castanospermine, a related analog, 6-epicastanospermine (Figure 1B), and deoxynojirimycin (Figure 1C), a polyhydroxy alkaloid occurring naturally

in certain species of *Bacillus* and roots of mulberry (Nash et al., 1985), were performed as previously described (Dreyer et al., 1981). Deoxynojirimycin was obtained through the courtesy of E. Truscheit and D. Schmidt, Bayer A.G. Castanospermine and 6-epicastanospermine were isolated from *Castanospermum australe* seeds (Molyneux et al., 1986). These compounds were bioassayed against *A. pisum*, whose host plants consist of a wide range of legumes, and *S. graminum* and *M. persicae*, which do not feed on legumes. Results were expressed as the concentration of alkaloid that deters aphid feeding by 50% in comparison to aphids feeding on control diets (ED_{50}). These concentrations were calculated by a probit-analysis program to generate a dose–response curve (Schoofs and Willhite, 1984).

RESULTS

The specific activities of the disaccharidases studied for each species of insect and the effect of castanospermine on enzyme activity (as expressed by concentration required for 50% inhibition) are shown in Table 1. There are a number of conspicuous characteristics related to differential insect disaccharidase activities and their inhibition by castanospermine. Firstly, only in the case of the Homoptera [i.e., the aphids (*M. persicae, A. pisum, T. maculata, S. graminum*) and the mealybug (*P. longispinus*)] were all five disaccharidases inhibited by castanospermine. Moreover, inhibition of sucrase activity by castanospermine was only observed for this group of sap-feeding insects. Secondly, cellobiase and lactase activities were inhibited by castanospermine in every insect examined with the exception of the *Astragalus*-adapted seed beetle, *A. aureolus*. Concentrations required for 50% inhibition of cellobiase-activity ranged from 3.2×10^{-5} M for the rice weevil to 2.5×10^{-7} M for larvae of *H. zea*, the greatest degree of enzyme inhibition by castanospermine observed. Lactase inhibition ranged from 1.5×10^{-4} M castanospermine for *H. brunneipennis* to 3.6×10^{-6} M castanospermine for *C. pomonella*.

The results also indicated that, in general, trehalose-hydrolyzing enzymes in the Coleoptera are not inhibited by castanospermine. The one exception was the bruchid, *A. aureolus*, from *Astragalus*, the trehalase from which showed only a relatively slight degree of inhibition (1×10^{-4} M). Conversely, trehalase activities in the Homoptera and, in general, in the Lepidoptera are inhibited by castanospermine. With the exception of the Homoptera, insect sucrase activity is apparently unaffected by castanospermine. Similarly, maltase activity is generally unaffected by castanospermine with exception of the Homoptera and two Lepidoptera, *H. zea* and *M. sexta*. The only predatory insect examined, *H. convergens*, had a significantly higher specific trehalase activity in comparison to the other herbivorous insects (i.e., 1.4 nkatals compared to a mean of 0.4 nkatals for the other insects). The high trehalase activity of this insect, in ad-

dition to the relatively high maltase and sucrase activities, may reflect the comparatively high metabolism of an active, predatory insect.

The effects of the three polyhydroxylated alkaloids on aphid feeding behavior are presented in Table 2. Castanospermine was the most active compound in deterring 50% of feeding of *A. pisum* at only 1×10^{-4} M in artificial diets. However, it was inactive against *S. graminum* and *M. persicae*. 6-Epi-castanospermine was only slightly active against greenbugs, with an ED_{50} of 5×10^{-3} M, and was inactive against the pea aphid. Deoxynojirimycin was nominally active only against green peach aphids at an ED_{50} of 2.5×10^{-3} M.

DISCUSSION

The results show that castanospermine, on a selective basis, can inhibit various disaccharidases of insects, but this inhibition is highly dependent on insect taxa and particular disaccharide substrate. This variable inhibition of insect disaccharidases by castanospermine is in contrast to the inhibition of all disaccharidases of mouse gut epithelium (Scofield et al., 1986). Moreover, there is no distinct correlation with the degree of enzyme inhibition in the insects studied, and their relationship with or adaptation to plants containing either castanospermine or other toxic alkaloids. For example, the inhibition by castanospermine of disaccharidases from the mealybug, *P. longispinus*, living on *Castanospermum* plants is qualitatively and quantitatively similar to the other sap-feeding insects (all the aphids) studied. The survival of the mealybugs on *Castanospermum* is particularly puzzling in view of the fact that sucrase-activity is inhibited by castanospermine in the mealybugs. Sucrose is the chief constituent ingested from the phloem by sap-feeding insects, and its hydrolysis is an important digestive step preceding either absorption of glucose or its transglucosylation into glucans for osmotic regulation (Fisher et al., 1984). Castanospermine occurs in the honeydew of mealybugs feeding on *C. australe* and, therefore, is presumably ingested from the phloem. Furthermore, castanospermine is not active as a feeding deterrent against the mealybug (Dreyer et al., 1987). Perhaps, in the mealybug, sucrase is localized in such a way that it is not exposed to castanospermine.

However, castanospermine was a potent feeding inhibitor against the pea aphid but ineffective towards the other two aphids. Because of this potent feeding deterrency by castanospermine and its structural and stereochemical relationship to glucose, it was anticipated that the monocyclic analog, deoxynojirimycin, would be highly active as a toxicant or feeding deterrent. Deoxynojirimycin is a known trehalase inhibitor (Murao and Miyata, 1980) and, hence, was predicted to have a profound effect on the aphids. Such proved not to be the case, inasmuch as it possessed only weak activity against green peach aphids and no activity against the other aphids tested. Furthermore,

TABLE 1. SPECIFIC ACTIVITIES (SA, PKATALS/MG PROTEIN) AND CONCENTRATIONS OF CASTANOSPERMINE FOR 50% INHIBITION (I_{50} MOLAR) OF ENZYMATIC HYDROLYSIS OF DISACCHARIDE SUBSTRATES BY VARIOUS INSECTS[a]

	Substrate									
	Cellobiose		Lactose		Maltose		Sucrose		Trehalose	
Insect	SA	I_{50}	SA	I_{50}	SA	I_{50}	SA	I_{50}	SA	I_{50}
Homoptera										
Aphididae										
Myzus persicae	156	6.0×10^{-6}	141	2.3×10^{-5}	256	4.1×10^{-6}	182	5.2×10^{-6}	594	3.8×10^{-5}
Therioaphis maculata	112	3.4×10^{-6}	185	2.1×10^{-5}	289	5.5×10^{-6}	127	4.1×10^{-6}	541	4.1×10^{-5}
Acyrthosiphon pisum	144	2.3×10^{-6}	173	9.2×10^{-6}	338	3.0×10^{-6}	150	2.1×10^{-6}	624	2.2×10^{-5}
Schizaphis graminum	289	8.1×10^{-6}	252	1.5×10^{-5}	404	2.2×10^{-6}	175	5.6×10^{-6}	638	3.1×10^{-5}
Pseudococcidae										
Pseudococcus longispinus	55	2.2×10^{-5}	41	2.3×10^{-5}	305	1.1×10^{-6}	127	1.4×10^{-5}	305	7.9×10^{-5}
Coleoptera										
Coccinellidae										
Hippodamia convergens	36	9.8×10^{-6}	81	5.6×10^{-5}	1098	NI	1851	NI	1357	NI
Bruchidae										
Acanthoscelides aureolus	693	4.5×10^{-6}	467	NI	744	NI	348	NI	467	9.9×10^{-5}
Curculionidae										
Hypera brunneipennis	671	1.3×10^{-5}	342	1.5×10^{-4}	582	NI	490	NI	356	NI
Rhynchophoridae										
Sitophilus oryzae	844	3.2×10^{-5}	789	8.3×10^{-5}	1098	NI	1155	NI	510	NI
S. zeamais	516	4.4×10^{-6}	601	2.3×10^{-5}	688	NI	1179	NI	343	NI

Lepidoptera										
Sphingidae										
Manduca sexta	31	5.0×10^{-6}	25	1.2×10^{-5}	178	3.4×10^{-5}	653	NI	555	9.5×10^{-5}
Noctuidae										
Spodoptera frugiperda	21	1.2×10^{-6}	19	7.6×10^{-6}	161	NI	451	NI	420	1.4×10^{-5}
Heliothis zea	8	2.5×10^{-7}	6	3.9×10^{-6}	128	6.5×10^{-5}	212	NI	413	4.0×10^{-5}
Pyralidae										
Amyelois transitella	59	2.1×10^{-5}	42	5.0×10^{-5}	209	NI	368	NI	179	NI
Tortricidae										
Cydia pomonella	24	2.5×10^{-6}	86	3.6×10^{-6}	115	NI	115	NI	67	2.5×10^{-5}
Choristoneura occidentalis	50	2.3×10^{-5}	73	1.6×10^{-5}	1323	NI	1448	NI	283	7.9×10^{-5}
Gelechiidae										
Pectinophora gossypiella	59	1.3×10^{-5}	92	3.7×10^{-6}	477	NI	704	NI	307	3.5×10^{-5}
Sitotroga cereallela	51	2.4×10^{-5}	22	8.3×10^{-6}	612	NI	877	NI	18	NI
Diptera										
Tephritidae										
Unknown sp.	25	6.3×10^{-6}	68	1.7×10^{-6}	102	NI	68	NI	189	2.8×10^{-5}

[a] NI = not inhibited; viz., 50% inhibition $\geq 2.5 \times 10^{-4}$ M castanospermine.

TABLE 2. CONCENTRATION (MOLAR) OF POLYHYDROXY ALKALOID TO DETER APHID FEEDING BY 50% (ED_{50}) IN COMPARISON TO CONTROLS

	ED_{50}		
Alkaloid	*Schizaphis graminum*	*Acyrthosiphon pisum*	*Myzus persicae*
Castanospermine	inactive[a]	1×10^{-4}	inactive
6-Epicastanospermine	5×10^{-3}	inactive	[b]
Deoxynojirimycin	inactive	inactive	2.5×10^{-3}

[a]Inactive = $ED_{50} \geq 5 \times 10^{-2}$ M.
[b]Not assayed due to insufficient alkaloid available.

change in the stereochemistry of castanospermine to 6-epicastanospermine, a mannose analog, also resulted in a loss of feeding-deterrent activity against pea aphids and a gain of ostensible deterrency against greenbugs. The potential role of these alkaloids in insect–plant interactions is therefore not readily apparent from the results.

The inhibition of trehalose hydrolysis by castanospermine occurred, generally, in the Homoptera and Lepidoptera and also in the one dipteran analyzed. Trehalase inhibition by castanospermine is of particular interest since trehalose is the chief metabolic source of energy in insect blood (Chefurka, 1965). Again, the pattern of inhibition of trehalose hydrolysis between the various insects showed no relationship to the degree of adaptiveness to plants containing glycosidase-inhibitory substances. Oddly, the seed beetle from *Astragalus* was the only beetle studied whose trehalase activity was inhibited by castanospermine. This insect and the pea aphid, whose trehalase activity was also inhibited, feed on *Astragalus* species which contain the α-mannosidase inhibitory indolizidine alkaloid, swainsonine (Figure 1D) (Molyneux and James, 1982).

Cellobiase was the only enzyme observed to be inhibited by castanospermine in all insects studied. Similarly, inhibition of lactase activity was observed in all insects with the intriguing exception of the seed beetle from *Astragalus*. Qualitatively, enzymes with cellobiase and lactase activities are widespread among insects and are believed to be the same enzyme (Morgan, 1975). Indeed, cellobiase and lactase are indistinguishable by discontinuous electrophoresis in the maize weevil (Campbell and Van Vleet, unpublished) and in larvae of a xylophagous cerambycid, *Rhagium inquisitor* L. (Chipoulet and Chararas, 1985). Our findings also show generally similar specific activities for hydrolysis of these substrates within the same species of insect. However, the inhibition of cellobiase by castanospermine and lack of inhibition of lactase activity in the seed beetle would indicate that in this insect there are two different enzymes responsible for hydrolysis of these substrates.

It is worth noting that insects belonging to the Rhyncophoridae, Curculionidae, Bruchidae, and Aphididae, known to possess endosymbiotes (Buchner, 1965), all had average cellobiase and lactase activities 14 and 8 times, respectively, higher than those insects studied which do not maintain endosymbiotes. The beetles and larvae of the Curculionidae, Rhynchophoridae, and Bruchidae generally bore into the interior of pods or seeds of their host plant while the Homoptera exhibit a specialized mode of feeding by using stylet-like mouth parts to reach the vascular bundle of the host plant (Pollard, 1973). Higher activities of cellobiase, for the ultimate breakdown of β-linked glucans (e.g., cellulose), and of lactase, for breakdown of β-linked galactans [e.g., the arabinogalactan side chains of pectic substances (Aspinall, 1981)], in these insects may reflect a broader spectrum of enzymes for the depolymerization of plant matrix polysaccharides. The relatively low cellobiase and lactase activities of the mealybug (which possesses endosymbiotes) may be a result of its feeding on *Castanospermum*. Castanospermine, continuously ingested by the mealybug while feeding from the phloem of *Castanospermum*, may have attenuated the activity of these enzymes, resulting in the lower specific activities observed in the homogenates. The lack of inhibition of lactase in the *Astragalus*-feeding seed beetle may represent some level of biochemical adaptation to indolizidine alkaloids.

Castanospermine appears to be a potent inhibitor of insect β-glucosidase and β-galactosidase activities and, on a more taxonomically limited basis, α-glucosidase activity. Recently, the imino sugars, 1,5-dideoxy-1,5-imino-D-mannitol (Evans et al., 1983) and 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (Evans et al., 1985) were also shown to inhibit insect glycosidase activity at approximately the same concentration as castanospermine. These groups of compounds (and/or their analogs) may be promising candidates for the inhibition of insect digestive glycosidase activity which ultimately would disrupt normal insect carbohydrate metabolism.

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CHEMICAL STIMULANTS AND DETERRENTS REGULATING ACCEPTANCE OR REJECTION OF CRUCIFERS BY CABBAGE BUTTERFLIES

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Abstract—Gravid *Pieris rapae* butterflies oviposit on many, but not all, crucifers. Rejection of *Erysimum cheiranthoides* and *Capsella bursa-pastoris* was initially explained by the presence of chemical deterrents in the plants. Analyses and bioassays of plant extracts indicated the absence of oviposition stimulants in *C. bursa-pastoris*, but similar chemical separation of *E. cheiranthoides* extracts revealed the presence of stimulants as well as deterrents. Choice tests illustrate how acceptance or rejection of a plant by an insect may depend on the balance of positive and negative chemical stimuli within the plant.

Key Words—Cabbage butterfly, *Pieris rapae*, Lepidoptera, Pieridae, oviposition, stimulants, deterrents, *Erysimum cheiranthoides*, *Capsella bursa-pastoris*.

INTRODUCTION

The host ranges of phytophagous insects are determined to a large extent by the presence or absence of specific chemicals in potential host plants (Thorsteinson, 1960; Städler, 1976). Discriminatory behavior has been linked to olfactory or contact chemoreception of attractants, repellents, stimulants, and deterrents (Dethier, 1947; Schoonhoven, 1968). The final response of an insect in accepting or rejecting a particular plant is thought to be mediated by a balance of sensory inputs from these positive and negative chemical stimuli in the plant (Dethier, 1982; Miller and Strickler, 1984). However, definitive proof of such a dynamic relationship has been difficult to obtain. One major problem is to determine whether a nonhost plant is avoided by a herbivore because of the presence of deterrents or a lack of stimulants.

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The cabbage butterfly, *Pieris rapae* L., specializes on members of the Cruciferae (= Brassicaceae) and a few related plant families that contain mustard oil glycosides (Verschaffelt, 1911; Ma and Schoonhoven, 1973). Yet several crucifers are unacceptable to this insect (Feeny, 1977), and the chemical basis of such discriminatory behavior is not clear. Previous studies on *P. rapae* have shown that recognition of host plants by ovipositing butterflies depends on the presence of water-soluble chemical stimulants that are detected by tarsal contact with the plant (Traynier, 1979; Renwick and Radke, 1983), but host plants may also contain lipid-soluble deterrents (Renwick and Radke, 1985). Under natural conditions, these compounds apparently do not interfere with recognition of host plants, probably because their concentration on the surface of undamaged leaves is negligible. However, in nonhost plants, additional water-soluble deterrents are present, and these could be responsible for rejection by gravid butterflies (Renwick and Radke, 1985).

The crucifers that are unacceptable to *P. rapae* include *Erysimum cheiranthoides* L. and *Capsella bursa-pastoris* L. Both of these species contain water-soluble deterrents to oviposition (Renwick and Radke, 1985). The study reported here was designed to determine whether these deterrents alone can explain avoidance of the plants, or if the stimulants necessary for host recognition by cabbage butterflies are lacking.

METHODS AND MATERIALS

Insects and Plants. Butterflies used for behavioral assays were from a colony started from field-collected insects each summer and maintained on cabbage plants. Seeds of *E. cheiranthoides* and *C. bursa-pastoris* were collected in Vermont and Connecticut, respectively, by Dr. Frances S. Chew.

Extraction of Plant Materials. Plants were grown from seed under uniform conditions in the greenhouse. After three to four weeks, leaves were harvested and immediately dropped into boiling ethanol to minimize enzyme degradation of constituents. After cooling, the tissue was homogenized in a Waring blender and the resulting macerate filtered through glass wool. The ethanol extract was evaporated to dryness and the residue was sequentially washed with hexane and water. The water extract was filtered, evaporated to 150 ml, and extracted three times with *n*-butanol. Standard cabbage extracts were prepared using the same sequence of boiling ethanol, homogenization, filtration, evaporation, lipid removal, and water extraction.

Stimulant Bioassays. The presence of stimulant was detected using artificial plants consisting of 77 × 64-mm green index cards supported on wooden stems (Renwick and Radke, 1983). The cards were painted with 0.5 ml extract at a concentration of 5 g original fresh wt/ml to approximate the concentration of material present in a plant. Control cards were painted with solvent alone. Five pairs of butterflies were presented with one test card surrounded by three

control cards in a 60 × 60 × 60-cm cage in a greenhouse with supplemental lighting. Eggs laid on each card were counted after 24 hr. The stimulatory effect was measured by comparison with that of standard cabbage extract offered to the same butterflies on preceding and following days. Control cards were included to ensure that no eggs were laid on substrates that lacked stimulant.

Deterrent Bioassays. Deterrent activity was assayed using identical cages in the greenhouse. Five pairs of butterflies were offered a choice of two cabbage plants. One was sprayed with 3 g equivalents (original fresh weight) of test plant extract in 100% or 70% methanol (depending on solubility). The control plant was sprayed with methanol alone. The plants were left in the cage for 4 hr during the peak daily oviposition period before counting the eggs.

RESULTS

Fractionation of polar extracts of the test plants by partitioning between water and *n*-butanol resulted in transfer of the deterrent into the butanol (Figure 1). The butanol extracts of both *E. cheiranthoides* and *C. bursa-pastoris* were just as deterrent as the original water extract, whereas the activity was almost completely removed from the postbutanol aqueous layer (Figure 1).

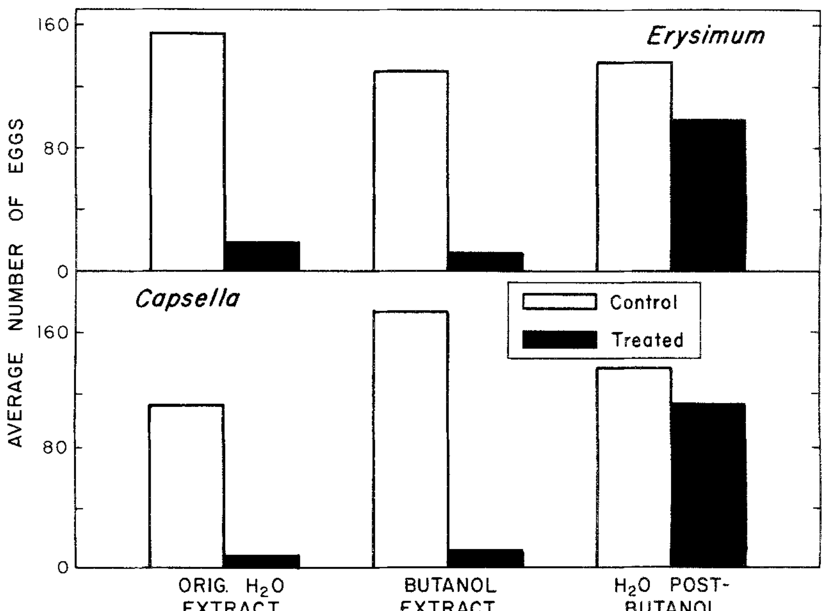

FIG. 1. Oviposition by *P. rapae* on cabbage plants treated with extracts of *Erysimum cheiranthoides* and *Capsella bursa-pastoris* or with solvent alone (controls) in choice assays.

TABLE 1. OVIPOSITION BY *P. rapae* ON CARDS TREATED WITH FRACTIONS OF EXTRACTS FROM *Erysimum cheiranthoides* AND *Capsella bursa-pastoris* COMPARED WITH STANDARD STIMULANT (H_2O EXTRACT OF CABBAGE)

Test material	No. of replications	Average No. eggs laid: Day 1 (cabbage standard)	Day 2 (test extract)	Day 3 (cabbage standard)	Relative stimulation (%)[a]
Erysimum cheiranthoides					
Orig. H_2O extract	7	92.7 (35.5)[b]	42.0 (51.8)	56.9 (27.7)	56
H_2O postbutanol	6	58.3 (28.3)	37.5 (40.2)	82.2 (28.8)	53
Butanol extract	9	76.5 (30.4)	27.0 (24.6)	68.1 (43.8)	37
Capsella bursa-pastoris					
Orig. H_2O extract	7	101.0 (38)	2.3 (3.5)	106 (23.5)	2.2
H_2O postbutanol	6	82.6 (17.1)	1.3 (2.8)	86.5 (27.6)	2.0
Butanol extract	5	137.6 (52.5)	0.6 (1.3)	154.2 (83)	0.4

[a]Relative stimulation (%) = No. eggs laid {day 2/[(day 1 + day 3)/2]} × 100, i.e., oviposition relative to mean oviposition on previous and following days in response to standard cabbage stimulant.
[b]Standard deviation is given in parentheses.

The same fractions from both plants were tested for stimulatory activity using artificial leaf bioassays. Activity was measured by comparison with standard cabbage extracts of known activity. The effect of possible changes in the butterflies' potential to lay eggs was eliminated by offering cabbage standard on days preceding and following the tests (Table 1). The results of these experiments clearly showed that the water-soluble fraction of *E. cheiranthoides* contains stimulant. Although less active than equivalent concentrations of cabbage extracts, this material was stimulatory before and after partitioning with butanol (Table 1). Some oviposition also occurred on cards treated with the butanol extract of *E. cheiranthoides* because of partial removal of the stimulant during the water–butanol partitioning.

Extracts of *C. bursa-pastoris* showed no significant stimulatory activity in any of the tests (Table 1).

DISCUSSION

The results indicate that the chemical explanation for avoidance of these two crucifers by cabbage butterflies is not the same. Since extracts of *C. bursa-pastoris* are not stimulatory, the presence of deterrent is not necessary for rejection of this plant. The lack of stimulant alone could account for unacceptability to landing butterflies. However, the presence of stimulant in *E. cheiran-*

thoides means that oviposition might be expected on this plant. Apparently the negative effect of the oviposition deterrent is sufficient to block the positive input from the stimulants. This result supports the suggestion of Jermy (1965) that the most potent stimuli inducing oviposition can be masked by inhibitory substances at an appropriate concentration.

Care is needed in interpretation of such laboratory results, especially when attempting to explain the factors that mediate oviposition in the field. Our green cards treated with standard cabbage extract are not as attractive as a host plant of comparable size. However, the concentrations of extracts used in these experiments were selected to represent levels of chemical stimuli comparable to those encountered by butterflies under natural conditions. Our efforts to mimic real plants are further complicated by the fact that little is known about the distribution of chemicals throughout the leaf. Since the ovipositional cues are contact stimuli, the active chemicals must be present at the leaf surface. Yet we have been unsuccessful in attempts to remove stimulant from cabbage leaves by solvent dipping (unpublished results). Despite these limitations in interpretation, we feel confident in concluding that we can readily determine whether stimulants are present in a plant. *P. rapae* does not lay eggs on blank green cards or cards that are treated with extracts of noncrucifer, nonhost plants (unpublished results).

The use of separate bioassay systems to detect oviposition stimulants and deterrents appears to be critical. The stimulant assay is particularly sensitive, and the use of three control cards in each cage emphasizes the ability of the butterflies to discriminate between low concentrations of active material or solvent alone (Renwick and Radke, 1983). Butanol extracts of *E. cheiranthoides*

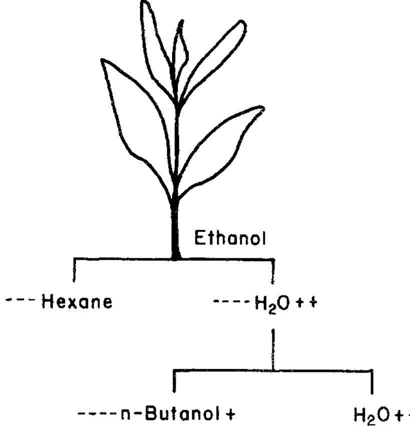

FIG. 2. Schematic representation of stimulant (+) and deterrent (−) activity of fractions from *Erysimum cheiranthoides* extracts affecting oviposition by *P. rapae*.

were slightly stimulatory when tested on green cards, even though most of the stimulant remained in the water fraction and despite the presence of deterrent (Table 1). When the same material was applied to cabbage plants, the deterrent effectively blocked the natural stimulant at the surface of the leaves (Figure 1). The reason for the lack of sensitivity to deterrent on the inert substrate provided by cards is not clear at this time. But the phenomenon does lend support to our conclusion that *Capsella* contains little or no stimulant since, even if other deterrents were present, they would probably have little effect.

The sequential extraction and the use of different bioassays for the study of *E. cheiranthoides* have effectively removed the mask from the stimulant which would not otherwise be detected (Figure 2). This may be the first demonstration that both oviposition stimulants and deterrents can actually occur in a plant that is rejected by an insect. The balance of such positive and negative signals within a plant is likely to play a major role in the acceptance or rejection of potential hosts by an insect.

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(Z)-11-OCTADECENYL ACETATE, AN AGGREGATION PHEROMONE IN *Drosophila simulans*

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Abstract—Existence of a male-produced pheromone, which attracts both males and females in a wind-tunnel olfactometer, has been demonstrated in *Drosophila simulans* (Sturtevant). A pheromone component was identified as (Z)-11-octadecenyl acetate (Z11-18:Ac), also known as *cis*-vaccenyl acetate. The pheromone is synergized by food volatiles. In bioassay ca. 1/1000 of a mature male equivalent of Z11-18:Ac is attractive and activity increases with increased amounts of Z11-18:Ac. Flies do not begin responding to Z11-18:Ac until after they have been away from food for at least 2 hr. Z11-18:Ac is transferred from the male to the female during mating, and the female emits the majority of the transferred Z11-18:Ac within 6 hr after mating.

Key Words—Diptera, Drosophilidae, *Drosophila simulans*, aggregation pheromone, (Z)-11-octadecenyl acetate, *cis*-vaccenyl acetate.

INTRODUCTION

Aggregation pheromones have previously been studied in seven *Drosophila* taxa: *D. virilis* (Bartelt and Jackson, 1984; Bartelt et al., 1985a); *D. a. americana, D. a. texana, D. novamexicana*, and *D. lummei* (Bartelt et al., 1986); *D. hydei* (Moats et al., 1987); and *D. melanogaster* (Bartelt et al., 1985b). In all these taxa, the pheromone component(s) are produced only by mature males and attract both males and females in a wind-tunnel olfactometer. This study was undertaken to determine whether an aggregation response could be demonstrated in *D. simulans* (Sturtevant), a close relative of *D. melanogaster*. In *D. melanogaster* a major aggregation pheromone component was identified as (Z)-

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11-octadecenyl acetate (Z11-18:Ac) (Bartelt et al., 1985b). This pheromone, in combination with food, substantially increased the attractiveness of food odors. Z11-18:Ac has been reported in *D. melanogaster* to mediate close-range behavior as well, by discouraging males from courting other males or recently mated females (Jallon et al., 1981). Because of the dual role of Z11-18:Ac in *D. melanogaster* and because *D. simulans* also possesses Z11-18:Ac (Jallon, 1984), it was hoped that *D. simulans* would provide further information on the function of Z11-18:Ac. Starting with crude extracts of mature flies and using bioassay to guide purification, Z11-18:Ac was identified as a major component of the aggregation pheromone of *D. simulans*. Other experiments related to aggregation in *D. simulans* are also discussed.

METHODS AND MATERIALS

Flies. D. simulans (strain 14021-0251.0) was obtained from the National *Drosophila* Species Resource Center at Bowling Green, Ohio. It was originally collected at Kenscoff, Haiti. Flies were reared on Instant *Drosophila* Medium Formula 4-24 (Carolina Biological Supply Co. Burlington, N.C.) in 1-liter jars or in 3.5 × 10-cm vials under a light–dark cycle of 16:8 hr and ambient laboratory temperatures.

Extracts and Chromatography. Flies were separated by sex when 0–6 hr old and extracted at that time or at 6–7 days of age by soaking them in hexane at room temperature for 24 hr. The crude extracts were separated by polarity on open columns of silicic acid (Bartelt and Jackson, 1984). Elution solvents were hexane; 10% ether in hexane; 50% ether in hexane; and 10% methanol in methylene chloride. Further purification of the male 10% ether–hexane fraction was done by preparative GC. Preparative and analytical GC were conducted as described previously (Bartelt and Jackson, 1984).

Bioassay. Bioassays were conducted in a wind-tunnel olfactometer (Bartelt and Jackson, 1984). The bioassay was used to screen chromatographic fractions for activity and to compare various synthetic and fly-derived preparations. Approximately 1000 flies (0–2 days old) were placed into the olfactometer 2 hr before tests would begin. Tests lasted 3 min and were separated by 7–10 min. When an experiment included more than two treatments, they were tested in pairs, in all possible combinations (a balanced incomplete block design). Generally, each experiment included a high control [fly-derived or synthetic compound(s) that bioassayed well] and a low control (solvent) in addition to the preparation(s) being evaluated. The activity level of a treatment was usually expressed relative to the high and low controls because catches to any one treatment varied from day to day, but ratios between treatments remained fairly constant. Analysis of bioassay data was by the method of Yates (1940), paired

t tests, or simple linear regression. The data were transformed to the log (X + 1) scale before analysis to stabilize variance.

Once it was established that the flies responded to Z11–18 : Ac, three additional bioassay experiments were conducted involving this compound. First, the relationship between magnitude of response and dose of Z11–18 : Ac was investigated. Doses ranged from 0.1 ng to 10,000 ng, by factors of 10. Each bioassay experiment contained two consecutive doses and a control. All possible pairs of consecutive doses were tested, and results are expressed relative to the activity (= 100) for 1000 ng. Second, the relationship between response to Z11–18 : Ac and time away from food was studied by measuring the bioassay response, compared to controls, every 15 min throughout the day. To distinguish between time-of-day effects and those due to time away from food, four replications of the experiment were begun at 0800 hr, while four others began at 1100 hr. Third, synergism between Z11–18 : Ac and food odors (from rearing medium inoculated with yeast) was investigated, throughout the day (three replications) and at the time of "peak" activity.

Z11–18:Ac Transfer. An experiment was conducted to test for the transfer of Z11–18 : Ac from males to females and for the subsequent release of the ester from the females. Virgin females (6–7 days old) were mated with virgin males of the same age. Immediately after mating, females were either extracted or placed into an empty glass vial or vial with food medium for 6 hr and then extracted. The vials were then rinsed with hexane to recover any Z11–18 : Ac which had been released (Bartelt et al., 1985b). To recover the Z11–18 : Ac quantitively, it was necessary to rinse the food vials again with methylene chloride. The mated males were treated in a similar fashion. Each extract represented 10 fly equivalents. As controls, comparable sets of extracts were obtained from males and females which had not been allowed to mate. The Z11–18 : Ac in the extracts was quantitated by GC relative to an internal standard (Bartelt et al., 1985b). The rearing medium did not contain compounds with the same GC retention as Z11–18 : Ac.

RESULTS

Identification of Pheromone Component. The male crude extract was clearly active in bioassay against a control ($P < 0.001$). For example, the mean catches for male extract and control were 13.9 and 1.4, respectively ($n = 16$). Flies attracted to the male extract were 54% female. Both sexes responded similarly to all preparations discussed in this report.

After fractionation of the male extract on silicic acid, only the 10% ether–hexane fraction was substantially different from the control ($P < 0.001$) in bioassay (Table 1). By capillary GC, the male 10% ether–hexane fraction was

TABLE 1. ACTIVITY OF SILICIC ACID FRACTION AND EXTRACTS RELATIVE TO MALE CRUDE EXTRACT

Fraction or extract[a]	Relative response[b]	
	Male extract	Female extract
Hexane	6.4*[c] (4.5, 3.0, 26.5)[d]	−0.7 (0.8, 0.9, 14.5)
10% ether–hexane	85.0***(26.4, 5.5, 30.1)	26.0***(12.9, 5.8, 32.7)
50% ether–hexane[e]	0.8 (1.7, 1.6, 9.1)	3.0 (4.2, 3.4, 29.0)
10% methanol–methylene chloride	2.6 (2.1, 1.6, 21.1)	15.7 (3.9, 2.5, 11.4)
Crude extract	100.0***	63.0***(9.3, 1.4, 13.9)

[a] All extracts and fractions were used at one fly equivalent per test.
[b] Relative response = (fraction − control)/(male crude extract − control) × 100.
[c] *and*** denote significance of t tests vs. controls at the 0.05 and 0.001 levels, respectively.
[d] Even catches ($N = 8$) for test treatment, control, and male crude extract, respectively, shown in parentheses.
[e] Because 50% ether–hexane solvent was attractive to the flies, this fraction was taken almost to dryness under nitrogen and back up in hexane.

86% one compound. Previous work with *D. melanogaster* (Bartelt et al., 1985b) and *D. simulans* (Jallon, 1984) suggested this was probably Z11–18 : Ac. GC retention time of fly-derived and synthetic Z11–18 : Ac (Sigma Chemical Co., St. Louis, Missouri), mass spectra, and double-bond location by ozonolysis-GC confirmed this identity. Young males 0–6 hr old possessed only trace amounts of Z11–18 : Ac and virgin females of any age contained none.

The crude extract, 10% ether–hexane fraction, synthetic Z11-18 : Ac (99 + % pure) and fly-derived Z11–18 : Ac (obtained from the 10% ether–hexane fraction by preparative GC, 98 + % pure) were compared by bioassay (all at 640 ng Z11–18 : Ac/test, ca. 1 fly equivalent, Table 2). All were significantly greater than the control ($P < 0.001$). The synthetic and fly-derived Z11–18 : Ac treatments were not significantly different, and Z11–18 : Ac accounted for all the activity of the male 10% ether–hexane fraction and crude extract. In this study the 10% ether–hexane fraction, synthetic Z11–18 : Ac, and fly-derived Z11–18 : Ac were significantly greater than the crude extract ($P < 0.05$).

Other Studies with Z11–18 : Ac. Response to Z11–18 : Ac increased with dose (Figure 1, slope significant, $P < 0.005$, simple linear regression). A dose of 1 ng, comparable to ca. 1/1000 of a male equivalent was significantly greater than the control ($P < 0.01$), although 0.1 ng was not. The 1000 ng and 10,000 ng doses were not significantly different from each other ($P > 0.2$), suggesting further increases in dose would cause little, if any, increase in response.

TABLE 2. SOURCES OF Z11-18:Ac COMPARED IN BIOASSAY

Source[a]	Mean bioassay catch[b] (N = 32)
Control	4.7a
Synthetic Z11-18:Ac	24.8c
Fly-derived Z11-18:Ac	25.0c
Male 10% ether-hexane	28.5c
Male crude extract	19.1b

[a]Compared on an equal-weight basis for Z11-18:Ac (640 ng/test).
[b]Means followed by a different letter were significantly different at the 0.05 level (LSD).

The responsiveness of the flies to Z11-18:Ac increased with time away from the rearing jars (Figure 2), becoming significantly greater than the control (P = 0.01) after 2 hr. The onset of response was accompanied by a change in behavior. When first introduced into the olfactometer, the flies remained on the floor and moved about very little. Within a few hours, the flies became more

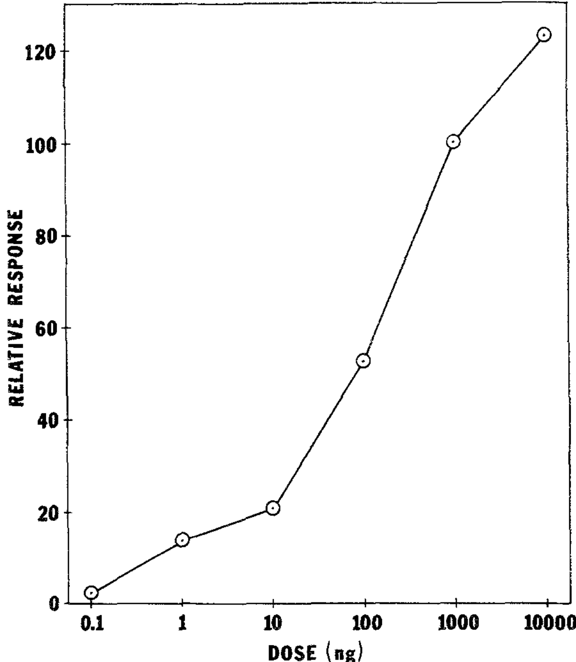

FIG. 1. Dose-response relationship for synthetic Z11-18:Ac. Relative responses are 100% for the 1000 ng dose and 0% for the controls. (See Table 1 for calculation of relative response.) Spacing along x axis is logarithmic.

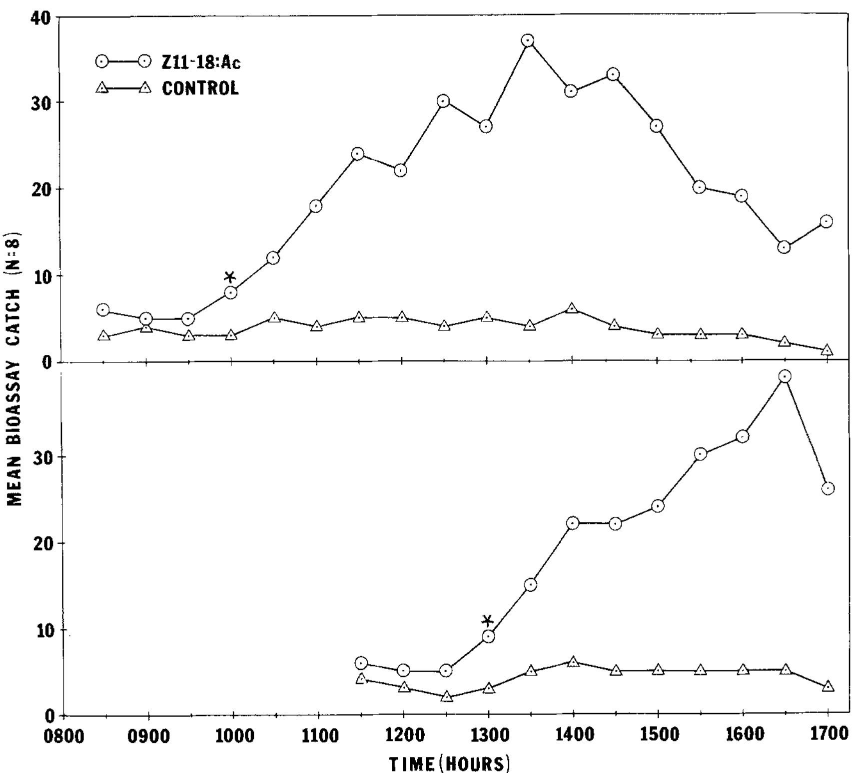

FIG. 2. Bioassay activity of Z11-18 : Ac vs. time after removing flies from food. Flies were placed in the olfactometer at 0800 hr (upper panel) or 1100 hr (lower panel). Synthetic Z11-18 : Ac was tested at 750 ng/test (ca. 1 fly equivalent). *Indicates when Z11-18 : Ac became significantly greater than the control ($P = 0.05$).

active and spent more time on the sides of the olfactometer and in flight. This response continued throughout the day, although the number caught per test decreased with time because fewer flies were left in the olfactometer. Bioassay behavior included flying upwind with a hovering, casting flight and descending into the vial or alighting on the vial and walking inside.

When a food source (Petri plate with rearing medium and yeast) was present in the downwind end of the olfactometer, the response to Z11-18 : Ac never commenced. After being tested for 6 hr, the mean catches for synthetic Z11-18 : Ac (750 ng/test) and control were 0.43 and 0.29, respectively ($N = 14$). Most of the flies spent the entire day at the food plate, moving or flying very little.

The onset of the responsiveness to Z11-18 : Ac appeared more related to time away from food than to time of day. The response to Z11-18 : Ac became

TABLE 3. SYNERGISM OF SYNTHETIC Z11-18:AC AND FERMENTED FOOD

Treatment	Mean bioassay catch[a] ($N = 18$)
Control	0.8a
Synthetic Z11-18:Ac[b]	10.6b
Food[c]	43.2c
Z11-18:Ac + food	103.6d

[a]Means followed by a different letter were significantly different in the log ($n + 1$) scale at the 0.001 level (LSD).
[b]Synthetic Z11-18:Ac used at 750 ng/test (ca. 1 fly equivalent).
[c]"Food" is 0.5 ml of *Drosophila* rearing medium inoculated with yeast 24 hr before bioassays began and placed in the bottom of a bioassay vial.

significantly greater than the control ($P = 0.01$) 2 hr after placing the flies into the olfactometer, regardless of whether this was done at 0800 or 1100 hr (Figure 2).

Although successful bioassays could be conducted with *D. simulans* in the absence of food, Z11-18:Ac was a potent synergist of the fermented rearing medium (Table 3). Z11-18:Ac increased the response to the food treatment by 60 flies per test, even though Z11-18:Ac alone caught only 10 flies per test. The treatment, Z11-18:Ac plus fermented food, was so attractive that flies responded to it above control levels within minutes of placing the flies into the olfactometer (Figure 3). Yet response to this treatment, as well as to the ester and fermented food alone, did increase strongly over time, consistent with earlier results (Figure 2). Catches for Z11-18:Ac alone were relatively few in this study, presumably due to the competition from the food-containing treatments. Nevertheless, Z11-18:Ac became significantly greater than the control 2 hr after tests began ($P = 0.05$), in agreement with earlier results.

Z11-18:Ac Transfer. Virgin females did not possess any Z11-18:Ac. However, males transferred approximately half of the Z11-18:Ac they possessed to the female during mating (Table 4). Within 6 hr after copulation, the female deposited the majority of the Z11-18:Ac transferred into a vial which contained rearing medium or even into an empty glass vial. The amount of Z11-18:Ac emitted by a recently mated female was ca. 10 times greater than that emitted by a virgin male. Few eggs were detected in the rearing medium vials after 6 hr, and none were in the empty vials.

Other Attractants. In bioassay, attraction to the female extract was significantly greater than the control (Table 1). Bioassay of all female silicic acid fractions indicated the 10% ether-hexane fraction was significantly above control levels ($P < 0.001$, Table 1), although not as active as the male-derived fraction. Thus a relatively nonpolar attractant in females of *D. simulans* is in-

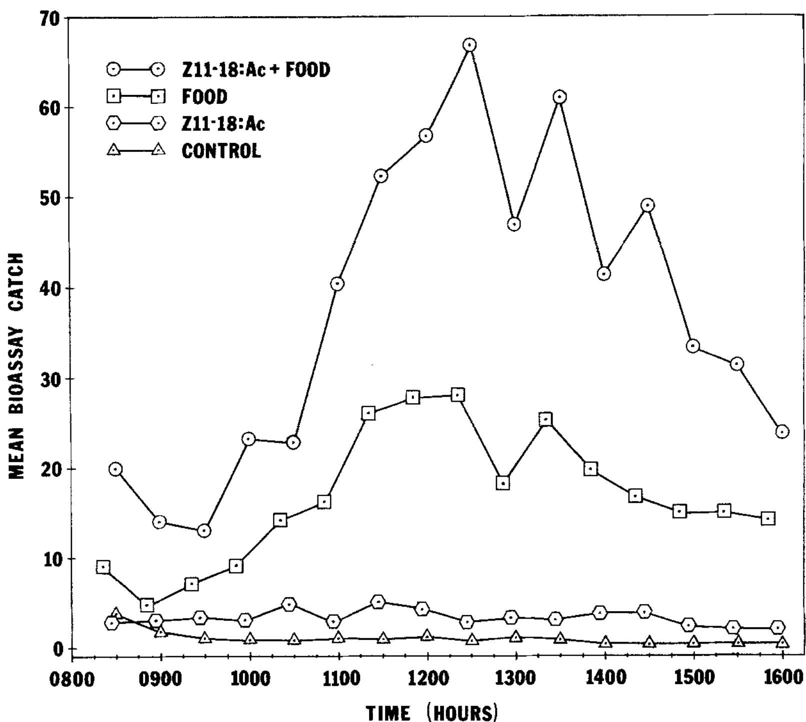

FIG. 3. Synergism of Z11–18 : Ac and fermented food over time. In each test, the treatment was bioassayed against a control. Except for controls, each point represents the mean of six catches (3 replications of experiment × 2 tests in each time interval). All control catches were combined to simplify the figure (N = 18); the means in any one time interval differed by no more than ±1 fly. Z11–18 : Ac was used at 750 ng/test (ca. 1 fly equivalent).

dicated (by GC no Z11–18 : Ac was detected). Identification of the attractant in this fraction is presently under investigation.

The male hexane fraction was also significantly greater than the control (P = 0.05) but accounted for only 6% of the activity of the male extract. The response was very subtle, and isolation of the active compound(s) under the present bioassay scheme would be very difficult.

The 10% methanol–methylene chloride fractions for both male and female were not significantly different from the control, although the extraction process for the crude extracts was geared toward the nonpolar attractants. A more thorough extraction may reveal active polar fractions, as observed in other *Drosophila* species (Bartelt et al., 1985a, 1986).

TABLE 4. MEAN AMOUNTS OF Z11-18:Ac EXTRACTED FROM MATED AND VIRGIN FLIES, AND FROM EMPTY VIALS OR FOOD VIALS WHICH THEY OCCUPIED FOR 6 HOURS

	Mean amount of Z11-18:Ac (ng/fly)			
Treatment	Mated male[a]	Mated female[a]	Virgin male	Virgin female
Flies immersed in hexane ($N = 4$)[b]				
Fly extract	386(±68)[c]	418(±112)	640(±137)	0
Flies in empty vial for 6 hr ($N = 2$ virgin, $N = 4$ mated)				
Fly extract	475(±65)	123(±81)	990(±438)	0
Vial rinse	30(±14)	158(±30)	0	0
Flies in food vial for 6 hr ($N = 2$ virgins, $N = 5$ mated)				
Fly extract	416(±89)	94(±38)	905(±78)	0
Vial rinse	48(±40)	188(±61)	15(±7)	0

[a] These flies either extracted or placed in vials for 6 hr immediately upon completion of mating.
[b] Each replication represents a group of 10 flies.
[c] Standard deviation.

DISCUSSION

Males of *D. simulans*, like *D. melanogaster*, possess Z11-18:Ac as a major pheromone component which attracts males and females in a wind-tunnel olfactometer. In *D. melanogaster*, Z11-18:Ac is found in the ejaculatory bulb, transferred to the female during mating (Brieger and Butterworth, 1970; Butterworth, 1969), and deposited by the female onto the rearing medium within 6 hr after completion of mating (Bartelt et al., 1985b). *D. simulans* is similar to *D. melanogaster* in the transfer and release of Z11-18:Ac. In both species, the mature male emits Z11-18:Ac in small quantities and a recently mated female in larger amounts. *D. simulans* is also similar to *D. virilis* in that both species must be deprived of food in order to be attracted to their aggregation pheromone in the olfactometer (Bartelt and Jackson, 1984).

Food or food odors are not needed in combination with Z11-18:Ac for a successful bioassay with *D. simulans*, as is the case for *D. melanogaster* (Bartelt et al., 1985b). Therefore, *D. simulans* would be the more convenient species in which to investigate additional, minor pheromone components.

In nature, mating, feeding, and oviposition occur at the same site. It is accepted that flies are attracted to food sites by odors (Spieth, 1974). We believe attraction to a site is a complex phenomenon, involving both food odors and fly-derived compounds. *D. simulans* males produce an attractant, Z11-18:Ac, that is a powerful synergist of food odors. One nanogram of Z11-18:Ac was sig-

nificantly more attractive than a control in our bioassay, and over a 6-hr period, a mature male or recently mated female is capable of emitting well over this amount at a food site. Since 10 times more Z11–18:Ac is emitted by recently mated females than virgin males, mating sites would be particularly attractive. Interestingly, Spence et al. (1984) found that male *D. simulans* are preferentially attracted to cylinders which had contained recently mated females compared to those with males or virgin females. Thus, a fly without a food source, moving through an area, would tend to alight preferentially at a food source which was already occupied by others of the species, especially if mating had taken place. Once at a food site, the flies would tend to remain on or near the food (Spieth, 1974). Flies in the olfactometer did not respond to Z11–18:Ac when given a food source or until deprived of food for a few hours, which indicates that responsiveness to the aggregation pheromone diminishes upon feeding.

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Announcement

The 1st International Conference on Classification, Phylogeny and Natural History of Scolytidae (Coleoptera)

To be held July 3–9, 1988, Vancouver, British Columbia, Canada, in conjunction with the XVIII International Congress of Entomology, will focus on current research relating to the morphology, systematics, phylogeny, zoogeography, ecology, physiology, and behavior of the Scolytidae. Potential participants and topics are now being solicited. Anyone interested in participating in the symposium is asked to notify one of the co-conveners listed below.

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Canada Agriculture
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G.N. Lanier
Environmental and Forest Biology
SUNY College of Environmental Science
and Forestry
Syracuse, New York 13210
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CYANOGENESIS—A GENERAL PHENOMENON IN THE LEPIDOPTERA?

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Abstract—There are two different pathways known to be used for the detoxification of hydrocyanic acid in insects, viz., rhodanese and β-cyano-L-alanine synthase. We consider the latter to be indicative for cyanogenesis, while rhodanese might, in general, play a more important role in sulfur transfer for protein synthesis. This paper reports on the distribution of β-cyano-L-alanine (BCA) in the Lepidoptera. First reports of cyanogenesis are presented for the following families: Papilionidae, Pieridae, Lycaenidae, Hesperiidae, Lymantriidae, Arctiidae, Notodontidae, Megalopygidae, Limacodidae, Cymatophoridae, Noctuidae, Geometridae, and Yponomeutidae. New and old records for three other families, the Nymphalidae, Zygaenidae, and Heterogynidae, are included to complete the present state of knowledge. Special emphasis has been laid on the Nymphalidae, where BCA has been detected in eight subfamilies. Taxonomic, geographic, and seasonal variation has been found in a number of cases. In all cases observed so far, the source of cyanogenesis in the Lepidoptera is most probably the cyanoglucosides linamarin and lotaustralin, although cyanogenesis based on mustard oil glucosides and cyclopentenoid glucosides might occur as well. BCA has been found in both cryptic and aposematic species, including taxa such as the Pieridae, Danainae, Ithomiinae, and Arctiidae, where the defensive biology is believed to be linked with other compounds, like mustard oil glucosides, cardenolides, or pyrrolizidine alkaloids. The ecological interaction and significance of such secondary compounds is not yet understood.

Key Words—Lepidoptera, chemical defense, cyanogenesis, cyanoglucosides, β-cyano-L-alanine, rhodanese, phytophagous insects.

INTRODUCTION

According to recent records, cyanogenesis in animals is restricted to a few cases in the Arthropoda, where examples are known from the Myriapoda, the Diplo-

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poda, and the orders Heteroptera, Coleoptera, and Lepidoptera in the Insecta (Davis and Nahrstedt, 1984). In the Myriapoda, Diplopoda, and Coleoptera (Cicindellinae and Chrysomelidae), HCN and benzaldehyde are produced by catabolic decomposition of mandelonitrile (benzaldehyde-α-hydroxynitrile) (Blum et al., 1981). In other insects, i.e., in *Acraea* and *Heliconius* butterflies (Nymphalidae) and in *Zygaena* moths (Zygaenidae), the source of cyanogenesis has been detected as the cyanoglucosides linamarin (2-β-D-glucopyranosyloxy-2-methylpropionitrile) and lotaustralin (2-β-D-glucopyranosyloxy-2-methyl-2*R*-butyronitrile) (Davis and Nahrstedt, 1979; Nahrstedt and Davis, 1983).

Most larval food plants of *Zygaena* moths and *Acraea* and *Heliconius* butterflies are themselves cyanogenic, but the source of cyanogenesis is different: in certain species of *Zygaena* the cyanoglucosides of the larval food plants (Fabaceae) and those of the insects are chemically identical, while the food plants of *Acraea* and *Heliconius* (Passifloraceae) usually contain cyanoglucosides with a cyclopentenoid aglycon (Davis and Nahrstedt, 1984). This indicates that larvae of *Zygaena*, *Heliconius*, and *Acraea* biosynthesize their cyanoglucosides independently from larval ingestion of cyanogenic food plants. In fact, Wray et al. (1983) have demonstrated that *Heliconius* and *Zygaena* use the same biosynthesis as plants that contain linamarin and lotaustralin. This synthesis starts from valine for linamarin and isoleucine for lotaustralin (Conn, 1979) (see Figure 1). The high amounts of cyanogenic compounds in these insects are generally thought to be linked with distastefulness to predators, which again is believed to be connected with the aposematic patterns of both the larvae and imagines.

Two different detoxification pathways for hydrocyanic acid have so far been demonstrated in insects (Figure 1).

1. The more commonly known, which had been favored especially in the older literature (Jones et al., 1962), is that found in vertebrates which produces thiocyanate (rhodanid) via the enzyme rhodanese (EC 2.8.1.1). The presence of this enzyme has been demonstrated by Beesley et al. (1985) to occur in a considerable number of phytophagous insects.

2. In other insects the catabolic reaction leads to β-cyano-L-alanine (BCA) via BCA synthase (EC 4.4.1.9). The presence of BCA in insects was first demonstrated by Duffey (1981) in larvae of *Heliothis zea* and *Spodoptera exigua* (Lepidoptera, Noctuidae) and in *Oncopeltus fasciatus* (Heteroptera, Lygaeidae). BCA has also been detected in the defensive secretion and tissues of *Zygaena* larvae, in *Zygaena* moths and in dried specimens of *Acraea* and *Heliconius* (Witthohn and Naumann, 1984a,b).

None of these detoxification systems is necessarily bound to the cyanoglucosides linamarin and lotaustralin; both could well be able to detoxify hydrocyanic acid released from any other cyanogenic precursor as well. It has also been argued that the role of rhodanese as a sulfur donor in protein synthesis

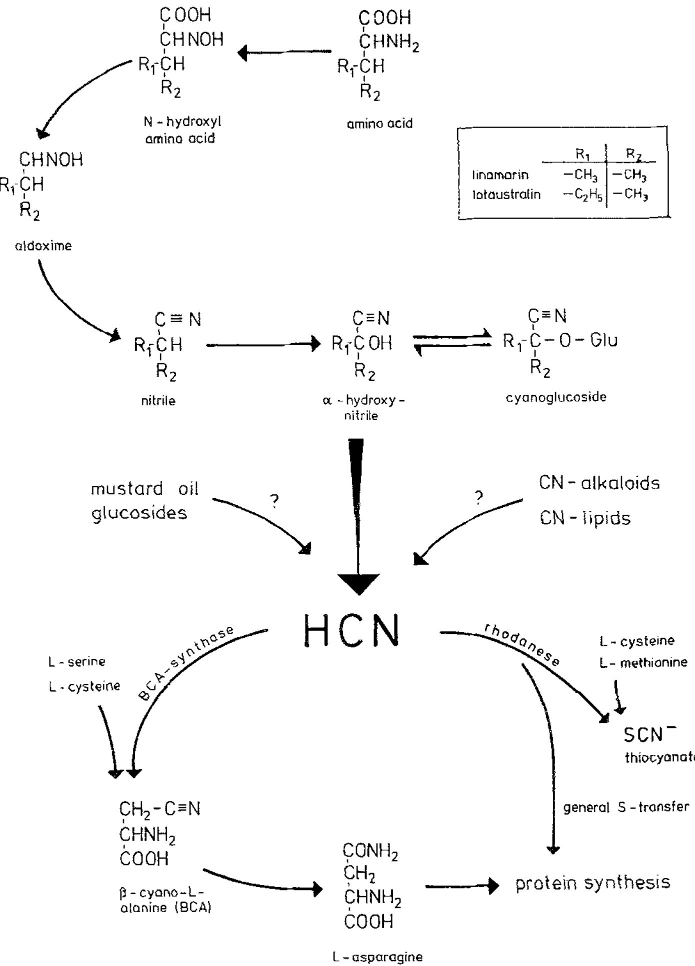

FIG. 1. Metabolic pathways for cyanoglucoside synthesis (modified, after Conn, 1979) and detoxification of hydrocyanic acid.

might be more important than its detoxifying properties (Volini and Alexander, 1981). If so, the presence of rhodanese does not necessarily imply that an organism is cyanogenic, but the evidence of BCA in animal tissues can generally be regarded as indicative for the presence of cyanogenic compounds. It is in this sense that we have used the test for BCA as an indicator for cyanogenesis in Lepidoptera in the present paper. Of course, the absence of BCA does not necessarily mean that the animal concerned is acyanogenic.

It has been shown that nonaposematic species of the Zygaenidae, especially those of the Zygaeninae, also contain the cyanoglucosides linamarin and lotaustralin (Davis and Nahrstedt, 1984; Witthohn and Naumann, 1984b), although like some aposematic species, they live on the same noncyanogenic food plants, the alkaloid-rich Celastraceae. Furthermore, we have previously demonstrated that cryptic species of the Zygaeninae detoxify hydrocyanic acid via BCA synthase, using the same method as their aposematic relatives. Similarly BCA has been demonstrated in some nymphalid genera unrelated to *Acraea* and *Heliconius* (viz., *Vanessa*, *Cynthia*, and *Maniola*). Of these, at least *Maniola* must be regarded as cryptic, although females of this species have been found to contain a histamine-like compound, leading to their rejection by a shama thrush (*Kittacincla malabaricus*) (Rothschild, 1985). Neither *Vanessa*, *Cynthia*, nor male *Maniola* butterflies have hitherto been thought to be chemically protected. Finally BCA has even been detected in the danaine butterfly *Danaus chrysippus*, a species which has been thought to be associated with cardioglucosides and pyrrolizidine alkaloids exclusively (Witthohn and Naumann, 1984b).

Following these results, we decided to investigate other families of butterflies for the presence of BCA and, having found it to be present in an astonishing number of cases, to extend the study to other lepidopterous families as well. The results of our research are presented in this paper.

METHODS AND MATERIALS

The tests were preferably carried out with fresh specimens which were either bred in captivity or caught in the wild. When such material was not available, specimens were either obtained from private collectors or from commercial dealers (Fa. Lörcher, Urach, F.R.G.). All tests on dried material reported here were done with specimens less than 5 years old, as we have found that BCA decomposes with time and its presence may be difficult to prove in older specimens, even of highly cyanogenic species. Thus, we could not demonstrate BCA in dried specimens of *Morpho* (Nymphalidae, Morphinae), while examples freshly emerged from imported pupae proved positive. The specimens were crushed and mixed with an aliquot solution of methanol–conc. aqueous ammonia (80:20), of which 10 μl were used for the tests. BCA was detected by two-dimensional thin-layer chromatography (TLC sheets 5 × 5 cm, Merck, silica gel 60, 0.2 mm), eluants: (1) chloroform, methanol, conc. aqueous ammonia (5:5:1); and (2) *n*-butanol, acetic acid, water (5:1:2). The sheet was sprayed with ninhydrin solution after drying. BCA could be identified by its characteristic blue color and its retention times: $R(F1) = 0.55$, $R(F2) = 0.19$. In a number of cases, the presence of the cyanoglucosides linamarin and lotaustralin was also examined by thin-layer chromatography with available lin-

amarin (Calbiochem) as reference; eluant ethylacetate–acetone–water (4 : 5 : 1), and spraying with anisaldehyde (Stahl and Kaltenbach, 1961).

Enzymatic activity, which must be responsible for the presence of BCA, was demonstrated following Henrickson and Conn (1969).

RESULTS

The results are given in Table 1. We have also provided information on the geographic origin of the specimens, the larval food-plant family, and whether cyanogenesis has been recorded in these food plants. These data have been taken from various literature sources.

According to the strength of the reaction, we have classified the concentration (c) of BCA into three groups: +: $c \leq 2$ μg/insect; ++: $2 \leq c \leq 20$ μg/insect; +++: $c \geq 20$ μg (175 nmol)/insect. The presence of the cyanoglucosides linamarin/lotaustralin has not been detected in all cases where BCA was found to be present, but they usually are present, when the reaction for BCA is strong (+++) and sometimes even when it is medium (++). Thus, the results are influenced by the fact that the test for the cyanoglucosides is less sensitive than that for BCA.

DISCUSSION

One might argue that the amounts of BCA detected in the different Lepidoptera could be due to postmortem microbial action and not to actual biosynthesis of the living insect. We have therefore compared the strength of the ninhydrin reaction for BCA in fresh and dried specimens of a number of species, including *Clossiana euphrosyne* (Nymphalidae), a number of *Zygaena* species and *Aglaope infausta* (Zygaenidae), and *Heterogynis penella* (Heterogynidae). There was no difference, and we therefore feel justified to presume that the amount of BCA contained in dried insects is, in fact, derived from the insect itself. We have furthermore demonstrated the presence of BCA synthase in *Clossiana euphrosyne* (Nymphalidae); *Pryeria sinica*, *Orna nebulosa*, *Zygaena trifolii*, *Rhagades pruni*, and *Aglaope infausta* (Zygaenidae); and *Heterogynis penella* (Heterogynidae). Of these, only *Zygaena trifolii* had been known to possess BCA synthase (Witthohn and Naumann, 1984a). These results suggest that BCA is actually biosynthesized by the insects themselves and is neither accumulated after larval or imaginal ingestion of food plants nor a result of microbial tissue decomposition.

Our data include the first records of cyanogenesis for another 14 lepidopterous families, i.e., the Papilionidae, Pieridae, Lycaenidae, Hesperiidae, Lymantriidae, Arctiidae, Notodontidae, Megalopygidae, Limacodidae, Cymato-

TABLE 1. DISTRIBUTION OF β-CYANO-L-ALANINE (BCA) IN LEPIDOPTERA[a]

Species	Origin	Stage	Preservation	BCA	LIN/ LOT	Larval food plant[b]
Papilionidae						
Trogonoptera brookiana Wall.	12	I	dr	–	–	Aristolochiaceae
Iphiclides podalirius L.	18	I	dr	+	–	Rosaceae
Zerynthia polyxena D. & Sch.	3	I	dr	–	–	Aristolochiaceae
Parnassius apollo L.	18	I	dr	+	–	Crassulaceae
	3	I	dr	–	–	
Pieridae						
Aporia crataegi L.[c]	18	I	dr	+	–	Rosaceae
[c]	7	I	fr	–	–	
Pieris brassicae L.[c]	7	L	fr	–	–	Brassicaceae
(gen.vern.)[c]	7	I	dr	++	–	
(gen.aest.)[c]	7	I	fr	–	–	
Pieris rapae L.	7	L	fr	–	–	Brassicaceae
	7	I	fr	–	–	
Pieris napi L.						
(gen.vern.)[c]	7	I	dr	++	–	Brassicaceae
(gen.aest.)[c]	18	I	dr	++	–	
Anthocharis euphenoides Stgr.	18	I	dr	–	–	Brassicaceae
Colias phicomone Esp.	10	I	dr	+	–	Fabaceae
Colias chrysotheme Esp.	8	I	dr	–	–	Fabaceae
Colias crocea Fourcr.[c]	18	I	dr	+	–	Fabaceae
[c]	7	I	dr	–	–	
Colias australis Vrty.	18	I	dr	+	–	Fabaceae
Gonepteryx rhamni L.	18	I	dr	+	–	Rhamnaceae
Gonepteryx cleopatra L.	18	I	dr	–	–	Rhamnaceae
Leptidea sinapis L.	18	I	dr	+	–	Fabaceae

Nymphalidae						
Apaturinae						
Apatura ilia L.[d]	7	I	dr	–	–	Salicaceae
Apatura ilia f. *clythie* D. & Sch.[d]	7	I	dr	–	–	Salicaceae
Limenitis reducta Stgr.	18	I	dr	+	+	Caprifoliaceae
Limenitis camilla L.	7	I	fr	–	–	Caprifoliaceae
	7	I	dr	–	–	
Nymphalinae						
Nymphalis polychlorus L.[c,d]	7	I	dr	–	–	Salicaceae
Inachis io L.[c]	18	I	dr	–	–	Urticaceae
	10	I	dr	–	–	
[c]	7	I	fr	–		
[c,d]	7	I	dr	–	–	
Vanessa atalanta L.[c,d]	18	I	dr	+	–	Urticaceae,
[c]	10	I	dr	+	–	Asteraceae
[c]	7	I	fr	–	–	
[c]	7	I	dr	–	–	
Cynthia cardui L.[c]	18	I	dr	+	–	Urticaceae,
[c]	7	I	dr	–	–	Asteraceae
Aglais urticae L.[c,d]	7	I	dr	–	–	Urticaceae
Polygonia c-album L.[c]	18	I	dr	+	–	Urticaceae
[d]	7	I	dr	–	–	
Araschnia levana L.[c,d]	7	I	dr	–	–	Urticaceae
Argynnis paphia L.[c]	18	I	dr	+	–	Violaceae
Mesoacidalia aglaia L.[c]	18	I	dr	++	–	Violaceae
Fabriciana adippe D. & Sch.[c]	18	I	dr	++	+	Violaceae
[c]	6	I	dr	+	–	
Fabriciana niobe L.[c]	18	I	dr	++	+	Violaceae
[c]	6	I	dr	+	–	
Issoria lathonia L.[c]	7	I	dr	+++	+	Violaceae, Fabaceae
Brenthis daphne D. & Sch.[c]	18	I	dr	++	+	Violaceae, Rosaceae
Brenthis ino Rott.[c]	18	I	dr	++	+	Rosaceae

TABLE 1. CONTINUED

Species	Origin	Stage	Preservation	BCA	LIN/ LOT	Larval food plant[b]
Clossiana selene D. & Sch.[c]	6	I	dr	+	−	Violaceae
Clossiana euphrosyne L.[c]	7	I	fr	+++	+	Violaceae
[c]	7	I	dr	+++	+	
Clossiana dia L.[c]	18	I	dr	+	+	Violaceae
Mellicta phoebe D. & Sch.[c]	18	I	dr	+	−	Asteraceae, Plantaginaceae
Mellicta dictynna Rott.[c]	18	I	dr	+	−	Valerianaceae, Plantaginaceae
Mellicta parthenoides Kef.[c]	18	I	dr	+	−	Dipsacaceae, Plantaginaceae
Danainae						
Danaus chrysippus L.[d]	17	I	dr	++	−	Asclepidiaceae
Ithomiinae						
Eutresis hypereia D. & Hew.	13	I	dr	−	−	Passifloraceae
Titorea harmonia Cr.	16	I	dr	+	−	Apocynaceae
Mechanitis cf. *polymnia* L.	16	I	dr	++	−	Solanaceae
Godyris zavaleta Hew.	16	I	dr	++	−	Solanaceae
Dircenna dero Hbn.	16	I	dr	++	−	Solanaceae
Dircenna cf. *loreta* Haensch	16	I	dr	+	−	Apocynaceae
Acraeinae						
Acraea horta L.[d]	4	I	dr	+++	+	Passifloraceae, Compositae
Acraea encedon L.	4	I	dr	++	+	Passifloraceae, Compositae
	17	I	dr	++	+	
Acraea igola Trimen	4	I	dr	+	+	Passifloraceae, Compositae
Acraea caldarena Hew.	17	I	dr	+	−	Passifloraceae

Acraea eponina Cr.	17	I	dr	++	–	Passifloraceae
Acraea natalica Bsd.	17	I	dr	++	–	Passifloraceae
Pardopsis punctatissima Bsd.	17	I	dr	+	+	Passifloraceae
Heliconiinae						
Philaetria dido L.	16	I	dr	–	–	Passifloraceae
Dryadula phaetusa L.	13	I	dr	++	+	Passifloraceae
Agraulis vanillae L.[d]	13	I	dr	++	+	Passifloraceae
	16	I	dr	++	+	
Podotriche telesiphe Hew.	16	I	dr	++	–	Passifloraceae
Eueides isabella Cr.	13	I	dr	++	+	Passifloraceae
Heliconius doris L.[d]	20	I	dr	+++	+	Passifloraceae
Heliconius charitonia L.[d]	20	I	dr	+++	+	Passifloraceae
Morphinae						
Morpho caternarius Per.	5	I	fr	+	–	Poaceae
Satyrinae						
Melanargia g. galathea L.[c]	7	I	dr	–	–	Poaceae
Melanargia g. lachesis Hb.[c]	18	I	dr	++	–	Poaceae
Hipparchia alcyone D. & Sch.	18	I	dr	+	–	Poaceae
Hipparchia semele L.	18	I	dr	+	–	Poaceae
	6	I	dr	+	–	
Satyrus ferula F.	10	I	dr	+	–	Poaceae
Brintesia circe F.	18	I	dr	++	–	Poaceae, Rosaceae
Erebia medusa D. & Sch.	7	I	dr	–	–	Poaceae
Erebia meolans Prun.	18	I	dr	++	–	Poaceae
Maniola jurtina L.[c]	18	I	dr	++	–	Poaceae
[c]	6	I	dr	+	–	
Aphantopus hyperantus L.	6	I	dr	+	–	Poaceae
Pyronia bathseba F.	18	I	dr	+	–	Poaceae
Coenonympha pamphilus L.[c]	6	I	dr	–	–	Poaceae
[c]	7	I	dr	–	–	
Coenonympha dorus Esp.	18	I	dr	+	–	Poaceae
Coenonympha arcania L.	18	I	dr	++	–	Poaceae
Lasiommata maera L.	18	I	dr	+	–	Poaceae

TABLE 1. CONTINUED

Species	Origin	Stage	Preservation	BCA	LIN/ LOT	Larval food plant[b]
Lycaenidae						
Nordmannia ilicis Esp.	7	I	dr	–	–	Fagaceae
Nordmannia esculi Hbn.	18	I	dr	+	–	Fagaceae
Lycaena phlaeas L.	7	I	dr	–	–	Polygonaceae
Heodes alciphron Rott.	18	I	dr	–	–	Polygonaceae
Palaeochrysophanus hippothoe L.	18	I	dr	+	–	Polygonaceae
	6	I	dr	+	–	
Cupido minimus Fuessl.	6	I	dr	+	–	Fabaceae
Plebejus argus L.	18	I	dr	+	–	Fabaceae
Aricia artaxerxes F.	6	I	dr	+	–	Geraniaceae, Rosaceae
Agriades pyrenaicus B.	18	I	dr	–	–	Fagaceae
Plebicula escheri Hbn.	18	I	dr	+	–	Fabaceae
Plebicula dorylas D. & Sch.	18	I	dr	–	–	Fabaceae, Lamiaceae
Plebicula amanda Schn.[c]	18	I	dr	–	–	Fabaceae
[c]	6	I	dr	–	–	
Lysandra sp.	18	I	dr	–	–	Fabaccae
Lysandra hispana H.Sch.	18	I	dr	–	–	Fabaceae
Polyommatus icarus Rott.[c]	7	I	dr	–	–	Fabaceae
[c]	6	I	dr	–	–	
Polyommatus eros O.	18	I	dr	+	–	Fabaceae
Hesperiidae						
Pyrgus sp.	18	I	dr	++	–	Rosaceae, Malvaceae

Erynnis tages L.	18	I	dr	++	–	Fabaceae, Apiaceae
Thymelicus actaeon Rott.	18	I	dr	++	–	Poaceae
Thymelicus lineola O.	18	I	dr	++	–	Poaceae
Thymelicus sylvestris P.[c]	18	I	dr	+	–	Poaceae
[c]	6	I	dr	–	–	
[c]	7	I	dr	–	–	
Ochlodes venatus Br. & Gr.	18	I	dr	++	–	Poaceae
Lymantriidae						
Lymantria monacha L.	7	I	fr	++	–	polyphagous
Arctiidae						
Phragmatobia fuliginosa L.[c]	7	I	dr	–	–	polyphagous
Parasemia plantaginis L.	18	I	dr	++	–	polyphagous
Spilosoma lutea Hfn.[c]	18	I	dr	++	–	polyphagous
[c]	7	I	fr	–	–	
Syntomidae						
Syntomis phegea L.	7	I	dr	–	–	polyphagous
Syntomis mogadorensis Blach.	14	I	fr	–	–	polyphagous
Notodontidae						
Stauropus fagi L.	18	I	dr	+	–	Fagaceae
Pheosia tremula Cl.	7	I	dr	–	–	Salicaceae
Pterostoma palpinum L.	18	I	dr	–	–	Salicaceae
Zygaenidae						
Procridinae						
Rhagades pruni D. & Sch.[d]	7	I	fr	+++	+	Ericaceae, Fagaceae
Adscita mannii Led.[d]	7	I	dr	+++	+	Cistaceae
Chalcosiinae						
Campylotes histrionicus Koll.[d]	9	I	dr	+++	+	Symplacoceae, Ericaceae
Agalope bifasciata Moore[d]	15	I	dr	+++	+	Aquifoliaceae, Rosaceae

TABLE 1. CONTINUED

Species	Origin	Stage	Preservation	BCA	LIN/ LOT	Larval food plant[b]
Elcysma westwoodi Voll.	11	L	dr	+++	+	Rosaceae
Aglaope infausta L.[d]	7	I	fr	+++	+	Rosaceae
	7	I	dr	+++	+	
Zygaeninae						
Pryeria sinica Moore[d]	11	I	dr	+++	+	Celastraceae
[d]	11	L	fr	+++	+	
Orna nebulosa Guerin[d]	17	I	dr	+++	+	Celastraceae
[d]	17	L	fr	+++	+	
Neurosymploca caffra L. (*auctorun*)[d]	17	I	dr	+++	+	Celastraceae
Praezygaena agria Dist.	17	I	fr	+++	+	Celastraceae
Reissita simonyi Rbl.[d]	21	I	dr	+++	+	Celastraceae
Epizygaenella cashmirensis Kll.[d]	1	I	dr	+++	+	Celastraceae
Zygaena orana Dup.[d]	2	I	dr	+++	+	Fabaceae
Z. carniolica Scop.[d]	7	I	dr	+++	+	Fabaceae
[d]	7	L	fr	+++	+	
Z. fausta L.[d]	7	I	dr	+++	+	Fabaceae
Z. viciae D. & Sch.[d]	7	I	dr	+++	+	Fabaceae
Z. dorycnii O.[d]	19	I	fr	+++	+	Fabaceae
Z. hippocrepidis Hb.[d]	18	I	dr	+++	+	Fabaceae
Z. transalpina Esp.[d]	7	I	dr	+++	+	Fabaceae
[d]	7	L	fr	+++	+	
Z. trifolii Esp.[d]	7	I	dr	+++	+	Fabaceae
[d]	7	L	fr	+++	+	
Z. lonicerae Sch.[d]	7	I	fr	+++	+	Fabaceae
Z. corsica Bsd.[d]	10	I	dr	+++	+	Asteraceae
Z. punctum O.[d]	10	I	dr	+++	+	Apiaceae

Anomoeotinae						
Anomoeotis levis Fld. & Fld.	17	I	dr	+++	+	Caesalpiniaceae
Heterogynidae						
Heterogynis penella Hbn.[d]	10	I	fr	+++	+	Fabaceae
	10	I	dr	+++	+	
Megalopygidae						
Trosia dimas	13	I	dr	++	+	polyphagous
Norape ovina Sepp	13	I	dr	++	+	polyphagous
Psycharium pellucens H.S.	17	L	dr	+++	+	Pinaceae
Limacodidae						
Tortricidia testacea Pack.	20	I	dr	++	–	–
Sphingidae						
Mimas tiliae L.	7	I	dr	–	–	Tiliaceae
Laothoe populi L.	7	I	dr	–	–	Salicaceae
Acherontia atropos L.	18	I	fr	–	–	Solanaceae
Cymatophoridae						
Thyatira batis L.	18	I	dr	+	–	Rosaceae
Palimpsestris ocularis L.	18	I	dr	+	–	Salicaceae
Drepanidae						
Drepana lacertinaria L.	7	I	dr	–	–	Betulaceae
Lasiocampidae						
Malacosoma neustria L.	18	I	dr	+	–	polyphagous, Rosaceae
Lasiocampa quercus L.	7	I	fr	–	–	polyphagous
Sesiidae						
Sesia apiformis Cl.	7	I	dr	–	–	Salicaceae
Noctuidae						
Scotia exclamationis L.	18	I	dr	–	–	polyphagous
Ochropleura plecta L.	18	I	dr	+	–	polyphagous
Noctua pronuba L.[c]	18	I	dr	++	–	polyphagous
[c]	7	I	dr	–	–	
Polia hepatica Cl.	18	I	dr	–	–	polyphagous
Polia nebulosa Hufn.	18	I	dr	+	–	polyphagous

TABLE 1. CONTINUED

Species	Origin	Stage	Preservation	BCA	LIN/ LOT	Larval food plant[b]
Phlogophora meticulosa L.	7	I	dr	–	–	polyphagous
Cosmia diffinis L.	18	I	dr	+	–	Ulmaceae
Apamaea monoglypha Hufn.	18	I	dr	+	–	Poaceae
Apamaea remissa Hbn.	7	I	dr	–	–	polyphagous
Oligia strigilis L.	7	I	dr	–	–	Poaceae
Meristis trigrammica Hufn.	7	I	fr	–	–	polyphagous
Autographa gamma L.	18	I	dr	–	–	polyphagous
Ectypa glyphica L.	18	I	dr	–	–	Fabacae
Hypena proboscidalis L.	7	I	dr	–	–	polyphagous
Geometridae						
Geometra papilionaria L.	7	I	dr	–	–	Betulaceae
Euphyia bilineata L.	18	I	dr	+	–	polyphagous
Eupithecia sp.	7	I	dr	–	–	–
Campaea margaritata L.	7	I	dr	–	–	Fagaceae
Bapta temerata D. & Sch.	18	I	dr	+++	–	Rosaceae, Fagaceae
Apeira syringaria L.	7	I	dr	–	–	polyphagous
Opistograptis luteolata L.	18	I	dr	–	–	polyphagous
Pseudopanthera macularia L.	18	I	dr	–	–	polyphagous
Macaria alternaria Hbn.	18	I	dr	–	–	polyphagous
Chiasmia clathrata L.	18	I	dr	+	–	Fabaceae
	7	I	dr	–	–	
Biston betularia L.	7	I	dr	–	–	polyphagous
Peribatodes rhomboidaria D. & Sch.	18	I	dr	+	–	polyphagous
Alcis repandata L.	18	I	dr	+	–	polyphagous
Ascotis selenaria D. & Sch.	18	I	dr	–	–	polyphagous

Boarmia sp.	18	I	dr	+	–	Ericaceae
Ematurga atomaria L.	18	I	dr	+	–	polyphagous
Pyralidae						
Pyrausta cingulata L.	7	I	dr	–	–	Poaceae
Crambus sp.	7	I	dr	–	–	Poaceae
Tortricidae						
Tortrix sp.	7	I	fr	–	–	Fabaceae
Yponomeutidae						
Yponomeuta padellus L.	7	I	dr	–	–	Salicaceae
Gymnogramma rufiventris Wlk.[e]	17	I	fr	+++	+	Anacardiaceae
	17	L	fr	+++	+	
Adelidae						
Nemophora degeerella L.	7	I	dr	–	–	–

[a]Origin: 1 Afghanistan, 2 Algeria, 3 Austria, 4 Botswana, 5 Brasil, 6 Denmark, 7 Germany, 8 Hungary, 9 India, 10 Italy, 11 Japan, 12 Malaysia, 13 Mexico, 14 Morocco, 15 Nepal, 16 Peru, 17 South Africa, 18 Spain, 19 Turkey, 20 USA, 21 Yemen. dr = air-dried specimens; fr = fresh specimens, usually bred. BCA concentration: – not detected; +: $c < 2$ μg/insect, ++: $2 < c < 20$ μg/insect, +++: $c > 20$ μg (175 mmol)/insect. LIN/LOT (linamarin/lotaustralin) test: – not detected, + present.

[b]Cyanogenesis has been recorded for the following plant families: Apiaceae (1 sp.), Asclepidiaceae (2 sp.), Asteraceae, Caprifoliaceae, Crassulaceae, Brassicaceae, Ericaceae, Fabaceae, Poaceae, Passifloraceae, Rosaceae, Tiliaceae, Ulmaceae (Hegnauer, 1962–1973; Conn, 1981a,b).

[c]3–6 specimens examined for each population.

[d]Previously recorded in Witthohn and Naumann (1984b).

[e]Probably to be transferred to Zygaenoidea (Kyrki, personal communication).

phoridae, Lasiocampidae, Noctuidae, Geometridae, and Yponomeutidae. Hitherto, cyanogenesis had only been known to occur in three families, viz., the Nymphalidae, Zygaenidae, and Heterogynidae (Witthohn and Naumann, 1984b, and references therein). The specimens used in the Heliconiinae and Acraeinae (Nymphalidae) had obviously lost some of their original amounts of BCA, as could be seen from the generally poor representation of amino acids in TLC. We expect very strong reactions (+++) for all species when fresh specimens are available. This will presumably apply also to those species of the Heliconiinae and Acraeinae which were negative for BCA.

The cyanogenic species *Gymnogramma rufiventris* will be excluded from the Yponomeutidae in the near future. It might be placed in the cyanogenic Zygaenoidea, but not in the Zygaenidae proper (Kyrki, 1984, personal communication). Larvae of this highly cyanogenic species possess lateral abdominal glands which can be extruded and are probably used to store or compartmentalize toxic—possibly cyanogenic—compounds (Naumann, unpublished). A similar, but not homologous, larval defensive system has been found in the Zygaeninae (Povolny and Weyda, 1981; Franzl and Naumann, 1984, 1985).

The number of species actually detoxifying cyanide via BCA synthase might still be greater than our results show: firstly, because the greatest need to detoxify cyanide might occur in the larval instars, which might lead to a reduction of the amount of stored BCA in the imagines, and secondly because an insect might detoxify HCN via BCA synthase but might not store the product of the reaction.

Based on these results, cyanogenesis can no longer be considered a phenomenon restricted to some aposematic species, such as *Zygaena*, *Acraea*, *Heliconius*, and their close relatives. However, there is still a general trend for some groups to show stronger reactions than others. A rather singular case is found in the geometrid moth *Bapta temerata*, a cryptic and mainly night-active species. Since the larval food-plant spectrum includes some cyanogenic Rosaceae, there may exist a connection between cyanogenesis of the larval food plant and that of the moth itself.

The degree of cyanogenesis as evident from the amount of stored BCA varies in a number of respects:

Variation within Lepidoptera. While certain taxa such as the Zygaenidae, Acraeinae, and Heliconiinae must be considered as highly cyanogenic at the family or subfamily level, other taxa such as the Nymphalinae and most other butterfly families show considerable variation which seems to be only partially connected with cyanogenesis of larval food plants. Thus, in the Nymphalidae, the subfamily Satyrinae are confined to the Poaceae, which are known to be cyanogenic. The imagines of this subfamily are cyanogenic at a rather low level. In the Nymphalinae, those species which live on the Violaceae, a plant family not known to be cyanogenic, show comparatively high concentrations of BCA,

as judged from the strength of the ninhydrin reaction. *Clossiana euphrosyne*, a species where BCA synthase was proved in a separate experiment, belongs to these species which are bound to the noncyanogenic Violaceae. Species of the same subfamily living on the acyanogenic Urticaceae or Asteraceae only occasionally contain BCA. It cannot be excluded, of course, that the genera *Vanessa*, *Cynthia*, and *Polygonia* do in fact accumulate cyanogenics from their larval food plants, but at very low concentrations, so that the amount of BCA cannot be detected by the usual analytical technique.

Geographic Variation. Geographic variation in the amount of allelochemicals is certainly not new and was demonstrated for the pyrrolizidine alkoloids in the monarch butterfly, *Danaus plexippus* as early as 1972 (Brower et al., 1972). In the present study an additional geographic pattern is apparent in *Parnassius apollo*, *Aporia crataegi*, *Colias crocea*, *Vanessa atalanta*, *Cynthia cardui*, *Polygonia c-album*, *Melanargia galathea*, *Thymelicus sylvestris*, *Spilosoma lutea*, and *Noctua pronuba*: central European specimens of all these species do not contain BCA, while Mediterranean ones do. In most cases we have examined several specimens of each species. An explanation for such a general pattern is still lacking.

Seasonal Variation. In *Pieris brassicae* our data suggest seasonal variation: members of the spring generation contain BCA, which is absent in the summer generation.

These three different types of variation, which were just discernible in the present study, will necessitate further research on an individual, population, seasonal, and geographic basis, preferably with respect to the larval and imaginal food plants of the various individuals tested.

As discussed above the presence of the cyanoglucosides linamarin/lotaustralin could only be demonstrated in a limited number of cases, because the TLC anisaldehyde reaction is much less sensitive than the TLC ninhydrin reaction used as a test for BCA. Nevertheless, we believe it to be most likely that these cyanoglucosides are the source of cyanogenesis, at least in all cases of the Rhopalocera and Zygaenoidea tested so far. This is indicated by the data on Zygaenidae, Acraeinae, and Heliconiinae supplied by Davis and Nahrstedt (1984), and by the fact that in the present paper the cyanoglucosides have been detected in some cases of related but cryptic species also storing BCA. One might even suppose that in the Lepidoptera the two mentioned cyanoglucosides are usually detoxified as in plants, i.e., via BCA synthase.

At present we have no evidence of an insect that would make use of both detoxification pathways, rhodanese *and* BCA-synthase, to some biologically significant extent. On the contrary, the Zygaenidae seem to use the BCA pathway exclusively, since Beesley et al. (1985) found practically no rhodanese in this group. The same seems to apply to *Erynnis tages* (Hesperiidae). Conversely, rhodanese has been demonstrated in sufficient amounts in the blue but-

terfly *Polyommatus icarus* (Parsons and Rothschild, 1964; Beesley et al., 1985), while BCA is obviously not stored.

Jones (1979) has argued that there might be a general need for phytophagous insects to detoxify HCN, because small amounts of cyanoglucosides seem to be present in nearly all angiosperms. Larvae of the southern army worm, *Spodoptera eridiana*, even use hydrocyanic acid as a feeding stimulant (Brattsten et al., 1983). It has been shown that rhodanese occurs in a considerable range of insects, but often in very small amounts only (Beesley et al., 1985). We suggested that BCA might be used as a stored product for the biosynthesis of asparagine and other amino acids (Witthohn and Naumann, 1984a). Similarly Volini and Alexander (1981) have stressed the possible importance of rhodanese for the biosynthesis of sulfur-containing amino acids. If these assumptions prove correct, then the products will be reintroduced into the primary metabolic process in both reactions.

With regard to the role of the cyanoglucosides linamarin and lotaustralin in the ecology of *Zygaena*, it is important to note that the parasitoid braconid wasp *Apanteles zygaenarum*, which lives almost exclusively in *Zygaena* species, does not use BCA synthase to detoxify linamarin, which is obviously acquired from the host's tissues (Davis and Nahrstedt, 1984). The same applies to the parasitic fly *Zenillia*, which also lives in *Zygaena* species and uses the rhodanese pathway (Jones et al., 1962).

Recently Davis and Nahrstedt (1984) have stressed the importance of delimiting the distribution of the cyanoglucosides in the Nymphalidae and suggested that cyanogenesis might be a taxonomically important character for the Zygaenidae. The same was proposed by Naumann (1985). It is now clear that cyanogenesis is not restricted to the Nymphalidae but occurs in all families of butterflies, where it is always based on the two cyanoglucosides linamarin and lotaustralin. It will now be of great importance to delimit the amount of cyanogenics acquired or synthesized by butterflies with respect to their geographic origin, seasonality, and the cyanoglucoside content of their food plants. In the Zygaenoidea strong cyanogenesis extends far beyond the family level and has now been shown to occur in the Limacodidae and Megalopygidae which, at present, are believed to belong to the Cossoidea, but which have also been associated with the Zygaenoidea in the past (Brock, 1971). As the Cossidae themselves are good candidates for cyanogenesis (because they are cyanide-resistant as well), the use of this character for taxonomic and phylogenetic purposes diminishes. On the other hand, the larval storage system for cyanogenic secretion might provide a useful character to delimit at least one major monophyletic entity within the Zygaenidae (Naumann et al., in preparation).

Thus we conclude that cyanogenesis is a widespread phenomenon in the Lepidoptera. It seems to be based mainly or possibly exclusively on the cyanoglucosides linamarin and lotaustralin, and the main detoxification pathway of

the Lepidoptera seems to be BCA synthase. We suggest cyanogenesis to be a basic characteristic of the Lepidoptera, but at present it is impossible to say whether this could, at the same time, be a synapomorphy of the taxon. The use of BCA synthase to detoxify HCN originating from mandelonitrile in the Chrysomelidae (Coleoptera) has been proposed by Blum et al. (1981). It can thus be assumed that a wide distribution of BCA could be found in other phytophagous insect orders as well. The presence of BCA and BCA synthase has recently been demonstrated in the chrysomelid beetle *Paropsis atomaria*, which uses mandelonitrile and prunasin as sources for hydrocyanic acid (Nahrstedt and Davis, 1986).

Rothschild (1985) has recently listed allelochemicals that have been recorded from various British Macrolepidoptera. When compared with the list presented above, it becomes obvious how little is known at present about the cooccurrence of several allelochemicals in nonglandular tissues and their possible ecological interaction. Rothschild rightly points out that one of the major deficiencies of this branch of insect chemical ecology is the present concentration of research on one or two major compounds, mainly cardenolides and pyrrolizidine alkoloids (Boppré, 1986), while very little is known about the ecological importance of other compounds, which sometimes cooccur. Thus, Muhtasib and Evans (1987) have demonstrated synergism in the defensive properties of linamarin and histamine. As for the cyanoglucosides, we shall report elsewhere on the reaction of various predators to the cyanogenic compounds linamarin and β-cyanoalanine.

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NOVEL PYRAZINES FROM THE HEAD OF AUSTRALIAN PONERINE ANT *Rhytidoponera metallica*

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Abstract—The novel pyrazines, (*E*)- and (*Z*)-5-methyl-3-(2-methylbutyl)-2-(3-methylbut-1-enyl)pyrazine, (*E*)- and (*Z*)-5-methyl-3-isopentyl-2-(3-methylbut-1-enyl)pyrazine, (*E*)- and (*Z*)-5-methyl-3-(2-methylbutyl)-2-(3-methylpent-1-enyl)pyrazine, (*E*)- and (*Z*)-5-methyl-3-isopentyl-2-(3-methylpent-1-enyl)pyrazine, together with the known pyrazines, 2,5-dimethyl-3-(2-methylbutyl)pyrazine and 2,5-dimethyl-3-isopentylpyrazine, have been identified from the head of the Australian ponerine ant *Rhytidoponera metallica*. Alkanes and alkenes, in small amounts, were also detected.

Key Words—Ant, *Rhytidoponera metallica*, pyrazine, GC-MS, Hymenoptera, Formicidae, ponerine.

INTRODUCTION

Chemical investigations on ants of the subfamily Ponerinae (Formicidae) have been notable for the fact that pyrazines have been detected in the heads of the majority of species from various genera to date examined, e.g., *Anochetus*, *Odontomachus*, *Ponera*, *Hypoponera*, *Rhytidoponera*, *Brachyponera*, *Mesoponera*, and *Dinoponera* (Attygale and Morgan, 1984; Fales et al., 1984; Herman et al., 1984). In most of the species that did not contain pyrazines, sulfides have been detected (Casnati et al., 1967; Crew and Fletcher, 1974; Longhurst et al., 1979, 1980). Although pyrazines have been detected in other subfamilies of the Formicidae (Dolichoderinae, Myrmicinae, Myrmeciinae, and Formi-

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cinae) (Attygale and Morgan, 1984; Brophy and Nelson, 1985), their occurrence is less common than in the Ponerinae.

Where activity tests have been carried out on the cephalic secretions (not only on the Ponerinae), alarm responses have been noted (Wheeler and Blum, 1973; Longhurst et al., 1978; Duffield et al., 1976; Howard et al., 1982; Brown and Moore, 1979). It is also of interest to note that an alarm pheromone is produced in the heads of doryline ants of the genera *Eciton*, *Nomamyrmex*, and *Labidus* and that in the case of *Eciton* and *Nomamyrmex*, the crushed worker heads emit a "meaty odor" assumed to be associated with the alarm substance (Brown, 1960). This observation is suggestive of the presence of pyrazines.

Ponerine ants of the genus *Rhytidoponera* have, to date, received relatively little attention. Two Australian species, *R. chalybaea* (at the time identified as *R. metallica*) (Brophy et al., 1981, 1984) and *R. aciculata* (Brophy et al., 1983), have been examined and both yielded 2,5-dimethyl-3-isopentylpyrazine (2) (the pyrazine most commonly detected in insects) and *R. chalybaea* also yielded 2,5-dimethyl-3-citronellylpyrazine. In each case the pyrazine(s) were isolated from the heads of the ants.

Study of *R. metallica* has been limited to an examination of the contents of its pygidial gland, which was shown to contain isogeraniol, *m*-hydroxybenzaldehyde, heptadecene, and heptadecadiene (Meinwald and Wiemer, 1983). We report here results of our investigations on secretions obtained from the heads of *R. metallica*.

METHODS AND MATERIALS

Identification of Ants. The ants used in this investigation were identified as *Rhytidoponera metallica* by Dr. R.F. Crozier, School of Zoology, University of New South Wales. Samples of the ants have been lodged with the Australian National Insect Collection, Canberra.

Extraction of R. metallica. The ants, which are approx. 8 mm in length and weighed approx. 5 mg, were collected in Centennial Park, Randwick, New South Wales. The heads of approx. 700 ants were removed and, after grinding with sodium sulfate, were extracted with dichloromethane (150 ml) in a Soxhlet apparatus for 10 hr. The extract was washed with sodium bicarbonate solution (2×20 ml) and water (2×20 ml) and dried over magnesium sulfate. Removal of the solvent yielded a pale yellow oil (51 mg) which was examined by combined gas chromatography–mass spectrometry (GC-MS).

Hydrogenation of Extract of R. metallica. A portion of the above extract of (20 mg) in ethanol (5 ml) was hydrogenated over Adams catalyst at room temperature and atmospheric pressure for 2 hr. Filtration through magnesium sulfate and removal of the ethanol gave a light yellow oil which was examined as above.

General Method for Synthesizing Alkenyl Pyrazines. The method generally follows that outlined by Wheeler et al. (1981). 2,5-Dimethyl-3-isopentylpyrazine and 2,5-dimethyl-3-(2-methylbutyl)pyrazine were prepared as previously described (Klein and Spoerri, 1951) in 19% and 10% yields, respectively by treatment of 2,5-dimethylpyrazine (Aldrich) with the appropriate alkyl lithium in petroleum ether (bp 40–60°C). The dimethylalkyl pyrazines (0.5 g, 0.03 mmol) so obtained were subsequently acylated with the desired ethyl ester at −78°C using lithium diisopropylamide as base, in 40–50% yields. Reduction of the pyrazyl ketones (0.1 g, 0.004 mmol) so formed with sodium borohydride in methanol at 40–50°C yielded the corresponding alcohols in 80–90% yields. The alcohols (0.08 g, 0.003 mmol) were treated with *p*-toluenesulfonyl chloride in the conventional manner to yield the tosyl esters which, without isolation, were refluxed in pyridine (15 hr) to yield the alkenyl pyrazines (70–80%) as pale yellow oils. In each case GC-MS examination of the synthetic pyrazines indicated that the ratio of *E* isomer to *Z* isomer was approx. 9 : 1.

E-5-Methyl-3-(2-methylbutyl)-2-(3-methylbut-1-enyl)pyrazine (5, Scheme 1): found 233.2031 (MH^+); calc. for $C_{15}H_{25}N_2$, 233.2067. [^{1}H]NMR (60 MHz) δ 0.78(d, J = 6 Hz, 3H); 1.02(d, J = 6 Hz, 6H); 1.10(t, J = 7 Hz, 3H); 1.2–

1 2

3 4

5 6

7 8

9 10

SCHEME 1.

1.8(m, 3H); 2.2(m, 1H); 2.41(s, 3H); 2.62(d, J = 8 Hz, 2H); 6.49(d, J = 15 Hz, 1H); 6.86(m, 1H); 8.18(s, 1H).

E-5-Methyl-3-isopentyl-2-(3-methylbut-1-enyl)pyrazine (6): found 233.2031 (MH^+); calc. for $C_{15}H_{25}N_2$, 233.2067. [^{1}H]NMR (60 MHz) δ 0.90(d, J = 6 Hz, 6H); 1.05(d, J = 7 Hz, 6H); 1.30–1.80(m, 3H); 2.35(m, 1H); 2.45(s, 3H); 2.78(m, 2H); 6.46(d, J = 16 Hz, 1H); 6.86(m, 1H); 8.16(s, 1H).

E-5-Methyl-3-(2-methylbutyl)-2-(3-methylpent-1-enyl)pyrazine (9): found 247.2179 (MH^+); calc. for $C_{16}H_{27}N_2$, 247.2174. [^{1}H]NMR (300 MHz) δ 0.86(d, J = 6 Hz, 3H); 0.91 (t, J = 7 Hz, 6H); 1.11 (d, J = 7 Hz, 3H); 1.19–1.48(m, 5H); 2.17–2.35(m, 1H); 2.49(s, 3H); 2.56–2.65(m, 1H); 2.82–2.90(m, 1H); 6.57(d, J = 16 Hz, 1H); 6.74(dd, J = 16, 8 Hz, 1H); 8.20(S, 1H).

E-5-Methyl-3-isopentyl-2-(3-methylpent-1-enyl)pyrazine (10): found 247.2155 (MH^+); calc. for $C_{16}H_{27}N_2$, 247.2174. [^{1}H]NMR (300 MHz) δ 0.91(t, J = 7 Hz, 3H); 0.96(d, J = 6 Hz, 6H); 1.11(d, J = 6 Hz, 3H); 1.40–1.59 (m, 4H); 1.62–1.70(m, 1H); 2.16–2.33(m, 1H); 2.48(s, 3H); 2.73–2.84(m, 2H); 6.54(d, J = 15 Hz, 1H); 6.73(dd, J = 15, 8 Hz, 1H); 8.19(s, 1H).

Hydrogenation of the synthetic pyrazines was carried out in the same manner as for the ant extract.

Interconversion of the synthetic *E* and *Z* isomers was achieved by irradiation of a deoxygenated dilute solution of the *E* pyrazine in pentane for 5 min at 300 nm.

Spectroscopy and Gas Chromatography. Mass spectra were obtained on an AEI MS12 mass spectrometer coupled to a Shimadzu GC6 AMP gas chromatograph. Separations were effected on SCOT columns, either (a) OV-1 (30 m × 0.5 mm) programmed from 90°C to 250°C at 5°C/min or (b) FFAP (85 m × 0.5 mm) programmed from 80°C to 225°C at 5°C/min, with helium as carrier gas. The mass spectrometer was operated at 8000 V and 70 eV ionizing voltage. Spectra were acquired and processed by a VG Digispec Display data system. Accurate mass measurements were obtained on an AEI MS902 mass spectrometer under chemical ionization conditions (isobutane reagent gas) using a chart timing method (Brophy et al., 1979). [^{1}H]NMR spectra were recorded in $CDCl_3$ solution on either a Varian EM 360 (60 MHz) or a Bruker CXP 300 (300 MHz) spectrometer.

RESULTS AND DISCUSSION

A GLC trace of the total extract of the heads of *R. metallica* is shown in Figure 1 and the assignments are given in Table 1. A chemical ionization mass spectrum of this crude extract was run using isobutane as reagent gas under conditions where all hydrocarbons fail to be protonated. This spectrum demonstrated the presence of components with molecular weights of 178, 232, 246, 264, and 278 and subsequent high-resolution mass spectrometry indicated that

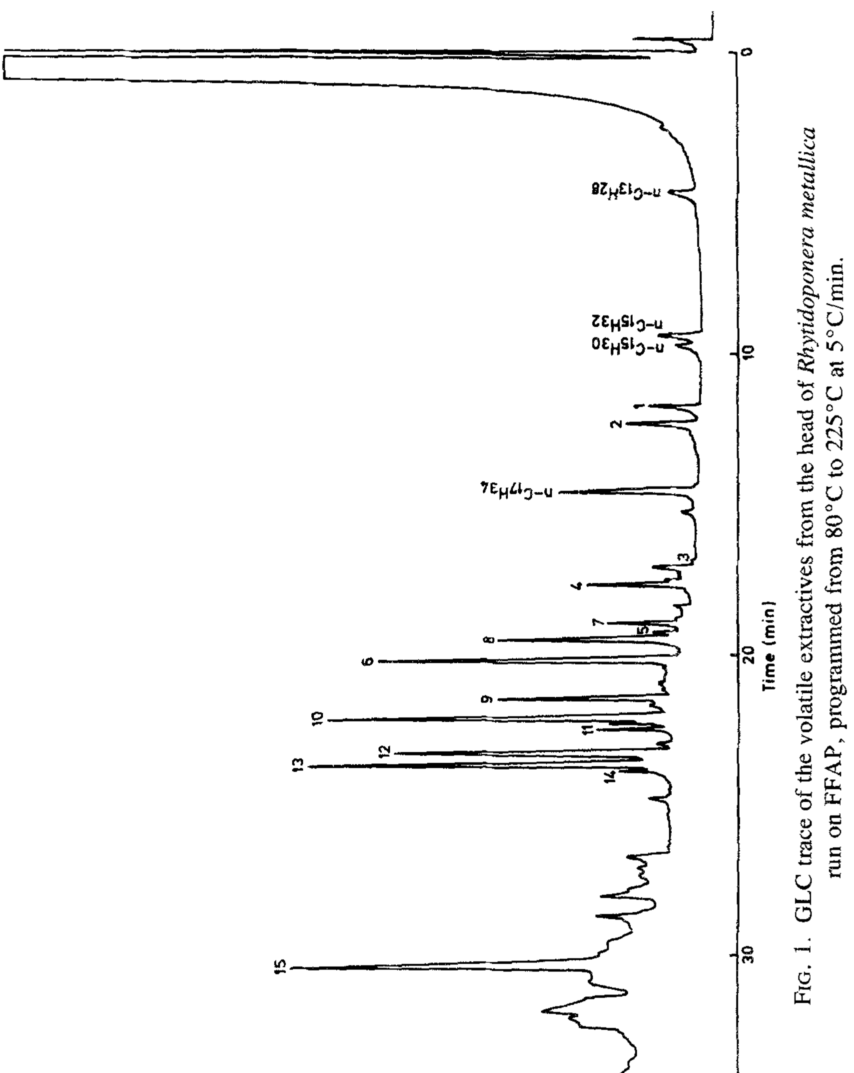

FIG. 1. GLC trace of the volatile extractives from the head of *Rhytidoponera metallica* run on FFAP, programmed from 80°C to 225°C at 5°C/min.

TABLE 1. COMPOUNDS IDENTIFIED IN HEAD OF *Rhytidoponera metallica*

Number	Compound
1	2,5-Dimethyl-3-(2-methylbutyl)pyrazine
2	2,5-Dimethyl-3-isopentylpyrazine
3	Z-5-Methyl-3-(2-methylbutyl)-2-(3-methylbut-1-enyl)pyrazine
4	Z-5-Methyl-3-isopentyl-2-(3-methylbut-1-enyl)pyrazine
5	E-5-Methyl-3-(2-methylbutyl)-2-(3-methylbut-1-enyl)pyrazine
6	E-5-Methyl-3-isopentyl-2-(3-methylbut-1-enyl)pyrazine
7	Z-5-Methyl-3-(2-methylbutyl)-2-(3-methylpent-1-enyl)pyrazine
8	Z-5-Methyl-3-isopentyl-2-(3-methylpent-1-enyl)pyrazine
9	E-5-Methyl-3-(2-methylbutyl)-2-(3-methylpent-1-enyl)pyrazine
10	E-5-Methyl-3-isopentyl-2-(3-methylpent-1-enyl)pyrazine
11	Unknown, suspected mol wt 264
12	$C_{16}H_{28}N_2O$
13	$C_{16}H_{28}N_2O$
14	Unknown, suspected mol wt 264
15	Diisobutyl phthalate
16	Dibutyl phthalate

the formulas for these ions were *m/z* 178, $C_{11}H_{18}N_2$; *m/z* 232, $C_{15}H_{24}N_2$; *m/z* 246, $C_{16}H_{26}N_2$; *m/z* 264, $C_{16}H_{28}N_2O$; and *m/z* 278, $C_{16}H_{22}O_4$, respectively. Subsequent GC-MS analysis of the extract under electron inpact (EI) conditions indicated that there were two compounds of mol wt 178, four compounds of mol wt 232, and four compounds of mol wt 246. In addition, at least two compounds which did not show molecular ions but whose molecular weight was assumed to be 264 were present, as well as two phthalate esters which were assumed to be butyl phthalates.

Hydrogenation of the crude extract led to a more simple GLC pattern (Figure 2, Table 2). Whereas the compounds of mol wt 178 remained unaltered, two peaks whose mass spectra showed mol wt 234 appeared at the expense of the four isomers of mol wt 232. Similarily, two new peaks whose mass spectra showed mol wt 248 appeared at the expense of the four of mol wt 246. The peaks suspected to be of mol wt 264 remained unchanged, as did those for the phthalate esters.

The mass spectra of peaks (a) and (b) of mol wt 234 in the hydrogenation mixture (see Table 3) indicated that the compounds were probably trisubstituted pyrazines with side chains of one, five, and five carbon atoms, while the peaks (c) and (d) of mol wt 248 appeared to be trisubstituted pyrazines with side chains of one, five, and six carbon atoms. The orientation of these groups in the two sets of hydrogenated pyrazines was not known.

The two pyrazines (1) and (2) of mol wt 178 were identified as 2,5-di-

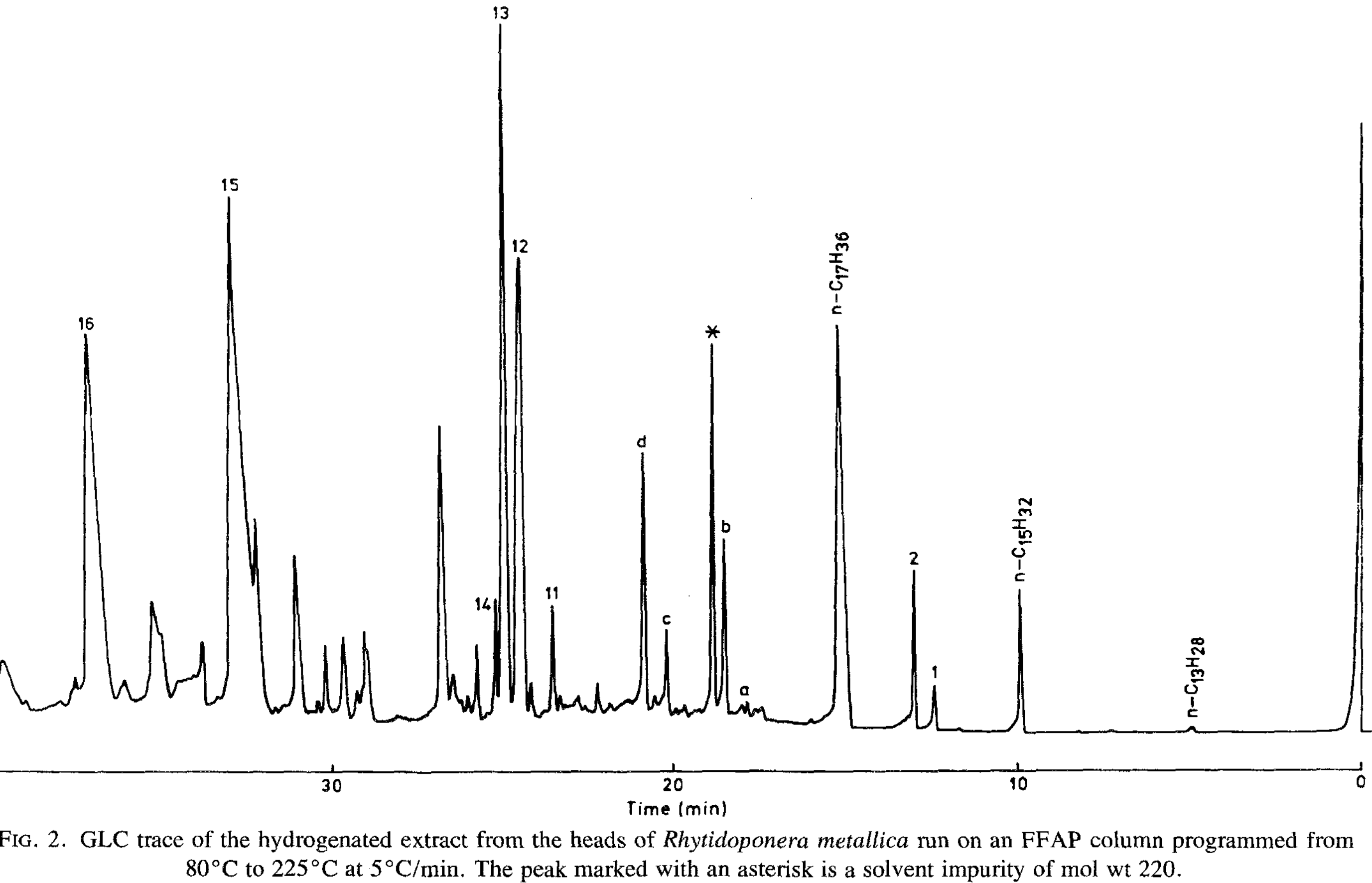

FIG. 2. GLC trace of the hydrogenated extract from the heads of *Rhytidoponera metallica* run on an FFAP column programmed from 80°C to 225°C at 5°C/min. The peak marked with an asterisk is a solvent impurity of mol wt 220.

TABLE 2. COMPOUNDS IDENTIFIED IN HYDROGENATED EXTRACT FROM HEAD OF *Rhytidoponera metallica*

Number	Compound
1	2,5-Dimethyl-3-(2-methylbutyl)pyrazine
2	2,5-Dimethyl-3-isopentylpyrazine
a	5-Methyl-2-isobutyl-3-(2-methylbutyl)pyrazine
b	5-Methyl-2,3-diisobutylpyrazine
c	5-Methyl-2-(3-methylpentyl)-3-(2-methylbutyl)pyrazine
d	5-Methyl-2-(3-methylpentyl)-3-isopentylpyrazine
11	Unknown, suspected mol wt 264
12	$C_{16}H_{28}N_2O$
13	$C_{16}H_{28}N_2O$
14	Unknown, suspected mol wt 264
15	Diisobutylphthalate
16	Dibutylphthalate

methyl-3-(2-methylbutyl)pyrazine and 2,5-dimethyl-3-isopentylpyrazine, respectively, on the basis of their mass spectra. This was confirmed by coinjection with authentic compounds on a FFAP column, known to be able to separate the 2,3,5 isomer from the slower eluting 2,3,6-trisubstituted pyrazines (Wheeler and Blum, 1973).

Since the two pyrazines of mol wt 178 had the 2,3,5 substitution, it was thought likely that the four suspected pyrazines of mol wt 232 and the four of mol wt 246 also had the same substitution pattern and also that one of the substituents may have been either the 2-methylbutyl or isopentyl group, i.e., that these eight unknown pyrazines involved further elaboration on the already identified pyrazines of mol wt 178. Moreover, the results from the hydrogenation experiment suggested these compounds to be dialkyl alkenyl pyrazines.

As noted above, hydrogenation of each of the pyrazines of mol wt 232 and 246 cause the uptake of one molar equivalent of hydrogen, indicating one of the side chains contained an olefinic bond. That this double bond was in a five-carbon side chain in compounds (3)–(6) and in the six-carbon side chain in compounds (7)–(10) was also deduced from their mass spectra (Table 3). Thus ions at m/z 176 and 190 for compounds (3)–(6) and (7)–(10), respectively, arose from loss of C_4H_8 by McLafferty-type rearrangement of a C-5 saturated side chain in each case. There was no significant peak corresponding to the loss of C_5H_{10} in compounds (7)–(10). Such a peak would be expected to occur if the olefinic bond were in the C-5 chain in compounds (7)–(10).

The relative placement of the two large side chains in (3)–(10) could be determined by consideration of the results obtained in the structural elucidation of 5-methyl-3-*n*-propyl-2-(1-butenyl)pyrazine from the ant *Aphaenogaster rudis*

TABLE 3. MASS SPECTRA OF PYRAZINES

Compound	Mass spectrum
1	(M^+)178(1%), 177(1), 163(8), 149(4), 135(0.5), 122(100)
2	(M^+)178(1%), 177(1), 163(8), 149(0.5), 135(10), 122(100)
3	(M^+)232(55%), 217(23), 203(13), 189(9), 177(20), 176(45), 161(100), 149(53), 147(30), 133(32), 122(8)
4	(M^+)232(53%), 217(19), 203(0.5), 189(33), 177(15), 176(58), 161(100), 147(28), 133(58), 122(3)
5	(M^+)232(15%), 217(9), 203(9), 189(10), 176(20), 161(23), 147(13), 133(100), 122(5)
6	(M^+)232(3%), 217(8), 189(11), 176(34), 161(30), 147(8), 133(100), 122(1)
7	(M^+)246(100%), 231(50), 217(50), 203(1), 190(25), 176(40), 175(57), 161(95), 147(40), 135(40), 133(35), 122(20)
8	(M^+)246(75%), 231(45), 217(20), 203(33), 190(45), 176(25), 175(80), 161(100), 147(27), 133(90), 122(25)
9	(M^+)246(7%), 231(5), 217(10), 190(24), 176(5), 175(8), 161(18), 147(23), 133(100), 122(3)
10	(M^+)246(5%), 231(10), 217(8), 203(7), 190(35), 176(3), 175(17), 161(23), 147(10), 133(100), 122(7)
11	231(3%), 208(19), 207(36), 190(15), 178(22), 163(17), 161(12), 149(100), 136(25), 135(20), 133(20), 122(41)
12	(M^+)264(0.5%), 249(1), 231(1), 208(13), 207(38), 190(7), 178(22), 177(6), 163(7), 161(7), 151(10), 149(16), 135(100), 122(23)
13	(M^+)264(0.2), 249(1), 235(0.5), 231(1), 208(13), 207(53), 190(5), 178(16), 163(6), 161(7), 151(8), 147(7), 135(100), 122(25)
14	208(8%), 207(38), 190(15), 163(5), 161(8), 151(11), 135(100), 122(27)
15	223(18%), 205(6), 189(1), 167(10), 150(20), 149(100), 104(12), 57(80)
16	239(1%), 238(1), 223(6), 205(6), 150(10), 149(100), 104(6), 57(25)
a	(M^+)234(2%), 219(7), 205(5), 191(15), 178(100), 163(45), 149(11), 135(15), 122(87)
b	(M^+)234(1%), 219(7), 191(12), 178(72), 163(40), 149(5), 135(25), 122(100)
c	(M^+)248(5%), 233(8), 219(13), 192(65), 191(28), 178(100), 177(30), 163(75), 149(17), 135(22), 122(93)
d	(M^+)248(3%), 233(6), 219(7), 205(9), 192(50), 191(17), 178(60), 177(20), 163(40), 149(10), 135(25), 122(100)

(Wheeler et al., 1982). It was observed that the occurrence of an alkenyl group adjacent to a large alkyl group (in this case *n*-propyl) diminished the intensity of the peak due to the McLafferty rearrangement. When the alkenyl group was adjacent to a methyl group or hydrogen, this rearrangement led to the base peak of the mass spectrum. It was also found that no matter what the substitution, the mass spectra of the *E* and *Z* alkenes differed quite considerably.

On the basis of these considerations candidate 2-alkenyl-3-alkyl-6-methyl- and 2-alkenyl-3-alkyl-5-methylpyrazines were synthesized for comparison with

the natural compounds. Full details of the compounds synthesized will be published elsewhere.

It was determined that compound (3) was (*Z*)-5-methyl-3-(2-methylbutyl)-2-(3-methylbut-1-enyl)pyrazine while peak (5) was (*E*)-5-methyl-3-(2-methylbutyl)-2-(3-methylbut-1-enyl)pyrazine. Compound (4) was (*Z*)-5-methyl-3-isopentyl-2-(3-methylbut-1-enyl)pyrazine and (6) was (*E*)-5-methyl-3-isopentyl-2-(3-methylbut-1-enyl)pyrazine. The *Z,E* relationship of these two pairs of compounds was shown by conversions of synthetic (5) into (3) and (6) into (4) by short irradiation of the *E* isomers. Similarly, the structure of compound (7) was shown to be (*Z*)-5-methyl-3-(2-methylbutyl)-2-(3-methylpent-1-enyl)pyrazine and (9) was (*E*)-5-methyl-3-(2-methylbutyl)-2-(3-methylpent-1-enyl)pyrazine, while (8) was (*Z*)-5-methyl-3-isopentyl-2-(3-methylpent-1-enyl)pyrazine and (10) was (*E*)-5-methyl-3-isopentyl-2-(3-methylpent-1-enyl)pyrazine. Once again the *E*, *Z* relationship of the pairs of isomers was shown by the photochemical conversion of the *E* into *Z* isomers. In every case mass spectra of the synthetic and natural compounds were identical, while the synthetic material enhanced the GLC peak of the natural material on a FFAP column.

The remaining compounds (11)–(14), of formula $C_{16}H_{28}N_2O$, whose mass spectra appear in Table 3 have so far eluded structural elucidation.

Also detected by GC-MS in the extract from the head were a series of *n*-alkanes and alkenes. These are indicated on Figure 1.

The mixture of 10 (possibly 14) pyrazines detected in the head of *R. metallica* represents the most complicated group of these compounds yet found; previously a series of five 2,6-dimethyl-3-*n*-alkylpyrazines had been found in the heads of an *Odontomarchus* species (Wheeler and Blum, 1973). Of the 10 compounds, 2,5-dimethyl-3-isopentylpyrazine (2) represents the pyrazine most commonly encountered in the Hymenoptera, having been identified from ants, bees, and wasps (Attygale and Morgan, 1984; Wheeler et al., 1982). 2,5-Dimethyl-3-(2-methylbutyl)pyrazine (1) has been detected once previously, from an Australian formicine ant of the genus *Calomyrmex* (Brown and Moore, 1979). In this ant it also cooccurs with 2,5-dimethyl-3-isopentylpyrazine.

Of the remaining eight pyrazines identified (3)–(10), no analogs as yet exist in nature. The presence of the more complex side chain in the 2 position so far has analogy only in 5-methyl-3-*n*-propyl-2-(1-butenyl)pyrazine, isolated from the heads of the myrmicine ant *Apaenogaster rudis* (Wheeler et al., 1982). The vagaries of the mass spectra of the various alkenyl pyrazines noted by Wheeler have been fully reflected in the mass spectra of the alkenyl pyrazines from *R. metallica*. It was not until the olefinic bond was hydrogenated that mass spectra more characteristic of alkyl pyrazines (Brophy and Cavill, 1980) were observed.

The only other olefinic pyrazines reported from the Hymenoptera have

been (*Z*)- and (*E*)-2,5-dimethyl-3-styrylpyrazines, isolated from the Argentine ant *Iridomyrmex humilis* (Cavill and Houghton, 1974). In this case it was shown, by extraction of the ants in the dark, that the $Z:E$ ratio was 1:3; extraction under ordinary laboratory conditions resulted in the detection of only the *Z* isomer. It was suggested that the natural compound in that ant was, in fact, the *E* isomer, with isomerization occurring during extraction and isolation.

With the olefinic pyrazines detected in *R. metallica*, the $Z:E$ isomer ratio was approx. 2:3, and this was reproducible over a number of extractions carried out under ordinary laboratory conditions. The synthetic *E* isomers could be converted to an approx. 1:1 mixture of *E* and *Z* isomers by irradiation at 300 nm for 5 min as noted above.

Of the two major remaining unidentified compounds, (12) and (13), the formulas of $C_{16}H_{28}N_2O$ and retention time on FFAP suggest an addition of the elements of methanol to the compounds (3)–(6). It is unlikely that these are artifacts since care had been taken to ensure that methanol did not come into contact with the extract. To date these compounds have eluded structural assignment.

The glandular origin of the pyrazines in the head of *R. metallica* has not been determined but, in light of previous reports, is assumed to be the mandibular gland. Behavioral tests have not been carried out.

The ubiquitous phthalate esters, (15) and (16), are considered to be artifacts, possibly having been acquired by the ant in the course of its food gathering, although contamination from solvents and glassware in the laboratory cannot be ruled out.

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EVIDENCE OF PHEROMONAL CONSTANCY AMONG SEXUAL AND ASEXUAL FEMALES IN A POPULATION OF FALL CANKERWORM, *Alsophila pometaria* (LEPIDOPTERA: GEOMETRIDAE)

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Abstract—The compounds (*Z,Z,Z*)-3,6,9-nonadecatriene (I), (*Z,Z,Z,E*)- (II), and (*Z,Z,Z,Z*)-3,6,9,11-nonadecatetraene (III) have been implicated as components of the female sex pheromone of the fall cankerworm. Chromatographic determination of the proportions of these compounds in individual females of sympatric asexual and sexual reproductive forms of the species, with concurrent analysis of the electrophoretic profiles of the same females, showed that the I : II : III proportion of compounds was constant across electrophoretically differing asexual genotypes and between these and the sexual form. Life-history characters, in contrast, typically show great variation among these genetic groups. The results indicate that pheromonal constancy is maintained in a reproductive system that is theoretically vulnerable to selective pressures that would lead to heterogeneity in the species' pheromonal communication channel.

Key Words—Lepidoptera, Geometridae, *Alsophila pometaria*, fall cankerworm, sex pheromones, genetic variation, pseudogamy.

INTRODUCTION

Although differentiation in female sex pheromones is a common isolating mechanism in Lepidoptera (Roelofs and Brown, 1982; Struble and Byers, 1985), we have as yet little understanding of how such pheromone systems evolve (Cardé

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and Baker, 1984). One unanswered question is the degree to which pheromone divergence results from selection, as imposed by factors such as overlapping signals from a related species, interception of the signal by predators, or physical characteristics of the habitat (Roelofs et al., 1982). Selection against deviant signals and receptors could be so strong as to prevent evolution of the pheromone channel except when gene frequencies are initially displaced by sampling error in founder populations (Wright, 1932; Templeton, 1981).

One gauge of the evolutionary plasticity of pheromone systems should be their intraspecific variability, but few relevant studies exist. Geographic variation in pheromone production or response has been reported for some species of Lepidoptera (Arn et al., 1982; Bailey et al., 1987; Klun and Cooperators, 1975; Klun and Maini, 1979), and intrapopulation variation has been examined in several others (Löfstedt et al., 1985; Miller and Roelofs, 1980). Single-locus polymorphism in pheromone isomeric composition was documented for the European corn borer (Klun and Maini, 1979), whereas quantitative heritability was demonstrated in the pink bollworm moth (Collins and Cardé, 1985).

Correlation of such intraspecific variation with inferred selective forces would provide evidence for the adaptive nature of pheromone differentiation, but it has rarely been sought. Character displacement of the pheromone composition has been suggested for a few species (Cardé et al., 1977; Greenfield and Karandinos, 1979). However, the only attempt to directly measure response to selection in the field was conducted by Haynes et al. (1984), who found no evidence of change in the pheromone system of the pink bolloworm moth after three to five years of field application of the species' pheromone as a mating suppressant.

We report a study of intraspecific variation of pheromone composition in the fall cankerworm (*Alsophila pometaria*), a geometrid moth that might be subject to continuous selection for novelty in the communication channel because of its unusual breeding system. The fall cankerworm has two female reproductive forms (Mitter and Futuyma, 1977). Eighty to ninety-nine percent of the individuals in most populations are pseudogamous asexual females, which must mate with conspecific males in order to reproduce but give rise to all-female progeny genetically identical to themselves, there being no paternal genetic contribution. The remaining 1–20% of these populations is composed of sexually reproducing males and females. The asexual females are genetically diverse due to repeated origin of asexual females from sexual progenitors (Harshman and Futuyma, 1985a), but one or a few widespread genotypes are typically dominant (Mitter and Futuyma, 1977; Harshman and Futuyma, 1985a,b; Mitter et al., 1987).

There are several reasons why selection in populations harboring pseudogamy might favor evolution of mating signals unique to the sexual form. Males make no genetic contribution to the offspring when they pair with asexual females. They should consequently face intense selection for discrimination

against asexual mates. Cankerworm females may also compete for the relatively scarce males (Harshman and Futuyma, 1985b), which make up only 1–10% of most fall cankerworm populations (Porter and Alden, 1924; Moore and Drooz, 1974; Mitter and Futuyma, 1977; Harshman and Futuyma, 1985b). Thus, any novel pheromone increasing the probability of male response might be strongly favored (West-Eberhard, 1984).

Evidence for mating discrimination favoring sexual females would also shed light on the puzzling dynamics of populations harboring pseudogamy. Under some conditions such discrimination could have the incidental effect of stabilizing the existence of these populations, which will tend to be driven to extinction by the superior reproductive potential of their all-female components (Kiester et al., 1981; Moore and McKay, 1971; Stenseth et al., 1985). Whether mating discrimination evolves may depend on such factors as the rate at which the dominant asexual genotypes can be replaced. If this turnover is rapid, there may be no divergence between the two reproductive forms for male discrimination to act on. Rare asexual variants might arise frequently in the fall cankerworm (Harshman and Futuyma 1985a), but how rapidly they could displace the dominant types is not clear.

We have sought to determine whether the fall cankerworm pheromone shows a pattern of variation suggestive of evolution under the pressure of pseudogamy. Such divergence could be manifest at several levels: as pheromonal difference between sexual and asexual forms; as variation among asexual genotypes, possibly reflecting the recency with which they have originated from the sexual form; or as geographic variation in the attractant blend resulting from independent evolution of the pheromone in different areas.

We therefore carried out a chromatographic analysis of the proportions of pheromone components in the glands of individual females of the sexual and asexual fractions of a fall cankerworm population near Beltsville, Maryland. We have also compared our findings with the pheromone composition determined for a fall cankerworm population at the opposite extreme of the species' range, near Saskatoon, Saskatchewan, Canada (Wong et al., 1984a). Wong et al. (1984a,b) indicated that the fall cankerworm pheromone is an approximately 25 : 60 : 15 mixture of *Z,Z,Z*-3,6,9-nonadecatriene (I), (*Z,Z,Z,E*)- (II), and (*Z,Z,Z,Z*)-3,6,9,11 nonadecatetraene (III). Components II and III, singly and in combination, attract males to traps. Compound II has greater attractiveness and was as attractive as any mixture of II and III. Component I is not attractive by itself, but is reported to synergize the effects of II and III (Wong et al., 1984a,b).

METHODS AND MATERIALS

Genetic Analysis. The moths analyzed in this study were laboratory-reared offspring of parents collected from three sites near Beltsville, Maryland. A con-

comitant survey of sex ratio, reproductive mode frequency, and genotypic composition at four electrophoretic loci (Mitter et al., 1987) showed the Beltsville population to be quite similar in breeding structure to intensively studied populations on Long Island, New York (Mitter et al., 1979; Harshman and Futuyma, 1985a,b). The proportion of females reproducing sexually was estimated from progeny testing to be 15–20%, while a sample of reared, wild-caught larvae contained 9% males. The asexual population was dominated by two very abundant genotypes (Table 1) (Mitter et al., 1987).

The individuals subjected to pheromone analysis were daughters of females collected in the winters of 1982–1983 and 1983–1984. The mode of reproduction of these females was determined from the sex ratios and electrophoretic profiles (at two polymorphic loci) of their progenies, following methods described by Mitter and Futuyma (1977) and Harshman and Futuyma (1985a).

Twelve sexually produced moths were assayed for pheromone composition, representing the offspring of eight different females. The 44 asexually produced females assayed represented 20 single-parent broods, and five different four-locus electrophoretic genotypes. The latter included the two abundant genotypes referred to above, plus three rare genotypes. Broods of asexual genotypes 1, 2, and 3 were assayed in a preliminary study in 1983. The larvae were mass-reared on cut foliage of *Prunus serotina* Ehrh. Additional broods of genotype 3, broods of genotypes 4 and 5, and sexual broods were assayed during more extensive progeny rearing in 1983, using "Fall Cankerworm Diet" supplied by Bioserv Inc. (Frenchtown, New Jersey), which follows the recipe of Fedde (1974).

Details of rearing procedures are given in Mitter et al. (1987). The virgin females used for pheromone analyses had eclosed an average of seven days earlier, with a range of 0–20 days. (Female fall cankerworms live 30 days or more at 4°C; unpublished observations.) After excision of the ovipositor for pheromone analysis, each female carcass was stored at −60°C until the specimen was subjected to electrophoresis. Electrophoretic methods and phenotype designations (as in Table 1) followed Mitter and Futuyma (1977) and Futuyma et al. (1981). Mothers and sibs of individuals subjected to pheromonal analysis were also electrophoresed to verify reproductive mode and genotype for each brood, as part of a larger genetic study (Mitter et al., 1987).

Chromatographic Analysis. Extracts of individual female pheromone glands were prepared by excising the ovipositor with fine dissecting scissors and soaking it for 3 min in 10 μl heptane contained in a conical microvial. The ovipositor was then removed from the solvent and 2–3 μl of the extract was injected onto a 60-m × 0.25-mm (ID) fused silica DB-1 open tubular capillary column (J&W Scientific Inc., Rancho Cordova, California) using the splitless injector (225°) of a Hewlett-Packard model 5840A gas chromatograph that was equipped with a flame ionization detector. Hydrogen, at a velocity of 50 cm/sec, was used as a carrier gas, and the column was temperature programmed:

120°C for 0.45 min after injection of a sample followed by heating to 175°C at 15°C/min.

The proportion of I, II, and III in each extract was calculated from the peak area recorded by the integrator of the gas chromatograph. Chromatographic peaks representing I, II, and III had 1867, 1903, and 1927 retention indices, respectively, on the DB-1 column. These retention indices were identical to samples of I, II, and III supplied by Dr. E.W. Underhill, National Research Council of Canada, Saskatoon, Saskatchewan, Canada. Combined gas chromatography-mass spectroscopy using the DB-1 column interfaced with a Finnigan model 6110 data system verified that the components at 1867, 1903, and 1927 retention indices in the combined extracts of four fall cankerworm ovipositors had mass fragmentation patterns identical to the authentic standards.

Statistical Analysis. Two aspects of pheromone composition were selected for statistical analysis. The proportion of the *Z,Z,Z,E* isomer (II) in the total of 3,6,9,11-nonadecatetraene (II + III) was examined because such isomeric ratios are of known behavioral importance in many other Lepidoptera. The proportion of the total pheromone made up by component I was also examined, because I is reported to have a qualitatively different behavioral effect from components II and III (Wong et al., 1984a, b).

Mean proportions among different genetic groups, consisting of the five asexual genotypes and the pooled sample of sexual females, were compared by analysis of variance, following arcsin square-root transformation.

RESULTS

Table 1 summarizes the chromatographic analyses of the ovipositor extracts of the Beltsville *A. pometaria* females. The data for 1983 and 1984 were combined because there was no statistical difference between years. Values are presented separately for the different electrophoretic classes of asexual females. The four-locus electrophoretic phenotype of each of those classes is also given, as is its frequency in samples of wild-collected larvae (Mitter et al., 1987).

The data of Table 1 indicate that the proportion of 3,6,9,11-nonadecatetraene comprised by the *Z,Z,Z,E* isomer (II) in the Beltsville samples does not vary significantly among the different genetic groups or between asexual females as a group and their sexual counterparts. The mean isomeric ratios for all genetic groups at Beltsville were also strikingly similar to the pooled value reported for the Saskatchewan population by Wong et al. (1984a).

The proportional amount of component I was also homogeneous across genetic groups at Beltsville. However, there was proportionally less of this component in Beltsville *A. pometaria* females than reported for Saskatchewan females (Wong et al., 1984a).

TABLE 1. PROPORTIONS OF THREE C_{19} OLEFINIC HYDROCARBONS IN FALL CANKERWORM OVIPOSITOR EXTRACTS, BY GENETIC GROUP[a]

Genetic group	Genotype frequency, Beltsville population	Broods assayed (*N*)	Individuals assayed (*N*)	Proportions of C_{19} olefinic hydrocarbons: $\frac{III}{I + II + III} \times 100\%$ $(\bar{X} \pm SE)$	$\frac{I}{I + II} \times 100\%$ $(\bar{X} \pm SE)$
Asexual genotype 1	<0.01	4	15	*not determined*[b]	79.8 ± 0.24a
Asexual genotype 2	<0.01	6	11	not determined[b]	79.5 ± 0.24a
Asexual genotype 3	0.40	6	13	10.1 ± 0.93a (*N*=6)	79.7 ± 0.95a
Asexual genotype 4	0.29	2	3	6.2 ± 1.33a (*N*=3)	80.0 ± 1.03a
Asexual genotype 5	0.06	2	2	6.7 ± 1.30a (*N*=2)	79.1 ± 1.45a
Asexual genotypes combined		20	44	8.4 ± 0.84a (*N*=11)	79.7 ± 0.30a
Sexuals, Beltsville		8	12	8.0 ± 1.37a (*N*=11)	80.3 ± 0.81a
Pooled sample (~5000 females), Saskatoon, Saskatchewan Wong et al., 1984a)				26.0	79.5

[a] I = (*Z,Z,Z,Z,E*)-3,6,9,11-C_{19}; II = (*Z,Z,Z,Z*)-3,6,9,11-C_{19}; III = (*Z,Z,Z*)-3,6,9-19_{19}. Values followed by the same letter are statistically indistinguishable at $\alpha = 0.05$. Genotype frequencies from Mitter et al. (1987).

[b] Compound III was not known to be a pheromonal component when these females were studied (1982–1983).

DISCUSSION

Our findings indicate that the proportion of the three known pheromonal components was essentially constant across a representative set of electrophoretically characterized sexual and asexual fall cankerworm genotypes at Beltsville. By contrast, asexual genotypes at Beltsville and elsewhere typically show marked differences in a wide range of other characters such as body size, phenology, host preference, and developmental response to crowding (Mitter et al., 1979, 1987; Schneider, 1980; Futuyma et al., 1981, 1984. Rare pheromonal variants may have been missed in our limited survey, and it is also possible that variation might exist in some as yet unknown component of the species' pheromone (Klun et al., 1979). Nonetheless, our findings suggest that the Beltsville fall cankerworm population is not undergoing continual evolution of the pheromone such as pseudogamy could theoretically produce. We find likewise no evidence of pheromone differences which could promote persistence of cankerworm populations by facilitating male preference for sexual females. Our observations are in general agreement with those of Harshman and Futuyma (1985b), who found a marginal indication of differential mating success among fall cankerworm genotypes in the wild, but no tendency for greater success by rare genotypes, which will presumably include the rare sexual individuals.

One explanation for these observations is that the rate of origin and turnover of asexual genotypes from the sexual population is sufficient to thwart the development of pheromonal divergence between the reproductive forms, despite the apparent persistence of the few widespread, dominant asexual genotypes (Harshman and Futuyma, 1985a; Mitter et al., 1979). The assumption that females compete for males might also be questioned, since mating frequency and longevity of males have not been studied in the field. Individual males have been observed to mate up to seven times in the laboratory (Moore and Drooz, 1974).

Another possibility is that episodes of pheromonal evolution driven by pseudogamy do occur but only sporadically, contingent on the appearance of appropriate pheromonal variants. Under this view, local fall cankerworm populations would most often show pheromonal constancy, but geographically separated populations might differ as a result of independent episodes of pheromonal evolution, which terminate when the asexual population gradually acquires the communication channel newly evolved by the sexual form. This is one of several explanations possible for the divergent proportions of 3,6,9-nonadecatriene observed in this study and by Wong et al. (1984a).

To summarize, the fall cankerworm population we studied, which is genetically diverse in reproductive mode and other characters, appears to be pheromonally constant despite the potential challenge of the pseudogamous breeding system. Our findings resemble those of Haynes et al. (1984), in indicating no pheromonal response to a presumed selection pressure.

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DISCRIMINATION OF OCCUPIED HOST FRUIT BY PLUM CURCULIO FEMALES (COLEOPTERA: CURCULIONIDAE)

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Abstract—Larval survival of plum curculios (PCs), *Conotrachelus nenuphar* (Herbst), was found to decrease with increasing egg density per fruit. Subsequently, we assayed PCs for propensity to avoid egg-laying at sites (immature plums) already occupied by conspecific eggs. Laboratory choice tests showed PCs made an equal number of visits to and ovipositions in fruit with a single oviposition as in clean fruit. Although there was a trend toward more visits to fruit which contained four or eight oviposition wounds and eggs or eight artificial punctures than to clean fruit, PCs oviposited less frequently into these than clean fruit. Results suggest that wounding of fruit may enhance the ability of ovipositing PCs to locate fruit, but at the same time may furnish cues allowing some degree of discrimination against heavily infested fruit for oviposition.

Key Words—Plum curculio, *Conotrachelus nenuphar* (Herbst), Coleoptera, Curculionidae, oviposition, host discrimination, host location.

INTRODUCTION

Mechanisms which allow resource partitioning and subsequent avoidance of overcrowding and competition among individuals within a host of limited resource capacity are often of positive selective value (Prokopy et al., 1984). Prokopy (1981) reviewed the literature on an array of insects which respond to marking pheromones to avoid ovipositing at sites infested with their own progeny or progeny of conspecifics. Chemical cues emitted from a host likewise

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may serve as signals of egg or larval occupancy (Rothschild and Schoonhoven, 1977; Renwick and Radke, 1981, 1985; Girolami et al., 1981; Saxena and Basit, 1982; Mitchell and Heath, 1985).

In recent years, we have been investigating partitioning of larval fruit resources by some of the major apple pests in northeastern North America. Thus far we have found that (1) the apple maggot fly, *Rhagoletis pomonella* (Walsh), lays down a pheromone after oviposition that deters conspecifics from laying additional eggs in the same host fruit (Prokopy, 1972); (2) ovipositing codling moths, *Cydia pomonella*, (L.) do not discriminate against occupied sites through chemical signals but may partition ovipositional resources via other mechanisms (Roitberg and Prokopy, 1982); and (3) the European apple sawfly, *Hoplocampa testudinea* (Klug), responds to wound exudates of host tissue in discriminating against apples already infested with conspecifics (Roitberg and Prokopy, 1984). To date, no quantitative work has been done to determine if a fourth major apple pest, the plum curculio (PC), *Conotrachelus nenuphar* (Herbst), assesses fruit for the presence of conspecifics.

PC adults overwinter in ground cover or soil in or near woods bordering host trees, yet little is known about how PCs locate and choose egg-laying sites upon spring emergence. We do know that within host trees, the majority of movement is by crawling on branches, stems, and petioles until a fruit is encountered (Owens et al., 1982).

Oviposition commences when a female first feeds under the skin of a fruit, forming a flap. She then reverses position and deposits an egg under the flap. After egg deposition, the female again reverses position and continues feeding, forming a large crescent of gouged tissue above the flap. This is thought to relieve pressure produced by proliferating cells of the growing fruit. PCs in nature have been observed ovipositing repeatedly into the same fruit (Quaintance and Jenne, 1912; Owens et al., 1982). This behavior suggests PCs may not discriminate strongly against fruit previously infested with conspecifics. On the other hand, Jacklin et al., (1968) found that PC larval crowding in apples reduced larval weight and rate of survival, hinting that a mechanism by which females could assess conspecific density within a host might be of selective advantage.

At least two other Curculionidae are known to partition resources by avoiding occupied hosts for oviposition. The boll weevil, *Anthomonus grandis grandis* (Boheman), has been shown to discriminate against cotton squares that contain one or more eggs (Stansly and Cate, 1984), and *Ceutorhynchus assimilis*, the cabbage seed weevil, lays down a marking pheromone after oviposition which acts as an oviposition deterrent (Kozlowski et al., 1984). Here we report evidence indicating that PCs can, in fact, recognize a high density of conspecifics within a host via cues emitted from oviposition and/or feeding puncture sites.

METHODS AND MATERIALS

Larval Survival. Studies were conducted in June 1982 in Amherst, Massachusetts, to determine to what degree larval density affected survival in fruit. Fruiting branches on unsprayed cultivated plum trees were bagged in spring prior to PC adult emergence to protect fruit from infestation. Upon spring migration, adults were collected from an unsprayed orchard by tapping limbs over a large white sheet onto which dislodged adults fell. PCs were placed in the bags and allowed to oviposit into fruit. Once fruit abscission began, all remaining fruit were picked because larvae in fruit that do not abscise prematurely usually do not survive (Levine and Hall, 1977). Fruit were sorted into groups with like number of oviposition scars (98% of the oviposition scars were found to contain a single egg). Egg density levels per fruit ranged from one to six. Fruit were placed in plastic 35-ml cups and held at 25°C, 50% relative humidity for 30 days. Once larvae emerged from the fruit, they were removed from the cups, and percent survival was recorded for each density level. Egg density and larval survival were compared using Spearman's rank correlation (Sokal and Rohlf, 1981).

Fruit Discrimination Study. Studies were conducted in June 1984 to determine the ability of PCs to discriminate against egg-occupied fruit. Tests were run under fluorescent light at 16:8 hr light-dark, 50–60% relative humidity, and at 24°C. Opaque dividers were placed between each cup or cage to visually isolate each PC. Mirrors were placed on the wall behind a row of cups or cages so that PCs could be viewed from all sides. Because PCs frequently feign death when disturbed, a white curtain was hung just below eye level of the observer to hide the observer's movements from PCs.

Females were pretested to standardize their physiological state of readiness for oviposition. An immature plum (ca. 17 mm diam.) was hung in the center of a 35-ml transparent plastic cup, and a single PC was introduced. Each PC was allowed one oviposition per fruit over a total of three fruit. After a PC completed oviposition, the fruit was replaced with a clean one. All pretests took place within 5 hr of testing. We maintain the importance of standardizing through pretests the physiological state of PCs for subsequent assay, yet also recognize that, in so doing, PCs could conceivably learn to accept fruit of a particular type, e.g., clean fruit.

Once pretested, a female was placed gently into a 5-dram vial on a test cage floor and allowed to remain undisturbed for 15 min before the vial was opened. Test cages were placed on a wooden platform constructed so that cages could be removed from below. Test cages were transparent cylindrical plastic tubes 11 cm high by 8.5 cm diam. (Figure 1). At the center of each cage was a wooden stand in which a stem and crosspieces made from plum twigs were mounted. Two clean fruit and two other fruit of like treatment (with one, four,

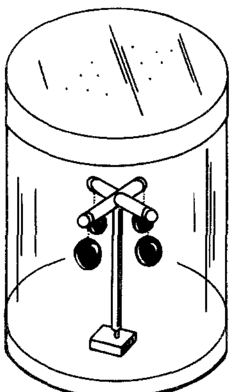

FIG. 1. Cage for PC fruit discrimination choice tests.

or eight ovipositions or eight artificial punctures) were hung at the ends of the crosspieces in an alternating fashion. Fruit were of the same size and type as in the pretest. Treatment fruit were prepared on the day of testing. Each of those to be infested was placed with one or two PCs in a 35-ml cup until the desired number of oviposition scars was observed. Artificial punctures were made with a scalpel to resemble natural oviposition scars in size and shape. Therefore, this treatment offered fruit in what appeared to us as being the same state as fruit which had received oviposition scars naturally except that all insect visitation previous to testing was eliminated.

We designated tests as beginning when a PC started crawling up the stem of the crosspiece and ending when the first oviposition was completed. We recorded the fruit type visited first by a PC (i.e., first visits), the total number of visits to each fruit type, and the fruit type in which oviposition occurred. If a PC fed on a fruit without ovipositing, the fruit was replaced with a new one of the same treatment. If a PC did not oviposit within 3 hr, it was removed and replaced with a new pretested individual. If a PC failed to oviposit, it was not included in the analysis of data. Test cages were rotated 45° after each test was completed to randomize treatment placement.

RESULTS

Larval survivorship was greatest, 36%, at a density of one egg per fruit (Table 1). With increasing egg density per fruit, larval survivorship progressively declined, reaching a low of 9% at six eggs per fruit.

PCs made an equal number of first visits, total visits, and ovipositions on clean fruit and fruit with one previous oviposition (Table 2). Every visit to a

TABLE 1. LARVAL SURVIVAL OF PLUM CURCULIO AT DIFFERENT EGG DENSITIES PER FRUIT[a]

Egg density per fruit	Total No. fruit	Eggs yielding larvae (%)
1	114	36
2	96	32
3	41	20
4	18	14
5	10	12
6	11	9

[a]Spearman's $r = -1.0$, $P < 0.001$.

TABLE 2. CHOICE-TEST RESPONSES OF FEMALE PLUM CURCULIOS TO IMMATURE PLUMS RECEIVING DIFFERENT TREATMENTS[a]

Treatment	No. PC tested	No. fruit visited first	Fruit visited first resulting in oviposition (%)	No. total visits to fruit	Total visits resulting in oviposition (%)
Clean vs. 1 oviposition wound	11	17a	100a	17a	100a
		17a	100a	17a	100a
Clean vs. 4 oviposition wounds	20	15a	100a	27a	63a
		22a	91a	38a	53a
Clean vs. 8 oviposition wounds	13	13a	92a	23a	78a
		21a	48b	39b	41b
Clean vs. 8 artificial punctures	14	9a	100a	19a	79a
		15a	60b	26a	35b

[a]Values within the same column within the same treatment comparison followed by the same letter are not significantly different at the 5% level according to a G test.

fruit in this choice test resulted in an oviposition. Thus, PCs did not discriminate in favor of or against infested fruit at a level of one egg per fruit. In all other choice tests, however, there was a greater number of first and total visits to insect- or artificially punctured fruit than to clean fruit. However, differences were significant only in the case of total visits to fruit with eight ovipositions vs. clean fruit, according to a *G* test for goodness of fit (Sokal and Rohlf, 1981). These results suggest that fruit wounded with four or eight ovipositions or eight artificial punctures had a positive effect on a female's propensity to visit that fruit. PCs had less propensity to oviposit in fruit with four or eight ovipositional punctures or eight artificial punctures than in clean fruit. There was a significant difference in the number of total visits which resulted in oviposition only in tests of eight oviposition wounds or artificial punctures vs clean fruit, according to a *G* test of independence (Sokal and Rohlf, 1981). The fact that ovipositional propensity was similar for fruit with eight ovipositional punctures as for fruit with eight artificial punctures suggests the stimulus leading to rejection of such punctured fruit for oviposition was of fruit and not insect origin.

DISCUSSION

The mechanism by which PCs locate and choose feeding and oviposition sites is not well understood. Preliminary olfactometer tests (Groden, Drummond, and Prokopy, unpublished) showed that female PCs crawled upwind more often toward chambers which contained apples than toward chambers containing moist cotton. This suggests that, at short range, PCs may respond positively to fruit volatiles to aid in fruit finding.

Physiological changes occur when a plant is wounded that may alter the nature or enhance the quantity of volatiles released. It is also known that volatile chemicals released from insect-damaged plant tissue can increase the attractiveness of damaged plants to some insects (Finch, 1980). Perhaps fruit volatiles which appear to aid PCs in host finding are modified in nature or enhanced in quantity in wounded tissue, leading to increased PC visitation to such fruit. It appears that volatiles from fruit with a single ovipositional puncture are not modified in quantity or quality so as to have a detectable effect on PC visitation, but volatiles released from fruit with four or more punctures do produce such an effect.

Prior to visiting fruit wounded with four or more punctures, several PCs appeared excited, crawling quickly up the main stem and then to the end of a crosspiece where fruit was hanging. However, PCs would sometimes stop before reaching the end of a crosspiece, display antennal waving, and then either continue on or change direction. Many insects align their body in the direction of an odor source upon detection of a gradient of odor molecules (Shorey, 1973; Kennedy, 1978). Conceivably, antennal waving could aid a PC in locating the

direction of wounded fruit by comparing the concentration of odor on each side of its body. Owens et al. (1982) noted PCs in nature making short pauses between bouts of crawling, accompanied by antennal waving and sometimes reorientation. Miller (1979) proposed that a stop-go pattern of walking, as observed in phorid flies, may serve a visual and/or chemosensory function, aiding in orientation to host resources. However, we believe that PCs' use of vision to locate fruit within a tree is limited.

PCs often crawled onto wounded fruit, fed in wounds, and then left without ovipositing. During several replicates, PCs also were seen feeding on the tips of freshly cut fruit petioles, suggesting that wounded tissue may stimulate feeding behavior. Owens et al. (1982) reported PCs feeding at petiole abscission scars and on ooze exuding from damaged fruit tissue.

Why did PCs leave without ovipositing after feeding on wounded fruit? Several hypotheses are possible. First, wounded fruit tissue may release compounds which deter oviposition. Second, perhaps only feeding behavior, not oviposition behavior, is stimulated by wounded fruit. Third, the complete oviposition sequence may somehow be interrupted after sensing nearby wounded tissue, causing a female to enter a feeding mode rather than continue in an oviposition mode. PCs may partition time spent exploring or feeding at potential oviposition sites according to their physiological state or previous host-searching experience. If the appropriate oviposition cues are not received within a particular window of time, PCs may leave in search of a more suitable fruit.

A mechanism for discrimination against occupied hosts may exist in PCs, but in our tests it was not as strongly manifested as in most other herbivorous insects exhibiting discrimination. Perhaps this is because PCs' ability to move within a tree is rather limited. When a PC locates a fruit, it may tend to oviposit regardless of the fruit's condition and regardless of the lower probability of progeny survival with increasing egg density, as a PC may not encounter another fruit very readily. In other words, PCs appear to have a comparatively limited ability to sample a wide range of fruit within a tree, leading to comparatively greater propensity to accept discovered fruit despite possible harmful consequences to progeny. Low larval survival, even at one egg per fruit, may be due to inadequate nutrients and/or presence of toxic secondary plant compounds within the host. PCs' expansion from wild plum to cultivated plum (within the past 150 years) may not yet have allowed sufficient time for high larval survival.

Since plant-insect interactions often involve three trophic levels (plant-herbivore-enemies) (Price et al., 1980), one must also consider ways in which utilization of chemical cues may aid PCs in predator avoidance. Faeth (1985) found that leafminers chose significantly more intact leaves for oviposition sites than damaged ones. Damaged leaves received more parasitism than intact ones. Such parasitism may have constituted selection pressure giving rise to individual leafminers which avoided leaves occupied by conspecifics. Perhaps the ovi-

positional response of PCs to damaged fruit may have been shaped, at least in part, by a similar sort of parasitoid or predator selection pressure.

In conclusion, resource partitioning via response to chemical cues from occupied sites is not always a "tight" system. When the dispersal power of ovipositing females is weak, avoidance of overcrowding of resources and attendant effects on progeny competition may not be under strong selection. It appears that ovipositing PCs do possess some ability to discriminate against egg-occupied fruit on the basis of high levels of fruit-originating stimuli emitted from wounds, but that behavioral traits coupled with ecological conditions do not favor pronounced expression of this ability.

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PRESENCE OF PTERIN PIGMENTS IN WINGS OF LIBYTHEIDAE BUTTERFLIES

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Abstract—A few pterin pigments were discovered in the wings of six of eight libytheid species tested, using thin-layer chromatography with 1% HCl in butanol as a solvent. A 10X sample of *Libytheana bachmanii larvata* (Strecker) produced xanthopterin, isoxanthopterin, erythropterin, and leucopterin. Leucopterin was absent in the other libytheids tested. Morphology and pterin pigment data from wings suggest a Pieridae ancestry for the Libytheidae from the region of the northern Neotropical realm (including the Greater Antilles).

Key Words—Pterin pigments, *Libythea, Libytheana*, Libytheidae, Lepidoptera, thin-layer chromatography.

INTRODUCTION

The isolated family Libytheidae (two genera: *Libythea*, Old World; *Libytheana*, New World) shows a morphological relationship to both the Nymphalidae (adults) and the Pieridae (immatures) but not to Riodinidae (Jordan, 1925; Kristensen, 1976; Shields, 1974). It was decided to test for the presence of pterin pigments in libytheid wing scales, since these pigments are universally distributed in pierid wings but have been shown to be lacking in the wings of all other butterfly families except erythropterin in the nymphalid *Heliconius* (Heliconiinae) and isoxanthopterin in Nymphalidae, some Lycaenidae, one Riodinidae, and one Papilionidae (Ford, 1947a,b; Baust, 1967; Rawson, 1968; Robinson, 1971). If Libytheidae is a phylogenetically transitional family between Pieridae (primitive) and Nymphalidae (advanced), as its morphology indicates, it might still contain an array of pterins, at least in trace amounts.

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In Pieridae wings, pterins are deposited on the canal walls in the scales during the pupal and teneral adult stages and give the white, yellow, and red coloration to the wings (Wigglesworth, 1939; Ziegler-Gunder, 1956). Since pterins are chemically related to uric acid, they are probably excretory products transported by the hemolymph (Ford, 1947a; Ziegler-Gunder, 1956). Small amounts of uric acid and other nitrogenous breakdown products also occur in Pieridae wings (Gates, 1947; Harmsen, 1966). Pieridae differs dramatically in the origin and structure of its wing-scale pigments from the other butterfly families (Ford, 1947a).

In insects, pterins are reported in the bodies of various Rhopalocera and Geometridae, *Ascalaphus* (Neuroptera), *Gaema* (Cicadidae), *Tettigonia* (jassid), various Hemiptera, *Vespa* (Hymenoptera), some Diptera, and *Bombyx* (Bombycidae) (Bartel et al., 1958; Rawson, 1968). Thus, within the Pterygota, pterins are present in some of the advanced infraclasses of Paraneoptera (hemipteroids) and Oligoneoptera (Coleoptera, Neuroptera, Lepidoptera, etc.) but are lacking in the more primitive infraclasses of Paleoptera (Ephemerida and Odonata) and Polyneoptera (orthopteroids) (see Fox and Fox, 1964, pp. 337–339).

METHODS AND MATERIALS

An attempt to identify pterins from eight of the 12 species of libytheids was made by removing both wings from one side of an individual specimen of each and macerating them in 1% HCl. The mixture was centrifuged twice at 5000 rpm for 7 min. The decanted supernatent was air-dried in test tubes, redissolved in 1% HCl, and 13–33 μl spotted on a Baker-Flex thin layer cellulose plate. The solvent of 1-butanol–acetic acid–water (4 : 1 : 1) follows Watt (1964) and Pfeiler (1970). Two wings of *Eurema nicippe* (Cramer) were similarly extracted and 10 μl spotted as above, as the control on the same plate. The TLC plate was allowed to equilibrate against the butanol solvent vapor for 1 hr. Then the butanol solvent was added and allowed to migrate up the plate for 3–4½ hr in a chromatography tank. The 1% HCl in butanol was found to yield a far greater number of pterins than when 1% NH_4OH in butanol was used.

Spots from the libytheid species were matched to the control in color and R_f values under UV light after drying. R_f values and colors under UV follow Harmsen (1966) and Pfeiler (1970). The pterin spots were located under short wavelength UV light (2000 Å) for xanthopterin and under long wavelength UV light (ca. 4000 Å) for the other pterins.

Because unusual amounts of xanthopterin occur in *Libytheana bachmanii larvata* Strecker from Ciudad Victoria, Mexico, it was decided to further isolate the pterins from this population by removing the wings from five specimens (22.1 mg), or approximately ten times the sample size of the other species

tested. The same procedures were used (93 μl spotted), with *Pieris rapae* (Linnaeus) as a control using the R_f values for its pterins in Pfeiler (1970), except this time propanol solvent (1-propanol–1% aqueous ammonia, 2:1) was run in one direction while the butanol solvent was run at 90° on the same plate. Finally, the spots isolated under UV were scraped off and run separately on another TLC plate in the butanol solvent only.

RESULTS

Pterins were entirely absent in the western Old World species *Libythea labdaca* Westwood (Uganda), *L. laius* Trimen (South Africa), and *L. celtis celtis* Fuessly (Spain). However, xanthopterin was present in *Libythea narina rohini* Marshall (Thailand), which lacked other pterins, and in *Libytheana bachmanii bachmanii, Libythea myrrha*, and *L. geoffroy*. Erythropterin was faintly present in *Libythea geoffroy orientalis* Godman & Salvin (Guadalcanal) but appeared fairly prominently in *L. celtis formosana* Fruhstorfer (Taiwan), *L. myrrha sanguinalis* Fruhstorfer (Thailand), *Libytheana carinenta* Cramer (Brazil), and *L. b. bachmanii* Kirtland (Tennessee). Leucopterin was not detected in any of the libytheids, and isoxanthopterin was absent in all except *L. celtis formosana* for this sample size.

The larger sample of *Libytheana bachmanii larvata* (three males, two females) collected in August 1937 at Ciudad Victoria, Mexico, contained a very prominent amount of xanthopterin (yellow green under UV of 2000 Å, R_f = 0.42) which decomposed to uric acid after several weeks, prominent leucopterin (pale blue, R_f = 0.06–0.08, 2000 Å), and a faint spot of isoxanthopterin (blue under UV of ca. 4000 Å, R_f = 0.27). Erythropterin (R_f = 0.05) was also present but sepiapterin was absent. Xanthopterin and sepiapterin have identical R_f values in butanol but not in propanol. The Ciudad Victoria *L. b. larvata* wings had brown mottling on a whitish background on the undersurface, while *L. b. bachmanii*'s underside contained much less white and more brown, also reflected in the pterin results.

DISCUSSION

Pterin pigments present in the wings of most libytheids strengthen a possible tie with Pieridae that is also suggested on morphological grounds. This ancestral type may have had a yellow (prominent), white, and red wing pattern judging by the pterin results for *L. b. larvata*.

The Cuban *Libytheana motya* Hubner (not tested here) is likely the most primitive libytheid because of its extensive white coloration; it appears related to nearby *L. bachmanii* and *L. terena* Godart, but not to *L. carinenta*. The next

least advanced libytheids are probably the worldwide brown forms (*L. bachmanii, L. terena, L. carinenta, L. fulvescens* Lathy, *L. laius, L. celtis, L. myrrha*) with *L. labdaca, L. narina*, and *L. geoffroy* the most advanced black (and iridescent) forms. This scheme is substantiated by the pterin pigment results presented here and the phylogeny of their *Celtis* host plants (Shields, 1986). Thus *L. motya* is the most pierid-like libytheid with its white coloration, while at the opposite end of the spectrum *L. geoffroy* is the most nymphalid-like. This trend from primitive to advanced also included a corresponding shortening of their elongated labial palpi. New World *Libytheana* genitalia are closest to pierids, while Old World *Libythea* genitalia are closer to nymphalids (Shields, 1985, 1986). It is noteworthy that African *Mylothris*, one of the pierids probably close to libytheids in relationship, contains a very prominent amount of xanthopterin (Harmsen, 1970), as did *L. b. larvata*. Another is *Melete* (Neotropical, 13 species) which approaches *Libytheana* in genitalia and palpi. Both *Mylothris* and *Melete* use Loranthaceae as larval food plants, a family occasionally parasitic on *Celtis* (Celtidaceae), the food plant of libytheids. Earliest *Celtis* fossils date from the uppermost Cenomanian 92–94 million years ago (lower Upper Cretaceous time) (Shields, 1986).

CONCLUSION

Six of eight Libytheidae species tested by TLC contained pterin pigments in their wings: xanthopterin (four species), erythropterin (five species), and isoxanthopterin (one species). Few or no pterins were found in the most advanced species. A 10-fold sample of *Libytheana bachmanii larvata* wings from northeastern Mexico contained large amounts of xanthopterin and leucopterin, lesser amounts of erythropterin and isoxanthopterin, and no sepiapterin. Only the butterfly family Pieridae is known so far to contain such an array of pterin pigments in their wings. The findings help to strengthen the ties between the Pieridae and Libytheidae that had been suggested on morphological and behavioral grounds. The Libytheidae, a transitional family between the Pieridae and Nymphalidae, probably arose from Pieridae (e.g., *Melete*) in the northern Neotropical realm no earlier than lower Upper Cretaceous times, the earliest age of its *Celtis* host-plant fossils.

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Heliothis subflexa (GN.) (LEPIDOPTERA: NOCTUIDAE): DEMONSTRATION OF OVIPOSITION STIMULANT FROM GROUNDCHERRY USING NOVEL BIOASSAY[1]

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Abstract—Methanol extracts of homogenized groundcherry, *Physalis angulata*, leaves increased egg deposition by *Heliothis subflexa* (Gn.) (HS) on treated tobacco plants (a nonhost) 8.5-fold over untreated controls. In dose-response tests using whole-leaf washes of groundcherry leaves, the threshold of positive response vs. no response to the chemical stimulant was within one log dose unit when compared to the controls. This response was consistent whether the chemical was evaluated on plants in greenhouse-cage tests or in an olfactometer using pieces of broadcloth as the oviposition substrate. The olfactometer used allows year-round study of the behavioral effects of plant allelochemics on insect oviposition behavior in a controlled environment.

Key Words—Groundcherry, *Physalis angulata*, *Heliothis subflexa*, Lepidoptera, Noctuidae, oviposition stimulant.

INTRODUCTION

Heliothis subflexa (Gn.) (HS) is an innocuous noctuid moth that feeds exclusively on groundcherry (*Physalis* spp.) (Brazzel et al., 1953). Although not a pest, it recently has become the subject of numerous hybridization studies between it and the tobacco budworm, *H. virescens* (F.), a broadly sympatric sibling species of great economic importance (Laster, 1972). The basis of these control studies lies in the production of sterile male and fertile female hybrids and back-cross progeny which, when released into a natural *H. virescens* (HV) population, should reduce the numbers of HV via the induction of male sterility.

[1]This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or the recommendation of its use by USDA.

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Although the role of sex pheromones has been documented in *H. subflexa* (Teal et al., 1981; Klun et al., 1982), virtually nothing is known as to how this and, indeed, most phytophagous insect species locate their host plants or the mechanisms (chemical or mechanical) responsible for inducing oviposition once the host is found. The reasons for the lack of fundamental information on such important behaviors can be attributed to many factors, not the least of which is the ever changing biochemistry of plants, especially annuals, as they pass through their life cycle from seed germination and plant emergence to maturity and senescence. Such rapid changes in the biochemistry of the plant have made the development of reliable indoor bioassays for oviposition stimulants especially difficult. Jackson et al. (1984) found that whole-leaf cuticular washes from NC 2326 tobacco leaves will stimulate HV oviposition on TI 1112, a resistant tobacco strain. The major components in the NC 2326 methanol–water soluble fraction of the whole-leaf wash that stimulated HV oviposition were the α- and β-4,8,13-duvatriene-1,3-diols. It is significant that this process of identification and verification of activity had to be carried out over several seasons in small field plots and screen cages in the greenhouse using potted plant material because of the lack of a bioassay system that would permit monitoring of progress in the identification procedure indoors under controlled environmental conditions.

The present study was designed to develop a bioassay system that would be applicable to chemicals affecting oviposition behavior in a variety of insects. We selected HS as the test subject because of its limited host range (groundcherry) (Brazzel et al., 1953) and also because of implications in the host-plant selection process, which might be exhibited as a result of hybridization between HS and its sibling species, HV (Laster, 1972).

METHODS AND MATERIALS

Groundcherry Extracts Using Different Solvents. The response of *H. subflexa* to extracts of groundcherry leaves and fruit (in bracts) was determined by homogenizing 100 g of fresh-weight material in a Waring® blender in 500 ml of distilled water, methanol, ethanol, acetone, or methylene chloride. Large particles were removed by filtering the extract through a white Viva® paper towel. All extracts, except the distilled water, were concentrated to near dryness in a rotary evaporator and brought back to 1-g equivalents per 1 ml of 3:1 acetone–distilled water for spraying onto potted tobacco plants (a nonhost). The test plants were ca. 40 cm in height, and they were stripped to four leaves, each about equal in size.

Each test was conducted in a natural-color Saran® field cage (round) 2.9 m in diameter and 1.2 m high with one vertical zipper. The bottom of the cage also was made of Saran screening. A test consisted of four treated plants placed

alternatively with four control plants (ca. 85 cm between plants) in a circular pattern inside each cage (four replications per night per cage). A compressed-air spray brush (Badger®, model 250) was used to apply the treatments (20 g equivalent test material in 20 ml of 3:1 acetone–distilled water per plant) in a fine mist at ca. 1500 hr. Control plants were sprayed with 20 ml of the solvent only. Test plants were sprayed on both the upper and lower sides of the leaves outside the cage and moved inside after drying. Two paper cups containing a cotton ball saturated with 10% honey–water solution were placed in the cage center along with ca. 50 pairs of 3- to 4-day-old moths, which had been held together in small cages indoors since emergence for mating. All moths were from a stock colony of HS reared in the laboratory on artificial diet. The number of eggs deposited on each plant was recorded the following morning, and the plants then were removed from the cage and destroyed. Additional moths were added as needed to maintain the test population at ca. 50 pairs. Each treatment was replicated 8–24 times, and means were separated using the unpaired t test (Steel and Torrie, 1960).

Evaluation of Extraction Methods. One-hundred grams of fresh ground-cherry leaves were homogenized in methanol in a Waring blender for 30 sec or washed for 10 or 30 sec in 500 ml of methanol. The liquid was filtered through a white Viva paper towel, concentrated to near dryness in a rotary evaporator, and brought back to 20-g equivalents in 20 ml of a 3:1 acetone–distilled water solution.

Potted cotton plants (ca. 40 cm high, a nonhost) were sprayed with 20 ml of the test material using a Badger air brush. The plants were stripped to six leaves about equal in size before spraying. Both upper and lower surfaces were sprayed. Control plants were sprayed with 20 ml of the acetone–distilled water solution only. The treated and control plants were placed in opposite corners of a 100-cm^2 × 70-cm-high screenwire cage with ca. 70 cm between plants. Twenty to 30 pairs of 3- to 4-day-old HS moths reared in the laboratory on artificial diet were released in each cage. Fresh insects were added daily to keep the moth population at the desired level. Each cage was equipped with two paper cups filled with cotton balls saturated with 10% honey–water solution as a food source for the moths.

All tests were carried out in the greenhouse under natural light at ca. 23°C at night, 27°C during the day, and ambient relative humidity of ca. 50–60%. The plants were sprayed at 1600 hr, allowed to dry, and immediately placed in the test cages. The cages then were draped with black polyethylene that covered the top and all four sides to the greenhouse floor. The polyethylene cover prevented influences from street lights in the test area vicinity and also prevented air movement through the cages. Thus, the plants were in an essentially static air environment for the test duration (ca. 16 hr). The number of eggs deposited on the treated and control plants were counted the following morning. The plants

were used only once, and each test was replicated eight to nine times. For statistical analysis, the data were transformed to arcsin $\sqrt{\text{percentage}}$; differences between treatment means were separated using the paired t test (Steel and Torrie, 1960).

Dose Response. The response of HS to different dosages of the oviposition stimulant was conducted under two different sets of conditions: first, in screen-wire cages in the greenhouse as described above for evaluating the leaf-extraction techniques [homogenized vs. whole-leaf wash (WLW)]; and second, in an olfactometer designed specifically for use in a controlled environmental chamber. Dosages evaluated in the greenhouse setting were 0.01-, 0.1-, 1.0-, 5.0-, 20-g equivalents in methanol WLW sprayed onto cotton plants in 20 ml of a 3:1 acetone–distilled water solution. Control plants were sprayed only with the solvent. Methods for handling plants and insects, the environmental conditions in the greenhouse, recording the number of eggs deposited on treatment and control plants, and transformation of the data were as described above. The data then were evaluated (6–13 replicates/dosage level) using regression analysis and the paired t test (Steel and Torrie, 1960).

An olfactometer was developed and tested for bioassaying the response of HS to the oviposition stimulant obtained from WLW of groundcherry (Figure 1). The olfactometer was constructed of 0.64-cm Plexiglas and consisted of a removable top plate, the insect holding chamber, and a base that connected the

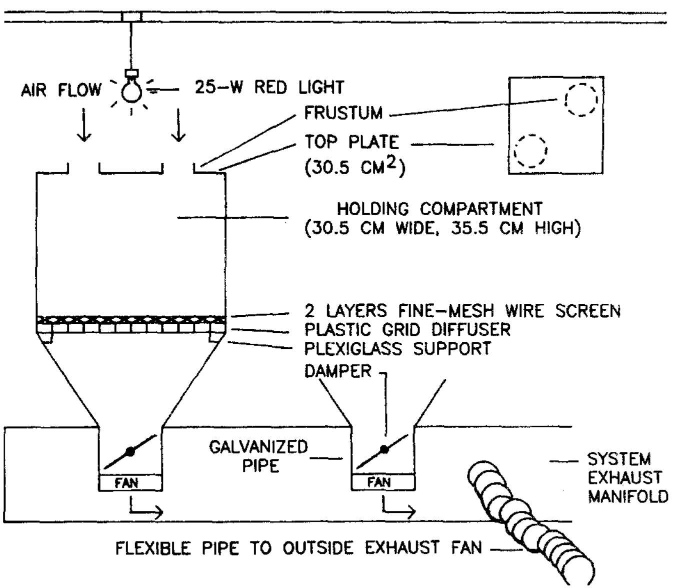

FIG. 1. Schematic of Plexiglas olfactometer (not to scale) for bioassaying oviposition stimulant (see text for dimensions and materials).

holding chamber to an exhaust manifold. The top plate (30.5 cm^2) had two 9.6-cm-diam. holes centered on a diagonal line equidistant from each other and the corners. A frustum (collar, 10.0-cm OD bottom, 9.2-cm OD top, 6.5 cm high) was glued to each hole. The frustums were made by cutting out the bottoms of clear plastic containers (Tri-State Molded Plastics, Inc., round container model T40C), and gluing the top edge of the containers to the Plexiglas top.

The square insect-holding compartment was 30.5 cm wide by 35.5 cm high and had 2.54 × 0.64-cm Plexiglas strips glued to the inside of the base to support a plastic grid cut from fluorescent light diffuser material (1.2 cm high with 1.4-cm^2 openings). The plastic grid was overlaid with two pieces of fine-mesh screen wire. The base of the holding chamber was connected to the exhaust manifold via a trapezoidal-shaped duct (34-cm high), which measured 30.5 cm at the top and narrowed to 20.5 cm at its base. The bottom plate of the duct had a 14.5-cm-diam. hole to which was connected a short length of galvanized stove pipe that was equipped with a damper that exited into the system manifold. The manifold was connected via a flexible plastic pipe to an exhaust fan that pulled air through the chamber from top to bottom and exhausted outside the holding room. An intake vent located in the wall (opposite wall from the exhaust vent) of the holding room allowed a continuous flow of fresh, unfiltered air into the room from the outside. Air flow through the oviposition chamber was adjusted with the damper and maintained at 0.24 m/sec when measured at the top of the frustum. The air exchange rate through the chamber was approximately once every 12 sec.

Eight of the oviposition chambers were fitted side by side on a wooden frame equipped with casters (Figure 2). Each chamber had a 25-W red light (GE®) suspended directly over its center ca. 12 cm from the Plexiglas top to provide a light source during the scotophase period (1100–2100 hr). The sides of the test chambers were covered with black construction paper to eliminate light reflections from the white walls of the test room through the Plexiglas holding chambers and from another bank of cages (there were two banks of eight cages each in the test room).

The bioassay chambers were located in an environmentally controlled room 3 m long, 2.6 m wide, and 2.1 m high. The room was equipped with an electric timer to turn the overhead fluorescent lights (two banks of two bulbs, 40 W each) on and off; a separate timer was used to turn the red lights over the oviposition chambers on and off. A heat pump/air-conditioner installed in one wall controlled the temperature in the room at ca. 28.6°C. A room humidifier maintained the relative humidity at ca. 50%.

Dosages of oviposition stimulant evaluated in the olfactometer were 0.001-, 0.01-, 0.1-, 1.0-, and 10.0-g equivalents of WLW in 1 ml methanol. The test material was dissolved in 1 ml methanol and pipetted in a circular pattern onto the center of a piece of white broadcloth (ca. 20 cm^2), the oviposition substrate. After drying, the broadcloth was fitted over the open end (ca.

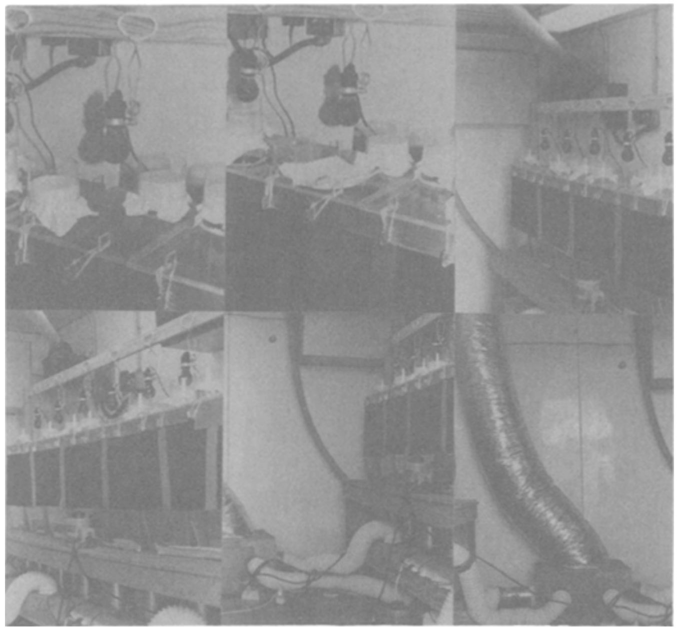

FIG. 2. Olfactometer system for testing oviposition stimulant chemical(s) for *Heliothis subflexa* extracted from groundcherry plants. Top left: individual chamber with oviposition substrates (two pieces of broadcloth) held in place with O-rings; top center: chamber with open (left) and covered port (right); top right: bank of eight bioassay chambers (five shown) with red lights and black construction paper on sides of chambers; bottom left: bank of bioassay chambers (six shown), with fresh air intake (upper left) and two exhaust pipes exiting from manifold (bottom); bottom center: exhaust manifold pipes entering collection chamber (lower left) for exhausting air outside of bioassay room; bottom right: air collection box with attached pipes from manifolds of bioassay chambers (two systems of eight compartments each); large flexible pipe leads to fan mounted outside of room, small flexible pipe (right side) is part of vacuum system used to remove moths from chambers when cleaning.

9.2 cm) of one of the frustums and secured into position with a plastic O-ring. A control cloth treated with 1 ml of methanol was fitted over the second frustum. A cotton ball soaked with 10 ml water was then placed on the top of each cloth and covered with a paper cup.

Each test cage contained 10 laboratory-reared female HS that were confined with eight males for two days for mating before being placed in the test chamber. The females were preconditioned through one complete light–dark cycle in the test chambers before being exposed to the test materials; thus, the females were 3 days old when first subjected to the treatments. The same moths

were subjected to different treatments (i.e., dosage levels) on days 4 and 5 before being replaced with new moths. The moths were supplied daily with fresh food (10% honey-water solution on a saturated cotton ball in a paper cup).

Treatment and control clothes (oviposition substrates) were replaced and repositioned twice daily at 1100 and 1500 hr. Treatments placed in position at 1500 hr were removed during the daylight phase the following morning (lights on at 2100 hr). The number of eggs deposited on each cloth (treatment and controls) was recorded and converted to percentages of the total number of eggs deposited in each chamber during each time period (11–21 replicates/dosage level). The data then were transformed to arcsin $\sqrt{\text{percentage}}$ and subjected to regression analysis and the paired t test (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Homogenate extracts of fresh groundcherry leaves in methanol increased egg deposition by HS on treated tobacco plants (a nonhost) in field cages 8.5 × over untreated controls; other solvent systems that showed significant oviposition stimulant activity from homogenized leaves included distilled water, ethanol, and methylene chloride (Table 1). By contrast, the highest levels of HS oviposition obtained from homogenates of groundcherry fruit was with dis-

TABLE 1. EFFECTS OF GROUNDCHERRY HOMOGENATE EXTRACTS (20 g EQUIVALENTS/PLANT) ON OVIPOSITION BY *Heliothis subflexa* ON TOBACCO (A NONHOST)

Solvent	Total eggs	Replicates	t value[a]	Increased oviposition[b]
Leaf extract				
Distilled water	542	8	7.34**	4.1X
Methanol	601	8	7.04**	8.5X
Ethanol	958	8	5.24**	2.4X
Acetone	482	12	1.98	0
Methylene chloride	500	8	3.15**	2.7X
Fruit extracts				
Distilled water	799	8	5.14**	4.1X
Methanol	979	8	2.16*	0.6X
Ethanol	2742	24	1.14	0
Acetone	177	12	0.59	0
Methylene chloride	1816	16	0.12	0

[a] ** Significant at $P < 0.01$; * significant at <0.05; absence of asterisk indicates means were not statistically different, unpaired t test.

[b] Increased oviposition = (eggs on treatment − eggs on control)/eggs on control.

tilled water (4.1×) and methanol (0.6×). None of the other solvent systems used to extract groundcherry fruit showed any significant increase in oviposition activity on treated plants vs. the controls.

Homogenate extracts of fresh groundcherry leaves were quite effective in stimulating HS oviposition (Table 1). However, a 30-sec WLW of fresh groundcherry leaves in methanol produced increased oviposition activity comparable to the homogenate leaf extract (Table 2). Although washing groundcherry leaves for 10 sec produced somewhat less activity than washing the leaves for 30 sec, the difference in increased oviposition activity was not significant (unpaired t test, 16 df, t = 1.1193). Nevertheless, the 30-sec WLW procedure was used routinely throughout the remainder of the tests and for preparation of materials used in the isolation and identification of chemical(s) (now in progress) responsible for the observed increases in oviposition. The WLW extract was virtually free of any foreign material except sand particles from the leaf-surface, which subsequently were removed via filtration through paper towels. We have stored the crude WLW extract (methanol) for more than 1 year in a freezer at 0°C without significant loss in oviposition stimulant activity.

In the dose–response tests (Figure 3), there was no significant relationship (regression analysis) between dose and the percentage of eggs deposited on treated cotton plants in cages or on broadcloth oviposition sheets in the olfactometer. In both tests, however, a significantly higher percentage of the total eggs deposited were recorded on treated plants or broadcloth vs. corresponding controls except at the lowest dosage levels (paired t test). The overall percentage of increase in oviposition was somewhat higher on treated cotton plants in the greenhouse test than in the olfactometer, although HS females typically

TABLE 2. EFFECTS OF DIFFERENT METHODS OF EXTRACTING OVIPOSITION STIMULANT FOR *Heliothis subflexa* FROM GROUNDCHERRY LEAVES

Extraction method[a]	Replicates	Total eggs	Increased oviposition on treated plants[b]	t value[c]
Leaves				
Homogenized	8	1691	15.9X	15.71**
Leaves washed				
10 sec	9	875	8.0X	12.79**
30 sec	9	1795	15.1X	21.27**

[a] Fresh leaves (100 g) were homogenized in methanol using a Waring blender for 30 sec or washed for 10 or 30 sec in 500 ml methanol. The liquid was filtered through a white Viva paper towel, concentrated to near dryness and then brought back to 20-g equivalents in 20 ml of a 3 : 1 acetone–distilled water solution that way sprayed on the test plant (cotton, a nonhost), with a compressed air sprayer. The controls were sprayed with 20 ml of the acetone–distilled water solution.
[b] Increased oviposition = (eggs on treatment − eggs on control)/eggs on control.
[c] **Significant at $P < 0.01$, paired t test.

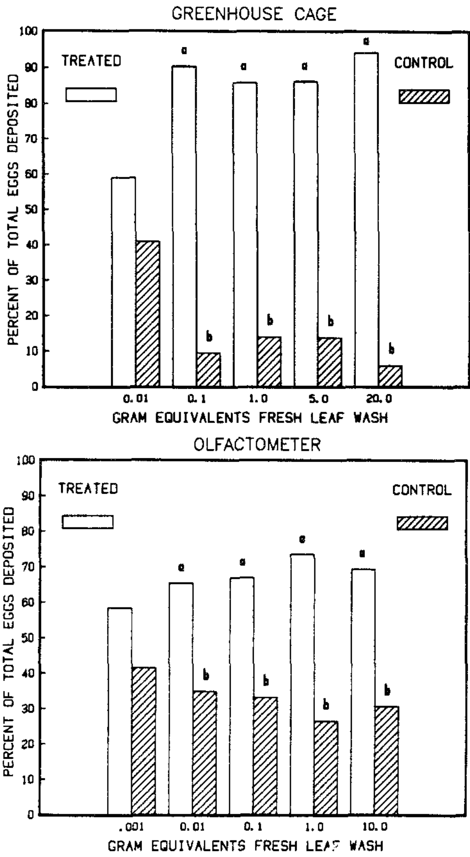

FIG. 3. Response of *Heliothis subflexa* females to different dosages of ovipositional stimulant extracted via whole-leaf washes in methanol in two different bioassay systems (greenhouse cage, top; olfactometer, bottom). Means within each dosage level followed by different letters are significantly different ($P < 0.01$), paired t test; absence of letters indicates no significant difference between means.

responded to a lower threshold dosage in the olfactometer (0.01-g equivalents) than in the greenhouse cage (0.1-g equivalent). Reasons for the difference in the two bioassay systems are unknown. Air movement, or the lack of it, possibly could explain some of the variation observed. The greenhouse tests were conducted in virtually static air (cages were covered with polyethylene), whereas the air in each chamber of the olfactometer was exchanged ca. every 12 sec.

The olfactometer described here offers the opportunity to study the behavioral effects of plant allelochemics on insect oviposition behavior in a controlled environment without the need for a constant supply of live plant material as oviposition substrate. Such an apparatus should speed the identification of these

chemicals, as it permits virtually year-round operation and is not dependent upon live plant materials, which are so costly to provide in time and funds.

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INHIBITION OF MALE RESPONSE OF DRUGSTORE BEETLES TO STEGOBINONE BY ITS ISOMER

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Abstract—Stegobinone, (2*S*,3*R*,1′*R*)-2,3,-dihydro-2,3,5-trimethyl-6-(1′-methyl-2′-oxobutyl)-4H-pyran-4-one, the sex pheromone of drugstore beetles (*Stegobium paniceum* L.), elicited the pheromonal response from the males of the species in our bioassay system; however, the synthesized diastereomeric mixture of this compound was actually inactive to the males. Although the stegobinone isolated from the beetles of this species had significant activity, its enantiomeric [(±)-2*S*,3*R*,1′*S*-] and diatereomeric [(±)-2*S*,3*S*,1′*RS*-] isomers were inactive. Adding the (±)-2*S*,3*R*,1′*S* isomer to stegobinone significantly reduced the male response. Furthermore, the activity of stegobinone vanished on keeping it at room temperature for two weeks. In such a stored stegobinone sample, the presence of 2*S*,3*R*,1′*S* isomer, the inhibitory component, was confirmed. This isomer might be produced by C-1′ epimerization during storage.

Key Words—Sex pheromone, drugstore beetle, Coleoptera, Anobiidae, *Stegobium paniceum* L., stegobinone, diastereoisomer inhibition, pheromonal activity.

INTRODUCTION

The drugstore beetle, *Stegobium paniceum* L., is a devastating pest of stored food and crops. The structure of its sex pheromone has been established as stegobinone, 2,3-dihydro-2,3,5-trimethyl-6-(1′-methyl-2′-oxobutyl)-4H-pyran-4-one (Kuwahara et al., 1975, 1978), whose absolute configuration at C-2, C-3, and C-1′ has been assigned 2*S*,3*R*,1′*R* on the basis of several stereoselective

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(2S,3R,1'R) (2S,3R,1'S) (2S,3S,1'R) (2S,3S,1'S)

(2R,3S,1'S) (2R,3S,1'R) (2R,3R,1'S) (2R,3R,1'R)

FIG. 1. Eight possible structures of stegobinone and diastereoisomers.

syntheses and its CD spectrum (Hoffmann et al., 1981). The amount of stegobinone was estimated at about 10^{-1} μg/female (Kuwahara et al., 1975). As stegobinone has three chiral centers at C-2, C-3, and C-1′, there are eight possible stereoisomers shown in Figure 1, in which the isomer in the upper row, such as (2*S*,3*R*,1′*R*) and the corresponding ones in the lower row, such as (2*R*,3*S*,1′*S*) are enantiomerically paired. Many synthetic approaches have been reported. Recently, Mori and Ebata (1986) accomplished the stereoselective synthesis of this compound. In early work, Ansell et al. (1979) synthesized two diastereomeric racemates, (±)-(2*S*,3*R*,1′*RS*)-stegobinone. The pheromonal activity of this diastereomeric mixture was at least 10^3 lower than that of the natural pheromone. This result suggested, among other possibilities, that one or more of the stereoisomers was inhibiting the response of male beetles to the active component.

Here, we report on an inhibition by one of the stegobinone isomers to the pheromonal activity of stegobinone.

METHODS AND MATERIALS

The insects were reared on a mouse diet at 28 ± 1°C and 60 ± 10% relative humidity. Males for bioassay were collected from cultures as papae and selected by observance of the posterior end of pupae (Azab, 1954). Selected male pupae were transferred to a separate room from that used for culturing and kept in a female-free incubator under the above conditions until bioassay, two to three days after adult emergence.

Instruments. Gas chromatography (GC) was performed using a Shimadzu 7A instrument equipped with FI detector and a 50-m × 0.2-mm-ID fused silica column coated with Carbowax 20 M. The column temperature was programmed

from 100 to 210°C at the rate of 2°C/min. Proton NMR spectra were obtained with a Bruker AM 500. GC mass spectra (GC-MS) were obtained on a Hitachi M 80 instrument equipped with the same column as above. High-performance liquid chromatography (HPLC) was performed with a Hitachi 655 constant flow pump, equipped with UVILOG-5A (Oyo-bunko kiki Co., Ltd., Tokyo) UV detector.

Stegobinone. Adult (150,000) drugstore beetles of mixed sex were extracted twice with hexane (1 liter × 2). After removing the solvent under reduced pressure at 40°C, 2.7 g of an oily residue was obtained. The extract was then charged on a silica gel column (3 cm ID × 27 cm) and fractionated with hexane–ether. Stegobinone (10 mg) was isolated from 30% ether–hexane effluent by HPLC (μ-Porsil, hexane–THF–MeOH, 6000:100:1). Although the obtained stegobinone (99.9% pure by G C) did not crystallize, all spectral data were identical with those reported previously (Kuwahare et al., 1978).

Diasstereoisomers. Diastereoisomers of stegobinone were synthesized by the method of Ono et al. (1983). Materials obtained consisted of eight diastereoisomers whose gas chromatogram is shown in Figure 2. The molar proportions of *SRR*, *SRS*, and *SSRS* were 3:4:4. The racemic mixtures and diastereoisomers used are designated herein as *SSR*, *SRS*, and *SSRS* which indicate enantiomeric mixtures of 2*S*,3*R*,1′*R* and 2*R*,3*S*,1′*S* isomers, 2*S*,3*R*,1′*S* and 2*R*,3*S*,1′*R* isomers, and 2*S*,3*S*,1′*RS* and 2*R*,3*R*,1′*RS* isomers, respectively.

SRR, SRS, *and* SSRS. Synthetic mixture of eight diastereoisomers of stegobinone, obtained by the method of Ono (1983), were separated by HPLC into three fractions (Figure 3). The first isomer eluted was *SSRS*, the second *SRS*, and the last *SRR*, whose relative configurations were established by comparing ^{1}H- and ^{13}CNMR spectra to those previously reported (Hoffmann et al., 1981). The purity of each group, determined by GC, was more than 99.9%.

Bioassay. The bioassay procedure utilized for another species of Anobiidae, the tobacco beetle, *Lasioderma serricorne* F., (Mori et al., 1985) was employed with several modifications. Large-size (90-mm-ID) plastic Petri dishes were used to avoid saturation with the test samples during the bioassay. A 90-mm-ID filter paper was tightly fitted on the floor of the Pertri dish. Ten virgin males were allowed to acclimate in each dish 3–4 hr prior to testing, and those individuals came to rest during that period. One microliter of hexane solution of test samples were dispensed onto a filter paper (5 × 25 mm, folded in W letter shape) by using disposable 5 μl pipets. After allowing the solvent to evaporate for ca. 10 sec, the sample-impregnated filter paper was immediately placed at the center of the Petri dish.

In the bioassay, the following three parameters were scored for 10 min: (1) attractiveness, total number of the males attracted on the sample-impregnated filter paper; (2) sex stimulation, total number of attempted couplings; (3) aggregation, maximum number of males that climbed on the sample-impregnated filter paper at the same time.

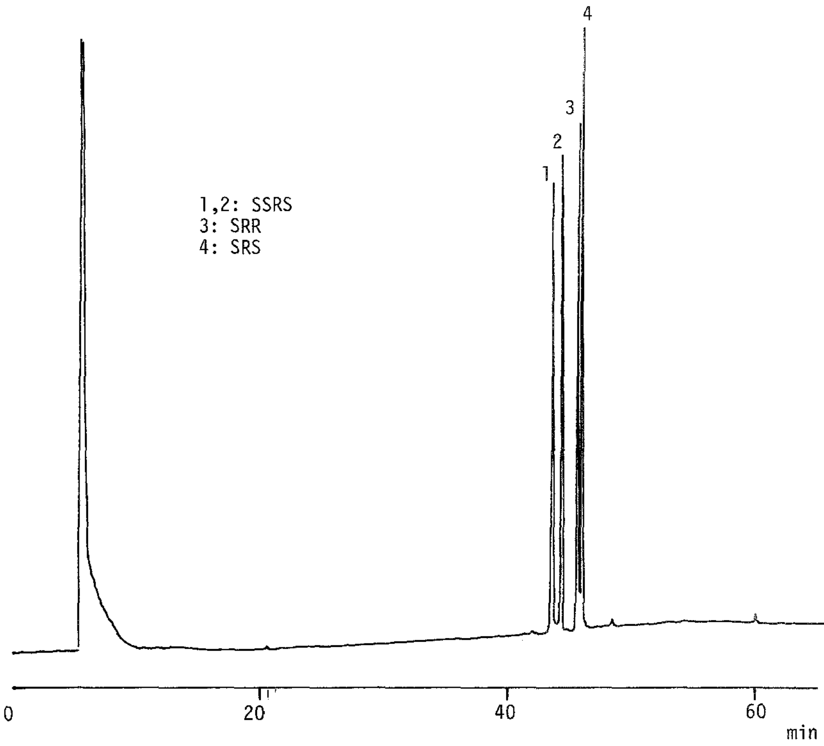

FIG. 2. Gas chromatogram of diasstereoisomers of stegobinone. conditions: column, 50 m × 0.2 mm ID fused silica coated with Carbowax 20 M; column temperature, 100–210°C (2°C/min); carrier gas, nitrogen at a flow rate of 1.1 ml/min; flame ionization detector.

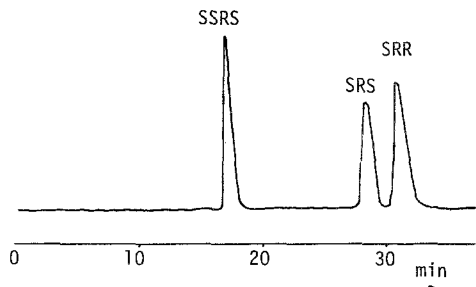

FIG. 3. HPLC of diastereomers of stegobinone. conditions: column, μ-Porasil (3.9 mm ID × 30 cm); mobile phase, hexane–THF–MeOH 6000 : 100 : 1; flow rate, 2.5 ml/min; detected by UV absorption at 281 nm.

A minimum of three and a maximum of nine replicates were run. Each male was used only once for the bioassay. The test room for the bioassay was illuminated by cool white fluorescent lighting. All bioassays were conducted at $28 \pm 1°C$ and $60 \pm 10\%$ relative humidity.

RESULTS AND DISCUSSION

Behavioral Aspects of Male Response to Stegobinone in Bioassay. Previous reports showed that stegobinone elicited precopulatory searching behavior. In our bioassay, not only searching but also the following behavior could be observed. The first response to stegobinone in the bioassay consisted of alerting followed by rapid movement around the stegobinone-impregnated filter paper with occasional arrest, during which antennal elevation and extension of the forelegs were observed. After this searching behavior, the males were attracted to the stegobinone-impregnated filter paper and climbed on it. When a male palpated another male, he mounted the other and extruded his aedeagus.

Male Response to Synthetic Stegobinone and Diastereoisomers. Responses of male drugstore beetles to stegobinone and diastereoisomers are shown in Table 1. In the replicated bioassay at 10^5–10 μg of tested sample, males showed a threshold (minimum dose per assay to elicit a pheromonal response) at 10^{-2} μg of stegobinone. Male beetles showed no response to the diastereoisomers. The most likely explanation for the lack of activity of the diastereoisomers seemed to be inhibition of the activity by one or more of the possible isomers in the diastereoisomers.

Male Response to SRR, SRS, *and* SSRS. As the diastereoisomers failed to elicit the pheromonal response from the males, activities of chromatographically separated *SRR*, *SRS*, and *SSRS* were examined. The results were summarized in Table 2. In the bioassay, 10^{-2} μg of *SRR* induced a positive response to the males, whereas *SRS* and *SSRS* failed to elicit the response from the males. The pheromonal activity of *SRR* suggested that its component enantiomer, *1R,2S,1'S* isomer, was not an inhibitor. Although the diastereomer separated by preparative TLC from synthetic stegobinone has been reported to have some pheromonal activity (Ansell et al., 1979), behavioral activity of *SRS*, or *SSRS* could not be observed.

Inhibition by SRS. Since *SRS* and *SSRS* were inactive, the effect of these isomers to the stegobinone was examined independently. Stegobinone was mixed individually with these isomers in 1000 : 1, 100 : 1, 10 : 1, and 1 : 1 ratios in which the amount of stegobinone was held constant at 0.1 μg. The results are summarized in Table 3. No apparent effect was observed for the mixture of *SSRS* and stegobinone, but *SRS* showed significant reduction of male response. As little as 10^{-3} μg of *SRS* was required to reduce the activity with every three

TABLE 1. MALE RESPONSE TO STEGOBINONE AND ITS DIASTEREOISOMERS

Concentration	Attractiveness (mean ± SD[a])		Sex Stimulation (mean ± SD)		Aggregation (mean ± SD)	
(μg)	Stegobinone	Diastereoisomers	Stegobinone	Diastereoisomers	Stegobinone	Diastereoisomers
10	23.0 ± 2.7	2.40 ± 3.36	6.67 ± 3.06	1.60 ± 1.14	2.00 ± 0	0.60 ± 0.89
1	39.5 ± 10.8	3.60 ± 4.83	10.75 ± 2.87	1.80 ± 0.84	4.25 ± 1.26	0.80 ± 0.45
10^{-1}	46.0 ± 16.3	0 ± 0	5.00 ± 3.16	0.80 ± 0.84	4.25 ± 1.26	0 ± 0
10^{-2}	18.0 ± 5.3	0.60 ± 0.55	3.33 ± 2.89	2.20 ± 1.48	2.00 ± 0	0.60 ± 0.55
10^{-3}	8.7 ± 4.6	0.80 ± 0.84	0.67 ± 1.15	0.60 ± 0.89	1.33 ± 0.58	0.80 ± 0.84
10^{-5}	2.7 ± 3.8	2.80 ± 2.05	0 ± 0	0.80 ± 0.84	1.00 ± 1.41	1.60 ± 0.89

[a] Standard deviation.

TABLE 2. MALE RESPONSE TO SRR, SRS, AND SSRS

Concentration (μg)	Attractiveness (mean ± SD)			Sex stimulation (mean ± SD)			Aggregation (mean ± SD)		
	SRR	SRS	SSRS	SRR	SRS	SSRS	SRR	SRS	SSRS
10	[a]	[a]	2.8 ± 3.8	[a]	[a]	2.2 ± 1.9	[a]	[a]	0.6 ± 0.9[b]
1	28.6 ± 11.2	18.9 ± 18.2	3.0 ± 4.1[b]	4.0 ± 2.5[b]	2.7 ± 2.8[b]	1.8 ± 1.3[b]	2.2 ± 0.8[b]	1.6 ± 1.4[b]	0.6 ± 0.9[b]
10^{-1}	32.9 ± 14.5	1.0 ± 1.3[b]	1.0 ± 1.2[b]	2.0 ± 1.7	0.7 ± 1.1[b]	1.6 ± 1.8	1.7 ± 0.8[b]	0.4 ± 0.5[b]	0.8 ± 0.8[b]
10^{-2}	27.7 ± 9.9	1.7 ± 2.3[b]	3.2 ± 3.1[b]	3.0 ± 3.0	1.8 ± 2.8	1.4 ± 1.3	2.0 ± 0	0.5 ± 0.6	1.0 ± 0.7
10^{-3}	14.4 ± 5.6	2.4 ± 5.9	1.8 ± 2.5[b]	1.2 ± 1.3	1.8 ± 1.8	1.0 ± 1.7	1.4 ± 0.6	0.4 ± 0.5	0.6 ± 0.6
10^{-5}	3.3 ± 3.2	0.3 ± 0.7	0.3 ± 0.5	2.7 ± 1.5[b]	1.1 ± 1.0	0.8 ± 1.0	1.3 ± 0.6	0.1 ± 0.4	0.3 ± 0.5

[a] Not tested.
[b] Differs significantly from natural stegobinone at the 5% level, Student's t test.

TABLE 3. EFFECT OF ENANTIOMERIC MIXTURE ADDITION ON PHEROMONAL ACTIVITY OF STEGOBINONE

Additive amount of isomer to 0.1 μg stegobinone (μg)	Attractiveness (mean ± SD)			Sex stimulation (mean ± SD)			Aggregation (mean ± SD)		
	SSRS	SRS	Reduction (%)[a]	SSRS	SRS	Reduction (%)[a]	SSRS	SRS	Reduction (%)[a]
0	46.0 ± 16.8	46.0 ± 16.8		5.00 ± 3.16	5.00 ± 3.16		4.25 ± 1.26	4.25 ± 1.26	
10^{-4}	[b]	38.7 ± 10.9	15.9	[b]	2.33 ± 1.75	53.4	[b]	1.67 ± 0.82[c]	60.7
10^{-3}	39.5 ± 13.4	16.6 ± 9.6[c]	63.9	5.75 ± 4.11	1.80 ± 1.84[c]	64.0	5.75 ± 4.11	1.68 ± 0.89[c]	60.5
10^{-2}	54.3 ± 18.1	3.7 ± 5.3[c]	92.0	7.50 ± 3.32	0.11 ± 0.33[c]	97.8	4.25 ± 1.71	0.78 ± 0.97[c]	99.3
10^{-1}	46.0 ± 8.9	0 ± 0[c]	100.0	11.6 ± 7.67	0 ± 0[c]	100.0	5.17 ± 1.47	0 ± 0[c]	100.0

[a] Percent reduction = [(each mean No. stegobinone − each No. SRS)/each mean No. stegobinone] × 100.
[b] Not tested.
[c] Differs significantly from control at 5% level, Student's t test.

TABLE 4. MALE RESPONSE TO EPIMERIZED STEGOBINONE

Concentration (μg)	Attractiveness (mean ± SD)		Sex stimulation (mean ± SD)		Aggregation (mean ± SD)	
	Natural	Epimerized	Natural	Epimerized	Natural	Epimerized
10	23.0 ± 2.7	4.3 ± 6.6[a]	6.67 ± 3.06	1.22 ± 1.56[a]	2.00 ± 0	0.67 ± 0.71[a]
1	39.5 ± 10.8	5.9 ± 5.3[a]	10.75 ± 2.87	1.45 ± 1.75[a]	4.25 ± 1.26	0.82 ± 0.67[a]
10^{-1}	46.0 ± 16.8	5.3 ± 4.7[a]	5.00 ± 3.16	5.00 ± 0.36[a]	4.25 ± 1.26	1.00 ± 0[a]
10^{-2}	18.0 ± 5.3	10.9 ± 9.6	3.33 ± 2.89	3.33 ± 0.82[a]	2.00 ± 0	1.64 ± 0.67
10^{-3}	8.7 ± 4.6	13.8 ± 4.1	0.67 ± 1.15	0.67 ± 0.71	1.33 ± 0.58	2.00 ± 0.71
10^{-5}	2.7 ± 3.8	6.0 ± 3.0	0 ± 0	0.75 ± 0.89	1.00 ± 1.41	1.63 ± 1.41

[a] Differs significantly from natural at the 5% level, Student's t test.

parameters. Considerably less than an equal amount of *SRS* entirely eliminated the pheromonal activity. This mixture did not induce rapid movement or antennal elevation on the males tested.

Inhibition by Epimerized Stegobinone. After stegobinone was kept for two weeks at room temperature, its activity was entirely lost. GC and GC-MS analysis showed the 10% presence of a stegobinone isomer, whose retention time on GC was identical with that of *SRS*. The amount of the isomer is 10% of the stegobinone. Samples of 10^{-5}, 10^{-3}, 10^{-2}, 10^{-1}, 1 and 10 μg of this partially epimerized stegobinone were bioassayed. The results are summarized in Table 4. Athough the amount of partially epimerized stegobinone was increased, behavioral activity could not be observed in 10–10^{-1} μg, and little activity could be observed in the assay of 10^{-2}–10^{-3} μg. The inhibitory activity of the isomer, whose amount was 10% of the stegobinone, might be below threshold in these bioassays. In the stegobinone molecule, the C-1′ position which is the α position to the C-2′ carbonyl group is easily epimerized. Therefore, the absolute configuration of this inhibitor was assumed to be 2*S*,3*R*,1′*S*.

The mating behavior of male drugstore beetles has been reported in detail (Ward, 1981). In our bioassay, it is shown that stegobinone significantly contributed to mating behavior, eliciting not only searching but also copulating. Several pheromones that contain asymmetric carbons have been identified, and in most cases only one enantiomer elicits a pheromonal response; all the other isomers were inactive. Furthermore, a certain enantiomer or isomer inhibited pheromonal activity in several cases; Tumlinson et al. (1977) reported that the attraction of male Japanese beetles (*Popillia japonica*) by the pheromone, (*R*,*Z*)-5-(1-decenyl)-dihydro-2(3H)-furanone, was inhibited by its enantiomer. Recently Mori et al. (1985) reported that the serricornin isomer inhibited the male response of cigarette beetles. In most cases, such isomers do not occur in nature, whereas the epimerization of stegobinone to its inhibitor took place naturally.

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STEGOBIOL, A NEW SEX PHEROMONE COMPONENT OF DRUGSTORE BEETLE (*Stegobium paniceum* L.)

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Abstract—New sex pheromone component of female *Stegobium paniceum* L. was isolated and identified as 2,3-dihydro-2,3,5-trimethyl-6-(1′-methyl-2′-hydroxybutyl)-4H-pyran-4-one, stegobiol, by spectral data and chemical conversion from stegobinone. Relative configuration at C-2, C-3, and C-1′ was determined as 2*S*,3*R*,1′*S* by the conversion from (2*S*,3*R*,1′*R*)-stegobinone. This new sex pheromone elicits the pheromonal response from the species.

Key Words—Sex pheromone, drugstore beetle, *Stegobium paniceum* L., Coleoptera, Anobiidae, stegobiol.

INTRODUCTION

The drugstore beetle, *Stegobium paniceum* L., is a devastating pest of stored grain. The structure of the sex pheromone has been established as 2,3-dihydro-2,3,5-trimethyl-6-(1′-methyl-2′-oxobutyl)-4H-pyran-4-one, **2** in Fig. 1, stegobinone (Kuwahara et al., 1975, 1978), and subsequent, study determined the absolute configuration at C-2, C-3, and C-1′ to be 2*S*,3*R*,1′*R* (Hoffmann et al., 1981). Stegobinone elicits precopulatory searching behavior from the males of the species (Kuwahara et al., 1975). Recently, we reported that stegobinone elicited not only searching but also mating behavior, and its pheromonal activity was inhibited by its 1′-epimer (Kodama et al., 1987). In the course of our study, we confirmed the presence of another sex pheromone component which we named stegobiol according to its structural similarity to stegobinone.

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Here, we report the isolation and structural determination of this new sex pheromone.

METHODS AND MATERIALS

The insects were reared on mouse diet at 28 ± 1°C and 60 ± 10% relative humidity. Male pupae were removed from cultures and isolated from females by observance of the characteristics described by Azab (1954). Selected male pupae were transferred into a separate room from that used for culturing and placed in a female-free incubator at 28 ± 1°C and 60 ± 10% relative humidity. The isolated insects were kept in the incubator until tested when 8–10 days old.

Instrumentation. Analytical gas chromatography (analytical GC) was performed using a Shimadzu 7A equipped with FI detector and a 50-m × 0.2-mm-ID fused silica column coated with Carbowax 20 M. The column temperature was programmed at 2°C/min from 100 to 210°C. High-performance liquid chromatography (HPLC) was performed with a Hitachi 655 constant flow pump, and for monitoring the column's effluent a UVILOG-5A UV detector (Oyo-Bunko Kiki Co., Ltd., Tokyo) was used. Mass and IR spectra were measured on a Hitachi M-80 and Nicolet 60SX instruments, respectively. Proton and ^{13}C NMR spectra were obtained with a Bruker AM 500 NMR spectrometers. The chemical shifts are expressed as ppm downfield from Me_4Si as an internal standard. Two-dimensional NMR experiments were performed on a Bruker AM 500 NMR spectrometer using available 2D software. The data size of the time domain of the correlated spectrum (COSY) was a 512 × 2048 matrix. To improve the spectral resolution, these data were multiplied in both directions with a sine bell function. Fourier transformation was performed with zero filling in *t*1 direction. Heteronuclear-shift-correlated 2D NMR was processed in a similar way. The data size of the time domains was 256 × 2K.

Isolation of Pheromone. Drugstore beetles of both sexes (150,000 adults) were extracted twice with hexane (1 liter, each). After removing the solvent

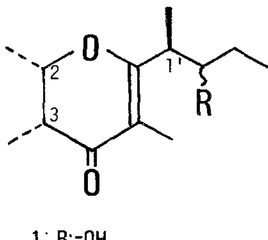

FIG. 1. The structure of the pheromones (**1**: stegobiol, **2**: stegobinone).

under reduced pressure at 40°C, 2.7 g of oily residue was obtained. The extracts were then charged on a silica gel column (3 cm ID × 27 cm) and fractionated with hexane, 10, 20, 30, 50, and 100% ether–hexane (250 ml each). Two substances eluted by 30% ether–hexane and ether elicited the pheromonal response from the males. Subsequent fractionation of the former by HPLC (μ-Porasil, 3.9 mm ID × 30 cm; mobile phase, hexane–THF–MeOH 6000:100:1; flow rate, 2.5 ml/min; detected by UV absorption at 281 nm) resulted in the isolation of stegobinone (10 mg). Although stegobinone obtained did not crystallize, all spectral data were identical with those reported previously (Kuwahara et al., 1978). The latter was successively fractionated by HPLC as shown in Figure 2. Active component (9.7 mg) was obtained from peak A and identified as 2,3-dihydro-2,3,5-trimethyl-6-(1′-methyl-2′-hydroxybutyl)-4H-pyran-4-one (stegobiol) **1**. $[\alpha]_D^{23} = -98.3°$ (c = 0.06, $CHCl_3$). HR-MS $C_{13}H_{22}O_3$ [found 226.1535, calc. 226.1567]. EI-MS (70 eV) m/z (%): 226(8, M^+), 168(88), 141(20), 139(17), 124(15), 113(70), 112(100), 109(27), 101(17), 85(17), 84(15), 83(87), 70(16), 59(49), 57(33), 56(37), 55(45), 43(41). IR ν_{max}: 1597 and 1652 (C=C—C=O), 3427 (OH) cm^{-1}. Proton NMR spectra are described in Table 1. ^{13}CNMR (125 MHz, $CDCl_3$): δ 197.1(C-4), 172.2(C-6), 109.5(C-5), 76.6((C-2), 75.5(C-2′), 43.8(C-3), 40.9(C-1′), 28.3(C-3′), 15.9(C-7), 14.8(C-10), 10.1(C-4), 9.4(C-8), 9.3(C-9).

Bioassay. Bioassay was performed by the procedure described before (Kodama et al., 1987).

Reduction of Stegobinone. A racemic mixture of stegobinone (22.6 g, 0.1 mol) synthesized by the method of Ono et al., (1983) was dissolved in ether (100 ml). Sodium borohydride ($NaBH_4$, 3.8 g, 0.1 mol) and zinc chloride

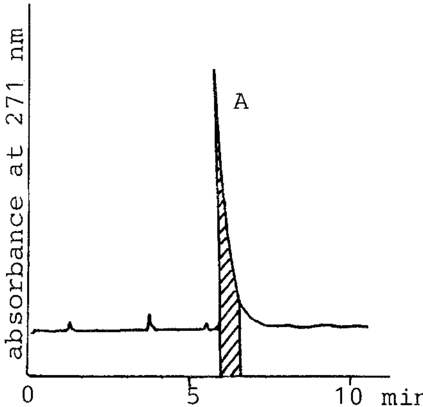

FIG. 2. High-performance liquid chromatogram of 100% ether fraction. Column, μ-Porasil (3.9 mm ID × 30 cm); mobile phase, 85% hexane–ether; flow rate, 3.0 ml/min; detected by UV absorption at 271 nm.

TABLE 1. [^{1}H] NMR ASSIGNMENTS FOR STEGOBIOL (500 MHz)

	Chemical shifts (ppm)	Coupling constants (Hz)
H-2	4.49 (dq)	$J_{2,3} = 3.4$, $J_{2,7} = 6.6$
H-3	2.38 (dq)	$J_{3,8} = 7.3$
Me-7	1.33 (d)	
Me-8	1.04 (d)	
Me-9	1.75 (s)	
Me-10	1.18 (d)	$J_{10,1'} = 7.1$
H-1′	2.86 (dq)	$J_{1',2'} = 6.8$
H-2′	3.58 (ddd)	$J_{2',3'\alpha} = 8.5$ and $J_{2',3'\beta} = 3.8$
H-3′α	1.42 (m)	
H-3′β	1.60 (ddq)	$J_{3'\alpha,\beta} = 14.0$, $J_{3'\beta,4'} = 7.4$
Me-4′	1.00 (t)	

($ZnCl_2$, 10.1 g, 0.1 mol) were added to this solution and subsequently stirred for 3 hr at room temperature. This suspended solution was then filtered and evaporated to give a diastereoisomeric mixture of stegobiol **1** (22 g). The starting materials, a racemic mixture of stegobinone, could not be detected in these products by GC and GC-MS. The diastereoisomeric mixture (5 g) was then charged on a silica gel column (4 cm ID × 30 cm) and fractionated with hexane, 10, 20, 30, 50, and 100% ether–hexane (500 ml each).

RESULTS

*Spectroscopic Analysis of **1**.* The most striking feature of the EI mass spectrum (EI-MS) of **1** is quite similar to that of stegobinone except for the M^+ peak at *m/z* 226. On the basis of an analysis of the high-resolution mass spectrum (HR-MS), the molecular formula of **1** was assigned as $C_{13}H_{22}O_3$. The IR spectrum showed the absorption of enone and hydroxy group. These data suggested the structure of **1** to be C-2′ hydroxy derivative of stegobinone. The assignment of the signals in ^{1}H- and ^{13}CNMR spectra gave the precise structure of **1**. The proton NMR spectrum of **1** at 500 MHz (Figure 3A) showed the clear separation of the resonance due to each proton. The lowest frequency doublet of quartet at δ 4.90 could be assigned to the proton H-2 which was bonded to oxygen atom. The position of the resonances due to H-3, Me-7, and Me-8 are clearly revealed by the contour plot (Figure 3B) from COSY experiment. Off-diagonal peaks correlate the H-2 resonance with the H-3 and Me-7 resonances which appear as a doublet of quartets at δ 2.38 and a doublet at δ 1.33 in Figure 3A, respectively, and H-3 resonance with Me-8 resonance, which

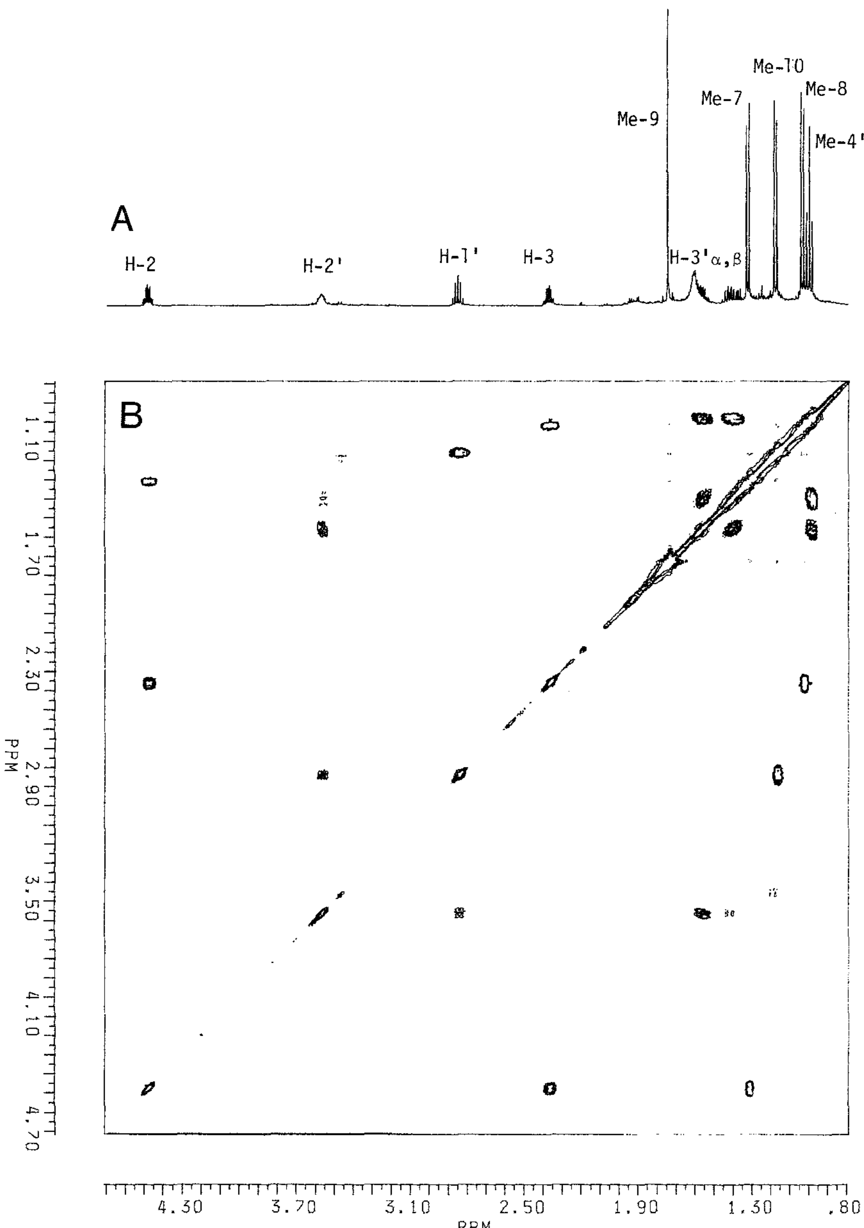

FIG. 3. (A) 500-MHz [¹H]NMR spectrum of **1** (in $CDCl_3$); (B) Two-dimensional [¹H]COSY NMR spectrum of **1**.

appears as doublet at δ 1.04 in Figure 3A. Also the position of the resonances due to Me-10, H-1′–H-3′α and β, and Me-4′ are clearly revealed. The highest frequency triplet at δ 1.00 can be assigned to Me-4′. Off-diagonal peaks correlated this resonance with the H-3′α and 3′β which appear as multiplet and doublet of doublet of quartet at δ 1.42 and δ 1.60, respectively. These resonances were correlated with the H-2′ resonance, which appears as a doublet of doublets at δ 3.58 in Figure 3A. An off-diagonal peak correlates the H-2′ resonance with H-1′ resonance which appears as a doublet of quartets at δ 2.86 and correlates with the Me-10 resonance appearing as a doublet at δ 1.18.

The carbon signals of **1** were assigned as follows. Resonances due to CH, CH_2, or CH_3 groups were identified from the spectra obtained using the DEPT pulse sequence. The assignment of resonances corresponding to carbon atoms was made by a heteronuclear correlation experiment. The assignments of the carbon signals, except for C-4, 5, and 6, were made by direct correlation with the resonances of the corresponding proton signals previously assigned. These spectra confirm the structure of **1** to be 2,3-dihydro-2,3,5-trimethyl-6-(1′-methyl-2′-hydroxybutyl)-4H-pyran-4-one.

Elucidation of Structure 1 by Chemical Conversion. Chemical reduction by the synthesized stegobinone afforded a diastereoisomeric mixture of **1**. Eight possible racemates in the diastereoisomeric mixture showed the corresponding eight peaks as shown in Figure 4. The mixture was further separated by silica-gel column chromatography as described above. The isomer eluted by 100% ether showed a single peak on the analytical gas chromatogram, which corresponded to peak 5 in Figure 4. Proton-, ^{13}C NMR and EI mass spectra of the isomer were identical with those of **1**.

Relative Configuration of 1. A resolution-enhanced one-dimensional proton-NMR spectrum showed the interproton coupling between H-2 and H-3. The $J_{2,3}$ (3.4Hz) value indicated the relative configuration between Me-7 and Me-8 to be *cis*. The relative stereochemistry at C-1′ was determined by the chemical conversion from 2,3-*cis*-stegobinone. Chromatographically separated (±)-2*S*,3*R*,1′*R*- and (±)-2*S*,3*R*,1′*S*-stegobinone were independently reduced by the manner described above to give (±)-2*S*,3*R*,1′*S*,2′*RS*- and (±)-2*S*,3*R*,1′*R*,2′*RS*-stegobiol, respectively. On the analytical gas chromatogram, the former showed two peaks corresponding to peaks 5 and 8, and the latter to peaks 3 and 7. The retention time of **1** on the gas chromatogram was identical with one of those of the former (peak 5). From the above observation, the relative configuration at C-2, C-3, and C-1′ was determined to be 2*S**,3*R*,1′*S*.

Biological Activity of Stegobiol. Responses of male drugstore beetles to stegobiol are summarized in Figure 5. In a replicated bioassay at 10^{-5}–10 μg dose, stegobiol showed the same level of biological activity as that of stegobinone, and the threshold of its activity was 10^{-3} μg. Although the parameters scored were almost the same between two pheromones, a small difference in

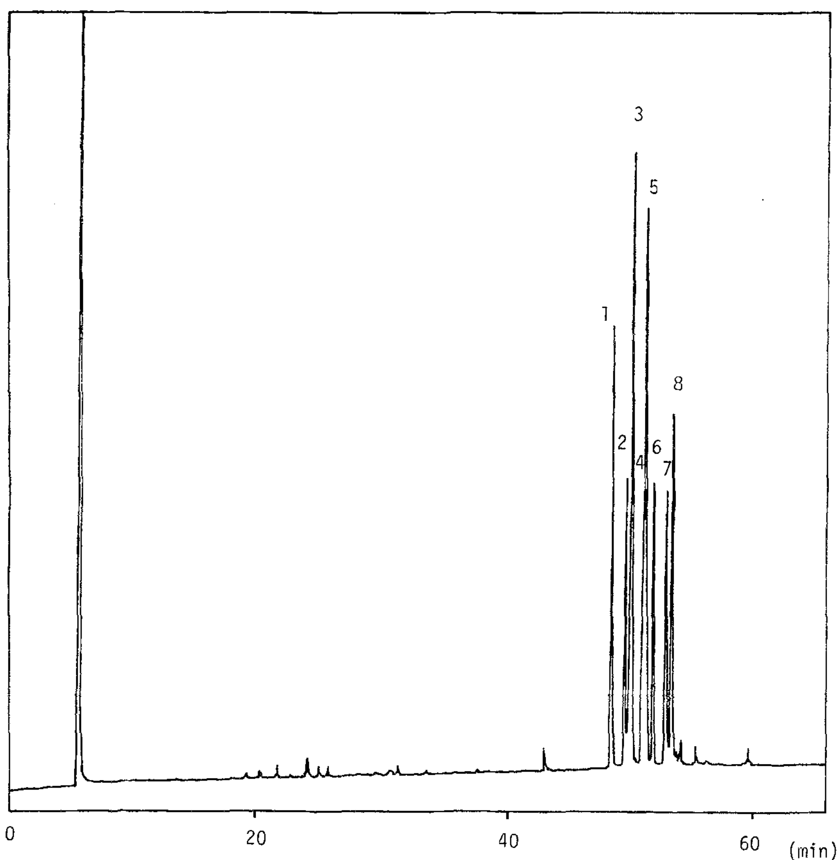

FIG. 4. Analytical gas chromatogram of the diastereoisomeric mixutre of **1**. Retention time and assignment: peak 1, 48.3 min, 2,3-*trans*; peak 2, 49.4 min, 2,3-*trans*; peak 3, 49.8 min, (+)-2*S*,3*R*,1′*R*; peak 4, 50.7 min, 2,3-*trans*; peak 5, 50.9 min, (+)-2*S*,3*R*,1′*S*; peak 6, 51.5 min, 2,3-*trans*; peak 7, 52.5 min, (+)-2*S*,3*R*,1′*R*; peak 8, 53.0 min, (+)-2*S*,3*R*,1′*S*. Peak 5 corresponded to that of stegobiol obtained from the male.

behavioral response was observed. In the bioassay of stegobiol, a few minutes passed before the males awakened, whereas stegobinone elicited immediate awakening. Once the males awakened, no difference of the response was observed between them. Pheromonal activity of synthetic stegobiol was also examined. Although a diastereoisomeric mixture showed no pheromonal activity, the column-chromatographically separated isomer corresponding to peak 5 on the analytical gas chromatogram elicited the pheromonal response from the males.

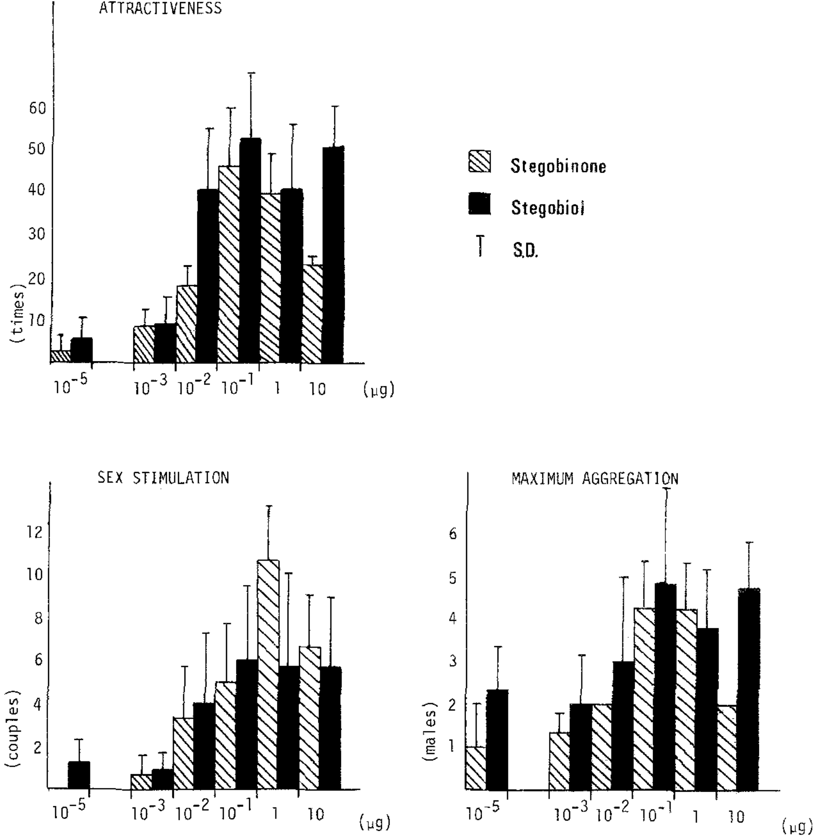

FIG. 5. The pheromonal activity of stegobiol and its diastereoisomeric mixture. The assay data for stegobinone are from a previous paper (Kodama et al., 1987).

DISCUSSION

We previously reported the mating behavior induced by stegobinone (Kodama et al., 1987). The newly identified pheromone, stegobiol, elicited the same response as stegobinone. Although the absolute configuration is still unknown, the structure of stegobiol is closely related to that of stegobinone in regard to relative stereochemistry at C-2, C-3, and C-1′. The stereochemistry at C-2, C-3, and C-1′ was thought to be necessary to elicit the pheromonal response.

We also reported that stegobinone is easy to epimerize to the C-1′ epimer that is the inhibitor, whereas stegobiol is stable and no epimerization could be observed under the same conditions. The diastereoisomeric mixture of stegobiol

elicited no response from the males. An isomer, which possessed the same relative chemistry as that of the stegobinone inhibitor, is also thought to inhibit the pheromonal activity. The volatility of two pheromones is thought to be considerably different because of the nature of C-2′ substituents. In the stegobiol molecule, the hydroxy substituent at C-2′ should depress its volatility. The difference of the response between two pheromones observed in the bioassay, the lag time before awakening, may be attributed to their volatility. These two pheromones may have independent roles in eliciting pheromonal activity, i.e., perhaps stegobiol and stegobinone and short-range and long-range sex attractants, respectively.

The absolute configuration of stegobiol, including the stereochemistry at C-2′, should be confirmed by synthesis.

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ALLELOPATHY IN AGROECOSYSTEMS: Wheat Phytotoxicity and its Possible Roles in Crop Rotation

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Abstract—The germination rates of cotton and wheat seeds were significantly affected by various extracts of wheat mulch and soils collected from the wheat field. This toxicity was even more pronounced against seedling growth. Five allelochemics: ferulic, *p*-coumaric, *p*-OH benzoic, syringic, and vanillic acids, were identified from the wheat mulch and its associated soil. Quantitatively, ferulic acid was found at higher concentrations than *p*-coumaric acid in the soil. Various concentrations of ferulic and *p*-coumaric acids were toxic to the growth of radish in a bioassay. The functional aspects of allelochemic transfer from decaying residue to soil and the subsequent microbial degradation within agroecosystems are discussed, particularly as they relate to wheat crop rotation, with wheat and cotton, in Pakistan.

Key Words—Allelopathy, ferulic acid, *p*-coumaric acid, *p*-OH benzoic acid, syringic acid, vanillic acid, *Triticum vulgare*, *Gossypisium hirsutum*.

INTRODUCTION

Rice (1984) reported that inhibitory effects (allelopathy) of crop plants on other crop plants have been reported as far back as 300 B.C. by Theophrastus. Unfortunately, research in allelopathy did not develop successionally, and only a few historical reports are found prior to the beginning of the twentieth century. Schreiner and Reed (1907) reported that the roots of wheat, oats, and other crop plants exude chemicals inhibitory to their own seedlings. Eveneri (1949) demonstrated the presence of seed germination inhibitors in several crop plants.

In the late 1940s, McCalla and his associates in Nebraska started their

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systematic studies with crop versus crop allelopathy. They isolated and quantified many allelochemics from the crop residues and their associated soils. Guenzi and McCalla (1962) tested various hot- and cold-water extracts of oats, corn, wheat, sorghum, sweetclover, and soybean and reported a wide range of inhibition of germination, shoot growth, and root growth of wheat. Subsequently, Guenzi and McCalla (1966a,b) isolated various allelochemics from crop residues and their associated soils. Wang et al. (1967) isolated five different phenolic acids from sugarcane-associated soils. The concentration of inhibitors found in many soils was sufficient to decrease the growth of wheat, corn, and soybean when applied to plants growing in nutrient culture solutions. Chou and Lin (1976) and Chou et al. (1981) reported that the decreases in rice productivity of crops following the first was due mainly to the allelopathic effects of decaying rice residues. Shilling et al. (1985) reported allelochemic activities in rye and wheat and alluded to their possible significance in no-till weed control.

It is clear that allelochemic interactions exist and play a major role in crop plants. Crop residue can be used to decrease and/or eliminate weeds from cropping areas, but they may also lower the productivity of subsequent crops of the same kind or of different crops used as rotational crops (Putnam and Duke, 1978; Chou et al., 1981; Lodhi, 1981; Rice, 1984; Putnam, 1985). In Pakistan, wheat crops are rotated in various combinations: wheat–fallow–wheat, wheat–cotton–fallow–wheat, rice–wheat–rice, wheat–green manuring–wheat, sugarcane–wheat–fallow–wheat, and wheat–legume–wheat. All rotations are based on region and season at the starting point of the rotation scheme; however, no data were found to show the effects of crop residue and their associated soils or crop–crop interaction of the various crop species. With this in mind, a project was initiated to establish systematic studies of the allelochemic behavior of crop species. This report deals mainly with wheat–cotton and wheat–wheat interactions.

METHODS AND MATERIALS

Location and Description of Study Area. A research plot was established at the Field Research Sites of the Nuclear Institute for Agriculture and Biology (NIAB) in Faisalabad, Pakistan. Six randomly located soil samples, 0–30 cm in depth, and six standing wheat mulch samples, from the same locations, were collected. All samples were air-dried to a constant weight at room temperature. Three composite samples were made by mixing two samples each of soil and wheat mulch. Test crop plants used in all experiments were the same as cultivated locally: *Triticum vulgare* Punjab 81 (wheat) and *Gossypium hirsutum* Niab 78 (cotton).

Effects of Wheat Mulch. Aqueous extracts were prepared with cold (20°C)

and hot (75 °C) water and treated separately for experimentation. Extracts were prepared by immersing the leaf mulch in water at the appropriate temperature for 10 min, grinding it in a blender for 5 min, and removing the particulate matter by centrifugation. Experimental dilutions were made at 1:10 and 1:20, leaf mulch–water, respectively. Twenty-five seeds each of the wheat and cotton were placed in each of three dishes containing filter paper saturated with 10 ml of distilled water (control), 10 ml of 1:10 extract solution or 10 ml of 1:20 extract solution. Seeds were allowed to germinate in the dark at room temperature. The percent germination index was calculated based on the formula $4(1g + 2g + 3g)$, where g represents the total germination each day.

To determine the allelopathic effects of wheat on wheat and cotton seedlings, plants were grown in a sand–vermiculite mixture for two weeks in Hoagland's nutrient solution (Hoagland and Arnon, 1950). Seedlings were then transferred to plastic vials containing one of the following solutions: 100% Hoagland's solution (control) or 1:10, 1:20, or 1:40, (test) mulch–Hoagland's solution, respectively. All extracts were prepared in Hoagland's solution to maintain the nutrient balance. Seedlings were allowed to grow in these solutions for five additional days, harvested, oven dried, and weighed.

Effects of Field Soil. Soil aqueous extracts were prepared with cold (20 °C) and hot (75 °C) water and treated separately for experimentation. Extracts were prepared by immersing soil samples in water at the selected temperature and magnetically stirring them for approximately 12 h. The soil was then removed by centrifugation. Experimental dilutions were made at 1:10 and 1:20, soil–water, respectively. To determine the percent germination index, experiments were conducted following the procedure described above.

Identification and Quantification of Phytotoxins. The procedures used to isolate the compounds were the same as used previously by Lodhi (1975a). Air-dried ground wheat or soil were separately hydrolyzed with 2 N NaOH in an autoclave for 45 min. The extracts were centrifuged and acidified to a pH 2.0 with HCl, then extracted with three half volumes of diethyl ether. The ether fractions were shaken with two half volumes of 5% $NaHCO_3$ and the ether fractions discarded. The alkaline portions were acidified again to pH 2.0 and reextracted with two half volumes of diethyl ether. The ether portions were evaporated to dryness and the residue taken up in 5 ml of 95% ethanol. The soil and mulch fractions were chromatographed in two dimensions using *n*-butanol–acetic acid–water (63:10:27 v/v/v, BAW), followed by 6% aqueous acetic acid (6% AA). The chromatograms were inspected under short and long UV. Compounds were marked under UV light and subsequently eluted with 95% ethanol and chromatographed in one dimension using several different solvent systems: BAW, 6% AA, upper phase of benzene–acetic acid–water (6:7:3 v/v/v, B_zAW), and sodium formate–formic acid–water (10:1:200 v/v/v, NaFW). The chromatograms were dipped in various reagents and maximum

absorption peaks in 95% ethanol determined with a spectrophotometer before and after the addition of two drops of 2 N NaOH to the cuvette.

Quantitative analysis of phytotoxins was done according to the procedure described by Lodhi (1975b). Known amounts of soil extracts were spotted on chromatography paper and developed as described above. Eluates of identified compounds were taken in known amounts of 95% ethanol and concentrations determined from the standard curves of known compounds. The standard curves were determined by spotting known concentrations of authentic samples on chromatography paper following the same procedure as used with the soil extracts. All standard curves were determined at the maximum absorption peaks of the authentic reagents.

Biological Activity of Quantified Phytotoxins. It was considered necessary to determine the biological activity of the compounds quantified from soil samples. Solutions of various concentrations of ferulic and *p*-coumaric acids in various concentrations were prepared with phosphate buffer, pH 5.65, to determine their biological activity against seed germination and radicle growth of the radish, a good indicator species (Lodhi, 1979). Known amounts of test buffer solutions were added to Petri plates containing 25 radish seeds on filter paper. Controls were treated in the same manner, except seeds were watered with toxin-free buffer solutions. After three days, germination was determined and each plate thinned, leaving only the 10 longest radicles per dish. Radicles were allowed to grow for two additional days, after which radicle growth was measured.

RESULTS

The germination rates of cotton and wheat seeds, as expressed by the percent germination index, were suppressed by the wheat mulch and soils taken from the wheat field (Table 1). Cold extracts inhibited the cotton germination rate more effectively than the hot extracts, whereas hot extracts (1 : 20) inhibited the wheat germination rate more effectively than the cold extracts. The two test concentrations, among themselves, were signifcantly different in a few cases in their effectiveness in inhibiting the germination rates (Table 1). Cold and hot soil extract effectiveness against cotton was significantly different between the two test concentrations. On the other hand, the wheat germination rate was significantly different between the two test concentrations using cold plant extracts (Table 1).

The toxicity of wheat was even more pronounced against seedling growth than germination (Table 2). Most seedlings in 1 : 10 mulch–Hoagland's solution died and were excluded from the data. Wheat and cotton seedling growth was significantly reduced in the remaining test concentrations (Table 2).

Allelochemics identified from mulch and associated soils were ferulic, *p*-

TABLE 1. EFFECTS OF VARIOUS EXTRACTS OF WHEAT MULCH AND ASSOCIATED SOIL ON GERMINATION INDEX (% GI) OF WHEAT AND COTTON ($N = 3$, MEAN ± SD)

Extract	Control % GI	Test % GI	
		1:20	1:10
Cotton			
Cold plant	324 ± 59.7	121 ± 12.0[a]	168 ± 43.3[a]
Hot plant	324 ± 59.7	250 ± 67.1	203 ± 26.6[a]
Cold soil	324 ± 59.7	117 ± 40.5[a,b]	237 ± 22.0
Hot soil	324 ± 59.7	320 ± 59.7[b]	124 ± 10.6[a]
Wheat			
Cold plant	527 ± 22.0	447 ± 6.1[a,b]	392 ± 18.3[a]
Hot plant	527 ± 22.0	401 ± 22.0[a]	413 ± 16.2[a]
Cold soil	527 ± 22.0	405 ± 43.9[a]	439 ± 26.6[a]
Hot soil	527 ± 22.0	324 ± 10.6[a]	460 ± 38.2[a]

[a] Statistical t value indicates significant difference between test and control values at $P \leq 0.05$ or better.
[b] Significantly different between tests at $P \leq 0.05$.

coumaric, *p*-OH benzoic, syringic, and vanillic acids (Table 3). The amount of ferulic acid isolated was calculated as 941 kg/ha; *p*-coumaric acid was 684 kg/ha. Para-OH benzoic, syringic, and vanillic acids were not measured after initial analysis due to their smaller amounts as compared to ferulic and *p*-coumaric acids (Figure 1). Other compounds were also isolated in soil and plant fractions, but no attempts were made to identify all compounds. Concentrations of ferulic and *p*-coumaric acids significantly reduced the germination and radicle growth of radish (Figure 1). Further, radicle growth was significantly different between test concentrations (Figure 1).

TABLE 2. EFFECTS OF WHEAT MULCH EXTRACT ON SEEDLING GROWTH OF TEST SPECIES ($N = 6$, MEAN mg ± SD)[a]

Treatment	Wheat	Cotton
Control	153 ± 13.2[b]	285 ± 26.7[b]
Test 1:20	100 ± 8.4	180 ± 23.5
Test 1:40	111 ± 11.2	188 ± 13.3

[a] Most seedlings in 1:10 ratio died.
[b] Statistical t value indicates significant difference between control and test values at $P \leq 0.05$ or better.

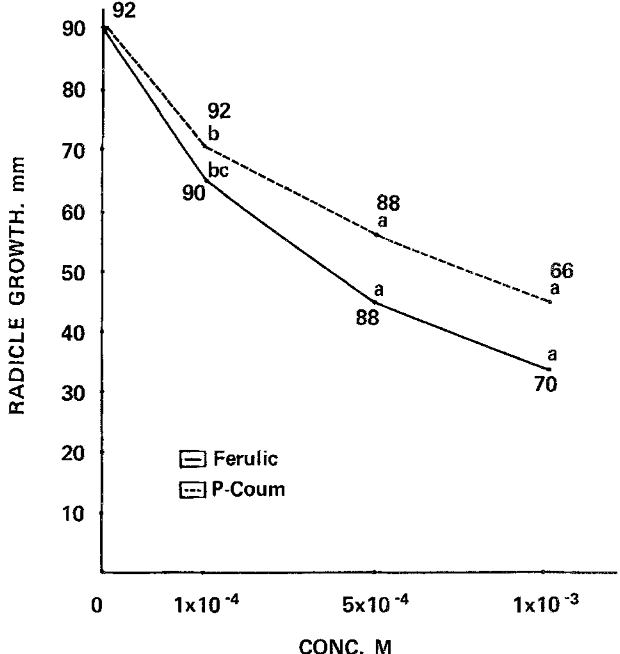

FIG. 1. Inhibition of germinations and radicle growth of radish. Numbers at each point represent percent of germination. [a]statistical t value indicates significant difference between control and test values at $P \leq 0.05$ or better; [b]significantly different between 1×10^{-4} and 1×10^{-3}; [c]significantly different between 1×10^{-4} and 5×10^{-4}

DISCUSSION

Experimental data clearly support the allelopathic impact of wheat on cotton and wheat autotoxicity, since competition was not a factor. Dilution of wheat mulch and soil extracts in buffered and nutrient-rich solutions inhibited the germination rates and seedling growth of wheat and cotton, and this clearly indicated the presence of allelochemics (Tables 1 and 2). In Punjab province, a wheat crop rotated with cotton and wheat (after fallow) would probably have an adverse effect on crop productivity.

There is growing evidence that many crop plants produce phytotoxic substances (Borner, 1960; Garb, 1961; McCalla and Haskins, 1964; Guenzi et al., 1967; Wang, et al., 1967; Putnam and Duke, 1978; Lodhi, 1981; Schumacher et al., 1983; Rice, 1984; Pope et al., 1985; Panasiuk et al., 1986). Kimber (1973) pointed out that subsequent low crop yields are generally attributed to nitrogen immobilization. His carefully designed experiments demonstrated that various combinations of wheat residues and nitrogen affect the yield of wheat

TABLE 3. PHYTOTOXINS IDENTIFIED FROM BOTH WHEAT MULCH AND ASSOCIATED SOIL

	Rf values[a]						UV Fluorescence[b]		Reagent colors[bc]		
Compounds	6% AA		BAW	BzAW	NaFW		Long	Short	PNit	Sulfa	Ferric
Paracoumaric acid	0.48	0.70	0.86	0.42	0.38	0.80					
Suspected *p*-coumaric acid	0.46	0.71	0.88	0.41	0.39	0.81	p, ab	p, ab	bn bk	or red	bl
Ferulic acid	0.32	0.69	0.84	0.64	0.22	0.64					
Suspected ferulic acid	0.34	0.68	0.84	0.65	0.25	0.65	bl	bl	f, bn bk	f, tan	bl
Para-OH benzoic acid		0.65	0.91	0.28		0.71					
Suspected *p*-OH benzoic acid		0.65	0.91	0.28		0.70	f, ab	p, ab	f, wine	or red	bl
Syringic acid		0.53	0.88	0.57		0.53					
Suspected syringic acid		0.51	0.87	0.56		0.50	—	l, ab	f, yell	f, or	bl
Vanillic acid		0.56	0.90	0.62		0.57					
Suspected vanillic acid		0.56	0.91	0.62		0.57	—	l, ab	f, yell	f, or	bl

[a] 6% AA = acetic acid; BAW = 63:10:27 butanol-acetic acid-water; BzAW = 6:7:3 upper phase of benzene-acetic acid-water; NaFW = 10:1:200 sodium formate: formic acid: water

[b] p = purple, ab = absorption, bl = blue, f = faint, l = lavender, bn = brown, bk = black, yell = yellow, or = orange.

[c] Diazotized *p*-nitraniline, diazotized sulfanilic acid, and ferric-potassium ferricyanide.

crops cultivated in the presence of straw residues. Further partitioning of experiments showed that the soil-surface straw very effectively depressed germination. Additionally, nitrogen immobilization affected yield the most when straw was mixed in the soil and adding more nitrogen did not minimize residue phytotoxicity. This supports our data, which showed reduced germination that occurred at a slower rate. Rapid germination rates play an important role in reducing the loss of seeds due to decay in soil and in providing more vigorous seedlings because of an early start.

The toxicity level was much more pronounced against seedling growth in cotton and wheat. A ratio of 1:10 (mulch) was too toxic and killed the test seedlings, whereas the same ratio only significantly reduced the germination rates of both test species (Tables 1 and 2). A concentration of 1:40 (mulch) was still strongly inhibitory ($P \leq 0.001$) to test seedling growth. This is an important point, which answers a frequently asked question about the retention time of allelochemics in the agro-, or natural, ecosystems. The various concentrations (1:10, 1:20, 1:40) used, represented possible toxicity retained during the course of mulch decay. Guenzi et al. (1967) reported changes in the phytotoxicity of decomposing wheat, and several other crops, after harvest. Phytotoxic levels in decomposing residue varied from crop to crop, but remained significant for several weeks. Loss of allelopathy from the mulch is simply (at least in part because some may decompose) a transfer of allelochemics to the associated soils. The process is not any different than the transfer of any other organic matter from crop/natural litter to soils in a natural or agroecosystem. Therefore, the presence of allelochemics in soils is as important as their presence in the crop litter (Lodhi 1975b, 1978).

Ferulic, *p*-coumaric, *p*-OH benzoic, syringic, and vanillic acids were identified in wheat residue and associated soil samples (Table 3). All five compounds are known to be allelopathic in natural and agroecosystems (Rice, 1984; Mandava, 1985). Ferulic and *p*-coumaric acids found in soil were in larger concentrations than *p*-OH benzoic, syringic, and vanillic acids. Further, the amount of ferulic acid was larger (941 kg/ha) than the amount of *p*-coumaric acid (684 kg/ha). These amounts seem to be higher than reported previously by Guenzi and McCalla (1966a); however, they reported higher amounts of ferulic and *p*-coumaric acids in the mulched plot than in the plowed plot. Lodhi (1975b, 1978) reported the cycling and larger amounts of many phenolics in soil systems throughout the year in forest communities. Furthermore, in a partially unharvested corn field, the amounts of ferulic and *p*-coumaric acids remained consistently high, comparable to the amounts reported in the current study (Lodhi, 1981); however, the amounts of phenolic compounds can vary due to cultivation or because of geographic, environmental, or ecological reasons. Additionally, the differences in phenolic profiles and concentrations can be due to eco-evolutionary reasons (Levin, 1971).

The mechanisms by which phytotoxins inhibit growth are not well understood. Allelochemics can target one or more physiological systems to block growth processes; therefore, a mixture of several allelochemics can be more detrimental than one inhibitor at the same concentration. Rasmussen and Einhellig (1977) reported that an equimolar mixture of ferulic and *p*-coumaric acids reduced sorghum germination and various growth factors of the seedlings more than either compound alone. In their systematic studies, Einhellig and Rasmussen (1978) reported on the synergistic action of vanillic and *p*-OH benzoic acids on sorghum growth at an equimolar concentration of 5×10^{-4}, which approximately resulted from a 10^{-3} M concentration of either phenol.

The dynamic nature of phenolics in soil is very complex. Phenolics can originate from microbial metabolism, lignin degradation, humate breakdown, etc. Paracoumaric acid can be oxidized by soil microbes into *p*-OH benzoic acid. Similarly, vanillic acid can be an oxidized product of ferulic acid (Wang et al., 1967; Turner and Rice, 1975). One should keep in mind that quantitative analysis of soil phenolics only represents the allelochemic functional status; ecologically speaking, the presence of the same phenolics in plants would be essential as an original source. Losses in the amounts of ferulic and *p*-coumaric acids do not necessarily represent a lessening in toxicity. Lowered amounts of ferulic and *p*-coumaric acids, combined with their oxidized products, vanillic and *p*-OH benzoic acids, collectively remained allelopathically active (Lodhi, 1978). Furthermore, the original syringic and *p*-OH benzoic acids constantly released from the residue into the soil will unquestionably accentuate the toxic nature of wheat mulch allelochemics.

It is clear that the involvement of biochemicals in an agroecosystem is a phenomenon that cannot be ignored, because these phytotoxins play a multipurpose role. Allelochemics can lower the yield of subsequent crops if proper rotation is not determined; yet they can also be used to minimize weed invasion and competition in crop fields (Lodhi, 1981). Such manipulations may assist in determining an effective (high yield) rotation of crops in mixed cropping. Thus it seems necessary to determine and exploit the primary roles of these secondary compounds classified as allelochemics.

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OLFACTORY RESPONSES OF A SPECIALIST AND A GENERALIST GRASSHOPPER TO VOLATILES OF *Artemisia ludoviciana* NUTT. (ASTERACEAE)

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Abstract—*Hypochlora alba* is a specialist grasshopper that lives and feeds almost exclusively on *Artemisia ludoviciana*, a plant that produces large amounts of allelochemics including a variety of monoterpenes. This plant is not a host for generalist grasshoppers such as *Melanoplus sanguinipes*. The role of olfaction in the grasshopper–plant relationship was investigated by comparing electroantennograms (EAGs) of males and females of both species generated by solvent-extracted volatiles from plant leaves and by major individual components. Volatiles of *A. ludoviciana* were identified by gas chromatography–mass spectrometry. The major components were 1,8-cineole, camphor, camphene, and borneol, while minor identified compounds were α- and β-pinene, α-thujene, myrcene, *p*-cymene, Artemisia ketone, α-thujone, and bornyl acetate. The EAGs (mV) of *H. alba* males to a range of concentrations of individual volatiles or the total plant extract were nearly double those of conspecific females or both sexes of *M. sanguinipes*. However, both sexes of *M. sanguinipes* were more sensitive than either sex of *H. alba* to geraniol, a monoterpene that commonly occurs in many plant species but is absent or is present in only trace amounts in *A. ludoviciana*. The increased sensitivity of *H. alba* males to the odor components of their host plant appears to be related to the greater number of certain olfactory chemoreceptors on male versus female antennae. The significance of this phenomenon is discussed.

Key Words—*Hypochlora alba*, *Melanoplus sanguinipes*, Orthoptera, Acrididae, *Artemisia ludoviciana*, electroantennogram, specialist insect, generalist insect, plant volatiles, grasshopper, monoterpenes.

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INTRODUCTION

Phytophagous insects have evolved different strategies for obtaining food. Generalists feed on a wide variety of plants, while specialists use only a single species or group of related species. The mechanisms of host plant location and nonhost avoidance involve many behavioral and sensory adaptations (Ahmad, 1983). Olfactory specializations of receptors in the peripheral nervous system have been found in several specialist species (reviewed by Mustaparta, 1984; May and Ahmad, 1983; Visser, 1983, 1986). A number of insects such as the Colorado potato beetle *Leptinotarsa decemlineata* (Visser, 1979), the carrot fly *Psila rosae* (Guerin and Visser, 1980), the oak flea weevil *Rhynchaenus quercus* (Kozlowski and Visser, 1981), the chrysomelid beetle *Trihabda bacharides* (Dickens and Boldt, 1985), the cereal aphid *Sitobion avenae* (Yan and Visser, 1982), and the boll weevil *Anthonomus grandis* (Dickens, 1984) have been found to be sensitive to certain volatiles typical of their host plants and to general green plant volatiles (Visser, 1979).

Hypochlora alba (Dodge) is a highly specialized grasshopper which feeds almost exclusively on Louisiana sagewort, *Artemisia ludoviciana* Nutt. (Knutson, 1982). This plant appears to be protected by both glandular and nonglandular trichomes and is fed upon very little by generalist grasshoppers such as the related *Melanoplus sanguinipes* (Fabr.) (Mulkern et al., 1969). This model system has been the subject of several previous studies emphasizing the physical defenses of the plant and adaptations of the specialist grasshopper to these defenses (Knutson, 1982; Smith and Grodowitz, 1983; Smith and Kreitner, 1983).

This study examines the role of olfaction in the adaptation of the specialist grasshopper, *H. alba*, to its host plant and, by use of the electroantennogram (EAG) technique (Schneider, 1957), the possibility of sensory specialization to volatiles of the host plant. This was done by comparing EAGs of specialist and generalist grasshoppers generated by volatiles from *A. ludoviciana* extract and by individual monoterpenes that are major components of the plant's odor.

METHODS AND MATERIALS

Analysis of Plant Volatiles. Two methods were used to collect the volatiles from *A. ludoviciana*. In the first, air was swept through a flask containing overdried (55°C), ground-up leaves and then through a glass U-tube (0.5 cm ID) immersed in liquid nitrogen. After trapping for 30 min, the tube was rinsed with 1 ml of dichloromethane. After separation of the water and solvent, the latter was drawn off, concentrated under nitrogen, and stored at −20°C for analysis. In the second method, specimens of *A. ludoviciana* were cut at the stem base and care was taken not to damage the leaves. These specimens were placed in a closed glass container which was purged with nitrogen at a rate of

1 liter/min through a trap containing 2 ml of Tenax. Volatiles were trapped for 6 hr before rinsing the Tenax with 4 ml of diethyl ether and concentrating to 0.5 ml under nitrogen. Volatiles were analyzed by capillary column gas chromatography (GC), and certain components were tentatively identified using retention times of standards. A Tracor 540 capillary GC with a 25-m BP-1 (SE-30 equivalent) vitreous silica 0.33-mm-ID column was used for analysis. Conditions were 50°C for 5 min, to 250°C for 5 min at a rate of 10°C/min. Helium was used as the carrier gas at a head pressure of 6 psi. Confirmation of structural identity of certain monoterpene components from the headspace volatiles was obtained by capillary gas chromatography–mass spectrometry (GC-MS) using a Hewlett Packard 5790A GC and 5790 MS detector.

Experimental Insects. Two species of grasshoppers were used in this investigation. The specialist, *H. alba,* was collected from a pasture 5 miles west of Brookville, Kansas. They were maintained in a rearing room and fed on freshly cut host plants until tested. The generalist, *M. sanguinipes* was from a nondiapausing laboratory colony that was reared in a greenhouse and fed seedling rye grass and ground Purina guinea-pig pellets.

Electroantennogram Techniques. Using adult insects, EAGs (in millivolts) were recorded from excised antennae of males and females of *H. alba* and *M. sanguinipes*. The distal tips (one segment or less) of the antennae were removed, and the antennae were severed from the head. Each antenna was placed between the tips of two Pasteur pipets containing cockroach saline II (Oakley and Schafer, 1978). Ag–AgCl wires were inserted into the saline from the other end of the pipet and connected to a Grass P-18 DC amplifier. The amplifier output was read on a Tektronix 564B storage oscilloscope.

Five test volatiles and a standard were used. The test volatiles were the monoterpenes found to be 5% or more of either of the two volatile collections. These were camphene (99%), camphor (97%), borneol (98%), (Aldrich Chemical Company), and 1,8-cineole (ca. 99%) provided by Dr. T. Mabry, University of Texas. The fifth test substance was a dichloromethane extract of *A. ludoviciana* leaves. The standard was the monoterpene geraniol (98%), also provided by Dr. Mabry. Each of these test volatiles was applied to a 1-cm^2 piece of filter paper at rates of 2, 20, and 200 ng in 20 μl of dichloromethane. The standard was used at the 200-ng rate. The filter paper was placed in the wide end of a Pasteur pipet. After the solvent evaporated, a 0.3-m length of Tygon tubing was attached. The pipets were positioned 1 cm from the antenna, and a syringe was attached to the other end of the tubing and used to blow a 0.5-ml puff over the antennae. A minimum of 30 sec was allowed between test readings.

The test pipets were coded by number so the identity of the substances was not known during the test. Only the identity of the standard was known, and this was used at the beginning of each series of 15 samples (five volatiles at three levels each) and after every fifth sample. Each of the five test responses

(millivolts) recorded between two standard responses was divided by an average of the latter to obtain readings relative to the standard. This procedure was used to compensate for decline in antennal response with time. These ratios were then multiplied by the initial response of the standard to obtain the final results in mV. Conversion to mV was necessary for comparisons between sexes and species. The series of 15 test odors was performed twice for every antenna, and the results were averaged. The data were evaluated by analysis of variance. Comparisons between the four test groups, and comparisons at the 200-ng level of the responses within a test group, were made using Duncan's multiple-range test.

RESULTS

Composition of Plant Volatiles. Capillary gas chromatograms of headspace volatiles collected from oven dried and whole fresh sage plants showed similarities in the compounds collected and their relative amounts (Figure 1). This indicates that most of the volatile terpenoids found in the sage are being released from undamaged plants. Table 1 shows compound identifications by GC-MS, and those identifications which have been confirmed by correspondence with retention times of monoterpene standards. In addition, the table contains the normalized peak area percentages of the compounds collected by the two methods. 1,8-Cineole was the major component, accounting for one third to one half of the volatiles. Other compounds constituting more than 5% of the volatiles collected by either of the two methods were camphor, camphene, borneol, and two unidentified compounds. Other monoterpenes identified in lesser amounts included α-pinene, p-cymene, myrcene, and bornyl acetate.

Electroantennograms Generated by Plant Volatiles. EAGs ranged from 0.06 to 1.26 mV for test substances while the response to an odorless air puff was ca. 0.07 mV (Table 2). No significant differences were found between the EAGs of male and female *M. sanguinipes* generated by any of the individual volatiles or the plant extract. Because of the similarity in responses between sexes of *M. sanguinipes*, interspecific comparisons were made by pooling the data from males and females of this species.

H. alba showed a sexual difference in its response to the host-plant volatiles (Table 2). Males had a significantly higher response to most of the volatiles except no significant difference was evident for any level of camphene, the lowest level of the extract of *A. ludoviciana*, and the control, geraniol. The strength of the EAGs of female *H. alba* was very similar to the responses of *M. sanguinipes*. Female *H. alba* had a significantly lower response only to the control, geraniol. Male *H. alba* showed statistically greater responses than *M.*

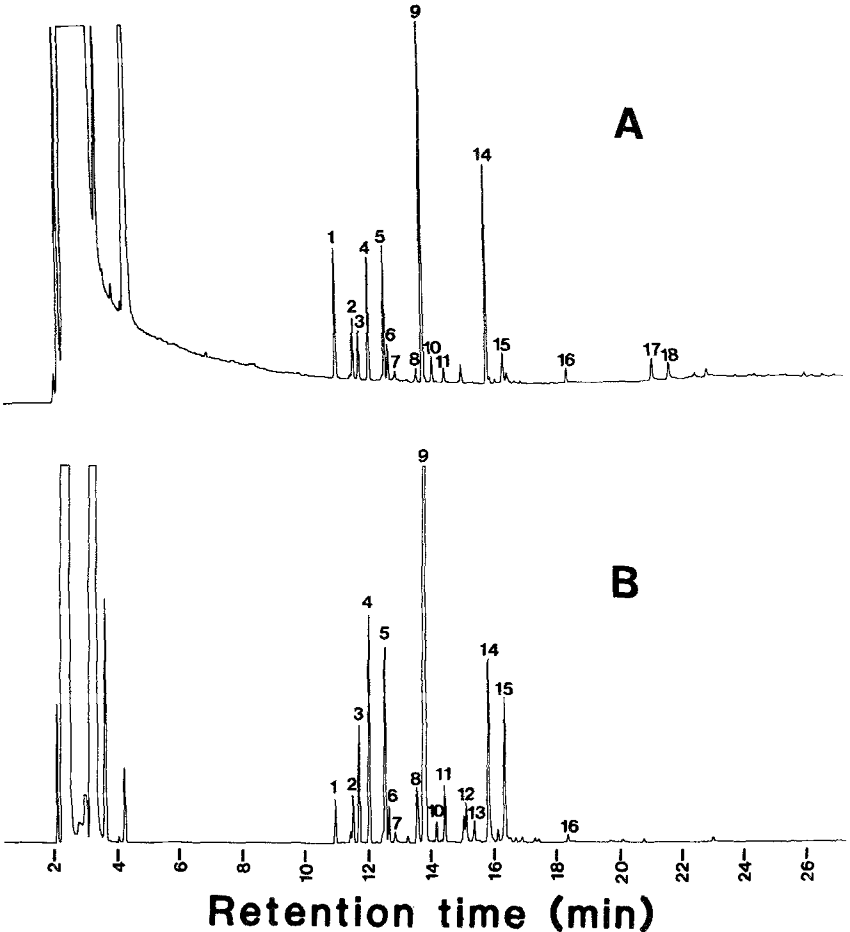

FIG. 1. Comparison of volatiles from (A) whole fresh plants and (B) oven dried foliage of *Artemisia ludoviciana* by capillary gas chromatography. Compounds assigned peak numbers are listed in Table 1. See Methods and Materials for details of analysis.

sanguinipes at all levels of camphor, 1,8-cineole, borneol, and extract, but no significant difference at any level of camphene.

Using the pooled data for *M. sanguinipes*, camphene, 1,8-cineole, and camphor gave the lowest responses. They were followed in increasing order by borneol, *A. ludoviciana* extract, and geraniol. This same pattern was followed closely by both male and female *H. alba* with geraniol as the major exception. This monoterpene gave a much more intense response in the generalist than in the specialist grasshopper (Table 2).

TABLE 1. CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY (GC-MS) OF HEADSPACE VOLATILES FROM DRIED AND FRESH FOLIAGE OF *Artemisia ludoviciana*

Peak number[a]	Identification by GC-MS	Retention time[b]	Normalized area (%)	
			Dried	Fresh
1	unknown (MW 136)	10.91	1.5	8.8
2	α-thujene	11.47	2.2	4.3
3	α-pinene	11.66 X	4.4	3.4
4	camphene	11.99 X	8.7	8.7
5	unknown (MW 136)	12.50	8.5	9.4
6	1-β-pinene	12.61	1.4	2.5
7	myrcene	12.81 X	0.5	1.1
8	*p*-cymene	13.5 X	3.2	1.2
9	1,8-cineole	13.7 X	44.4	30.6
10	*Artemisia* ketone	14.1	1.0	1.9
11	unknown (MW 154)	14.39	2.5	1.2
12	α-thujone	15.00	2.8	1.8
13	unknown (MW 150)	15.32	1.1	—
14	camphor	15.79 X	8.7	14.7
15	borneol	16.3 X	6.8	3.3
16	bornyl acetate	18.31 X	0.3	1.1
17	sesquiterpene (MW 204)	21.1	—	2.1
18	sesquiterpene (MW 204)	21.68	—	1.8

[a] Peak numbers correspond to Figure 1.
[b] X denotes additional confirmation of identification by matching the retention time with that of a standard. See Methods and Materials for details of analysis.

DISCUSSION

The monoterpenes identified in this study as major volatiles from *A. ludoviciana* match closely those identified by Stangl and Greger (1980). They list camphor and 1,8-cineole as occurring in relatively high concentrations, with lesser amounts of camphene, borneol, α- and β-pinene, and myrcene. They do not list α-thujone or *Artemisia* ketone as being found in this species. The authors state that cineole, thujane, and camphane derivatives are characteristic of sections *Artemisia* and *Abrotanum*. *A. ludoviciana* is placed in either of these two groups by various systematic schemes.

H. alba has evolved a monophagous relationship with its host plant *A. ludoviciana* and has adapted to its host in many ways. It is a brachypterous species with highly cryptic coloration and form while on its host. From the evidence presented here, it appears that only the male of this species has evolved significant changes in the peripheral olfactory system that result in increased

TABLE 2. COMPARISON OF EAGS (mV) OF MALE AND FEMALE (DATA POOLED) *Melanoplus sanguinipes* (S) AND MALE (M) AND FEMALE (F) *Hypochlora alba* (A) TO VOLATILES OF *Artemisia ludoviciana*

Volatile	Amount (ng)[a]	EAGs[b]			Comparisons[c]		
		S	FA	MA	S–FA	FA–MA	MA–S
Camphene	2	0.06	0.07	0.07			
	20	0.09	0.07	0.13			
	200	0.17ab	0.11a	0.23a			
Cineole	2	0.07	0.08	0.20		X	X
	20	0.10	0.08	0.30		X	X
	200	0.15a	0.26ab	0.47b		X	X
Camphor	2	0.08	0.08	0.17		X	X
	20	0.08	0.09	0.29		X	X
	200	0.26ab	0.24ab	0.66b		X	X
Borneol	2	0.12	0.10	0.26		X	X
	20	0.25	0.25	0.54		X	X
	200	0.37b	0.42bc	0.94c		X	X
Extract	2	0.08	0.11	0.16			X
	20	0.18	0.26	0.45		X	X
	200	0.65c	0.55c	1.17d		X	X
Geraniol	200	1.18d	0.42bc	0.62b	X		X

[a] See Methods and Materials for explanation of amounts.
[b] Values (200 ng) within a column followed by the same letters are not significantly different ($P < 0.05$) by Duncan's multiple-range test. Data are means from 10 male and 8 female antennae of *H. alba*, and 6 male and 5 female antennae of *M. sanguinipes*.
[c] X represents statistical difference ($P < 0.05$) by Duncan's multiple-range test.

sensitivity to the plant odor. However, it is possible that differences in individual receptors may exist which would not be apparent in EAG measurements since EAGs are a summation of the response of all receptors on the antennae. EAGs from female *H. alba* showed no differences from the responses of the nonadapted generalist *M. sanguinipes*. In addition, the generalist showed no sexual differences in its sensitivity to the plant volatiles tested. Male *H. alba*, however, showed significantly greater responses than conspecific females and both sexes of *M. sanguinipes*.

The sexual difference in EAGs is correlated with a difference in the number of certain antennal olfactory sensilla (Bland, 1982). Male *H. alba* have 50–80% more sensilla basiconica types I–III than females. Sensilla basiconica type IV, sensilla trichodea, and sensilla coeloconica are present in more equal numbers between sexes. Thus it appears that the sensilla basiconica types I–III may be involved in the reception of host-plant volatiles for this species. The sensilla basiconica have been shown to respond to host odors in other insects such as

the white pine weevil (Mustaparta, 1975) and the Colorado potato beetle (Ma and Visser, 1978).

Few EAG studies have examined sexual differences in responses to host-plant volatiles (Den Otter et al., 1978; Dickens, 1984; Guerin and Visser, 1980; Kozlowski and Visser, 1981; Visser, 1979). In the species studied, sexual differences in EAGs usually occur when the female needs to locate a host plant for oviposition. In species where the adults feed on the host, sexual differences in olfactory sensitivity are minimal. Chapman (1982) states that differences in numbers of antennal olfactory sensilla between sexes usually occur in species where the feeding habits of the sexes differ or when males are attracted to females by pheromones. He also summarizes data on numbers of olfactory receptors on other Acrididae, including *M. sanguinipes*, and shows no sexual differences except in *Truxalis nasuta*, an African species whose biology is still largely unknown. Thus, it appears that *H. alba* is relatively unique in having adults with similar feeding habits, yet having more antennal sensilla sensitive to host volatiles on the males than on the females.

One possible explanation is that females sequester the host volatiles for use as sex pheromones. This seems unlikely since the volatiles from surrounding host plants would probably mask such a pheromone. Another explanation for sexual differences in sensilla numbers may also relate to male behavior in locating females. The host plant propagates primarily by rhizomes, resulting in a patchy distribution. Eggs are laid in the soil within these patches and females need not leave a patch during their lifetimes. Males, however, may travel between patches in search of receptive females. Dispersion between host patches would also have genetic advantages by decreasing inbreeding. The increased sensitivity of males to host-plant volatiles would aid in location of other patches. This hypothesis is supported by the darker green, less camouflaged coloration of the males. The greenish-white color of the females makes excellent camouflage while on the host but would be more conspicuous for males traveling the darker vegetation between patches of sage. This type of behavior pattern has been found in the milkweed beetle *Tetraopes tetraophthalmus*. Studying the movement patterns of adult beetles, McCauley et al. (1981) found the males to be more vagile than the females. The host plant of this species also spreads by rhizomes and has a patchy distribution.

The extract from sage leaves containing a mixture of monoterpenes generally gave significantly greater responses in both grasshopper species than did the major monoterpenes tested individually. These data suggest that a mixture of volatiles making up the plant odor is more stimulatory than any of the individual components, as has been demonstrated with other species (Visser, 1986). Minor components not tested may also be highly stimulatory as was shown with the carrot fly, *Psila rosae* (Guerin et al., 1983). Borneol tended to be the most stimulatory of the individual components tested.

The response to geraniol in relation to the other volatiles showed a large difference between the generalist and specialist grasshoppers. Geraniol elicited a much stronger response in *M. sanguinipes* than did the other monoterpenes or the sage extract. Conversely, geraniol elicited a medium response in *H. alba* and was the only monoterpene that showed no sexual difference in the EAGs. Therefore, the greater olfactory sensitivity of male *H. alba* appears to be specific to its host volatiles, while the generalist grasshopper is more sensitive to geraniol, a widespread monoterpene in plants (Loomis and Croteau, 1980), as a possible host location stimulus.

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BEHAVIORAL AND PHYSIOLOGICAL RESPONSES OF CABBAGE LOOPER, *Trichoplusia ni* (HÜBNER), TO STEAM DISTILLATES FROM RESISTANT VERSUS SUSCEPTIBLE SOYBEAN PLANTS

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Abstract—Soybean plant volatiles, extracted as steam distillates, significantly affected the behavior and biology of the cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae). Distillates from the susceptible Davis variety attracted *T. ni* larvae and female adults, whereas those from resistant PI 227687 plants repelled them. When mixed in an artificial diet, steam distillates from PI 227687 plants caused mortality of first-instar larvae. Adults emerging from pupae topically treated with 5 μg of such PI 227687 extractable showed developmental abnormalities. Larval feeding was significantly less on Davis leaves treated with PI 227687 volatiles as compared to solvent (acetone) or such Davis extractables. However, Davis volatiles on PI 227687 leaves did not increase larval feeding. HPLC analyses of steam distillates from susceptible Davis versus resistant PI 227687 indicated differences.

Key Words—Allelochemicals, cabbage looper, *Glycine max*, Lepidoptera, Noctuidae, plant resistance, plant volatiles, soybeans, steam distillates, *Trichoplusia ni*.

INTRODUCTION

Among soybean [*Glycine max* (L.) Merr.] genotypes which bear resistance to insects and to cabbage looper [*Trichoplusia ni* (Hübner)] in particular, plant

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introduction (PI) 227687 is one of the more resistant (Luedders and Dickerson, 1977). *T. ni* thrives on numerous commercial varieties such as Davis, but feeds less, grows more slowly, and shows poorer survival on resistant PI 227687 plants (Khan et al., 1986a, b).

Chemical parameters contributing to PI 227687 resistance to insects may include plant volatiles. Such compounds contribute to rice resistance to a leafhopper, *Nephotettix virescens* (Distant) (Khan and Saxena, 1985). When steam distillates from a resistant rice cultivar were applied to a susceptible cultivar, *N. virescens* feeding behavior was disrupted. Steam distillates from resistant versus susceptible rice cultivars also had differing effects on the biology and behavior of *Nilaparvata lugens* (Stål) and *Sogatella furcifera* (Horváth) planthopper pests (Saxena and Okech, 1985; Khan and Saxena, 1986).

In the present study, plant volatiles, extracted as steam distillates from resistant PI 227687 versus susceptible Davis plants, were investigated for differential effects on behavioral and physiological responses by *T. ni*.

METHODS AND MATERIALS

Plants and Insects. Seeds of resistant soybean PI 227687 were obtained from Dr. Curtis Williams, Jacob Hartz Seed Co., Stuttgart, Arkansas. The commerical cultivar Davis was used as an insect-susceptible control. Soybean plants were grown in insect-proof, nylon-screen cages in a greenhouse at the University of Wisconsin at Madison.

Seeds were treated with Thiram (Science Products Company, Inc., Chicago, Illinois) and were germinated in moistened, sterilized vermiculite in flats. Germinated seedlings were transplanted at the first-leaf (V1) stage into 20-cm-diam. autoclaved earthen pots (two plants per pot) containing a sterilized potting mixture (field soil, sand, vermiculite, 2 : 1 : 1). To supplement the natural light, plants were kept under a 16-hr photophase using Metalarc high-intensity (1000-W), full-spectrum metal halide lighting (Duke et al., 1974). The plants were uniformly watered twice a day and were maintained at 27 ± 5°C and 60 ± 10% relative humidity.

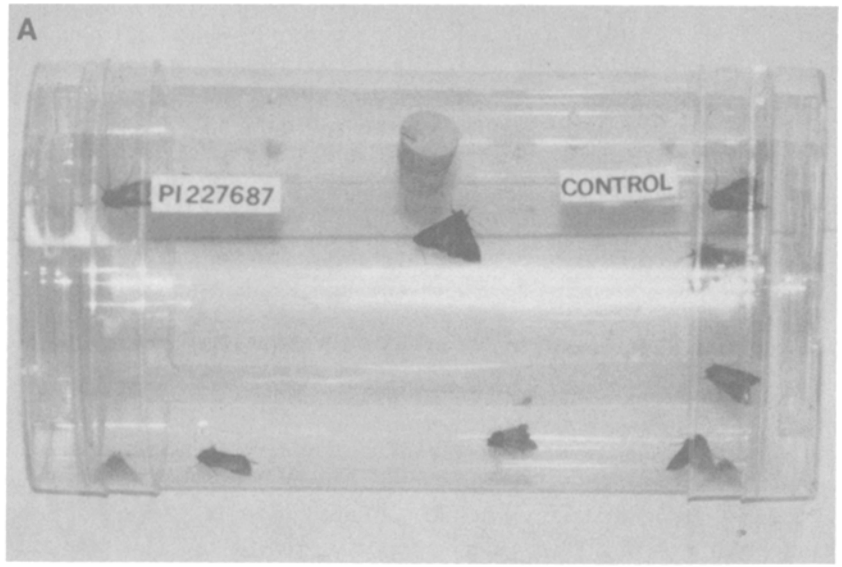
The colony of *T. ni* was maintained in the laboratory on a pinto bean-based artificial diet (Shorey and Hale, 1965) at 27 ± 2°C and 50 ± 10% relative humidity with a photoperiod of 14 : 10 hr light–dark.

Steam Distillation and Extraction of Plant Tissues. Leaves of 10-week-old soybean plants of resistant PI 227687 or susceptible Davis were harvested and ground without added solvent in an electric grinder (Waring Products Corporation, New York, New York). Each resultant 200-g sample was steam distilled for 3 hr according to Saxena and Okech (1985) and yielded approximately 900 ml of distillates. Distillates were extracted with diethyl ether (300 ml distillate–100 ml diethyl ether) by shaking the mixture in a 1000-ml separatory funnel for

5 min. Essential oils and other volatiles were absorbed by the diethyl ether. The aqueous layer was discarded. Ether extractables were pooled from several samples in a 500-ml beaker and dried with 100 g anhydrous sodium sulfate. Excess ether was removed from the resultant mixture in a fume hood to yield a final volume of approximately 25 ml. This final volume was covered with aluminum foil and held overnight to allow the sodium sulfate to absorb further traces of water from the extractables. Each sample was next reduced by evaporation to 10 ml and decanted into a preweighed glass vial. The vial was covered with perforated aluminum foil and the ether was evaporated under vacuum in a desiccator to yield a yellow oily residue. The sample vial was then reweighed, sealed under nitrogen, and kept at −30°C.

Orientation of Female Adults. Orientational responses of *T. ni* adult females to odors extracted from resistant and susceptible soybean plants were assayed in horizontal, cylindrical Mylar plastic (21-cm-long, 14-cm-diam.) chambers closed at each end with nylon net (Figure 1A) (adapted from Khan and Saxena, 1986). Treatment chemicals (4000 ppm in 0.25 ml paraffin oil) were sprayed on a 9-cm-diam. filter-paper assay disc using a spray atomizer (Sigma Chemical Company, St. Louis, Missouri). Control filter-paper discs were sprayed only with 0.25 ml paraffin oil. An extract- or solvent- (control) treated filter-paper disc was attached singly to the center of a 14-cm-diam. Petri-dish lid. Each dish lid had four 2-cm-diam. "air" holes positioned equidistantly on a circle 3 cm away from the center of the lid. A lid bearing a treated disc covered one end of each assay chamber, and one bearing a control (solvent) disc covered the opposite end. Ten 1-day-old *T. ni* females were introduced into the middle of each test chamber, and insect responses were observed for 30 min. Females thus could perceive odors, but could not contact the "source" filter-paper disc due to an intervening nylon-net wall. Percentages of arrivals of *T. ni* adult females on the nylon-net wall adjacent to the extract-treated filter paper disc were calculated as $100A/(A + B)$, where A is the number of arrivals on the wall facing the extract-treated filter-paper disc; and B is the number of arrivals on the wall facing the control filter-paper disc (Saxena et al., 1974; Obata et al., 1983).

Orientation of Larvae. Orientational responses of *T. ni* larvae to odors from resistant PI 227687 and susceptible Davis soybean plants were measured in a horizontal 15-cm-long, 3-cm-diam. cylindrical glass chamber enclosed by nylon-net ends (Figure 1B). Treatment chemicals (4000 ppm in 100 μl paraffin oil) were applied to a 2.5 cm-diam. filter-paper disc using a microsyringe. Control treatment for each assay was 100 μl paraffin oil. Each extract- or solvent- (control) treated filter-paper disc was attached singly to the center of the inside bottom of a plastic cup (4 cm high, 3.5 cm diam.) having four 0.5-cm-diam. airflow holes arranged equidistantly in a circle both on the bottom and the side. One plastic cup, with either an attached extract- or solvent- (control) treated filter-paper disc, was snug-fitted over each end of the assay chamber. Ten third-



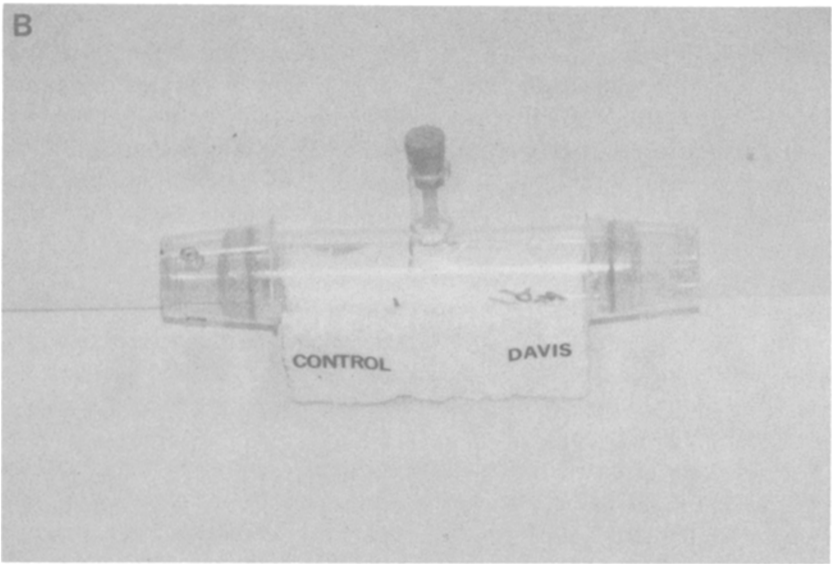


FIG. 1. Chambers for testing orientational responses of *T. ni* female adults (A) and larvae (B).

instar *T. ni* larvae were introduced at the midpoint in the assay chamber, and insect responses were observed for 2 hr. Percentages of arrivals of *T. ni* larvae on the nylon-net end adjacent to the extract-treated filter-paper disc were calculated using the same formula as with adult females.

Toxicity of Steam Distillates to First-Instar Larvae. Toxicities of steam distillates from resistant versus susceptible soybean plants were determined by incorporating diethyl ether extractables from PI 227687 or Davis tissues into a standardized artificial diet (Shorey and Hale, 1965). Twenty-five grams of the artificial diet, containing 25 μl of 1000, 2000, or 4000 ppm extractables from PI 227687 or Davis dissolved in acetone or just acetone (control) (HPLC grade, Aldrich Chemical Co., Milwaukee, Wisconsin), were placed in a plastic cup (4-cm-high, 3.5-cm-diam.). Acetone was evaporated from such diets by placing it in plastic cups under vacuum for 24 hr. Ten first-instar *T. ni* larvae were released in each cup, and it was enclosed with a perforated lid. Cups were maintained at 27 ± 2°C and 60 ± 5% relative humidity. Larval mortality was observed at 24-hr intervals for seven days.

Effects of Steam Distillates on Larval Development and Adult Emergence. Diethyl ether extractables from steam distillates from resistant PI 227687 or susceptible Davis soybean plants were diluted with acetone (HPLC grade, Aldrich Chemical Co.) to yield a 5 μg extractable/1 μl solution. Third-instar larvae and pupae of *T. ni* were individually treated topically, using a microsyringe, with 1 μl extractables on the dorsum of their first thoracic segment. Insects were similarly treated with 1 μl acetone (control solvent). Test larvae were maintained on the standardized artificial diet in a randomized complete block design at 27 ± 2°C and 65 ± 5% relative humidity until they pupated. Pupae were maintained similarly until emergence of adults.

Feeding Assays with Steam Distillates. Cabbage looper feeding was assayed with 18-mm-diam. leaf discs of susceptible Davis or resistant PI 227687 plants as the substrate. Discs were presented in a choice test in a 9-cm-diam. Petri-dish arena (Khan et al., 1986a, b). Five leaf discs from Davis or PI 227687 plants were positioned equidistantly in a circle with their abaxial side up. Four of the five discs randomly received 20 μl of 500, 1000, 2000, or 4000 ppm diethyl ether extractables from steam distillates from Davis or PI 227687 plants on their abaxial surface and were air dried for 5 min before being placed into a bioassay. The fifth leaf disc was treated only with 20 μl acetone (solvent control). Before exposing to *T. ni* larvae for feeding, the area of each leaf disc was measured in square centimeters using an automatic area meter (model LI-3100, LI-COR, Inc., Lincoln, Nebraska). Ten, 2-hr starved but water-satiated, third-instar *T. ni* larvae were placed in each five-treatment-choice assay arena. Bioassays were run for 20 hr at 27 ± 2°C and 65 ± 5% relative humidity. After termination of each experiment, the leaf area was again measured, and the area of feeding by *T. ni* larvae on each leaf disc was determined as the square centimeter difference.

High-Performance Liquid Chromatography of Soybean Plant Volatiles. Qualitative analysis of steam distillates was conducted by high-performance liquid chromatography (HPLC) using a prepacked silica column (25-cm-long, 4.6-mm-diam., packed with 5-μm-diam. silica particles) (Ultrasphere, Beckman Inc., Berkeley, California) and a variable UV spectrophotometer detector (model 100-40, Hitachi, Tokyo, Japan). The steam distillate from each variety was dissolved in hexane:2-propanol (95:5) (HPLC grade, Aldrich Chemical Co.) to a concentration of 10 mg/ml or 10,000 ppm. Twenty microliters of the solution were then injected per HPLC analysis. Operating conditions were: temperature, ambient; mobile phase, hexane:2-propanol at 96:4 for 1 min and then 95:5 for 9 min; flow rate, 1 ml/min; column pressure, 420 psi. The resolved peaks were not identified but were numbered according to order of elution. Steam distillates from each variety were analyzed at least five times.

Diethyl ether extractables from the steam distillates from resistant PI 227687 or susceptible Davis plants were collected as three HPLC fractions (peaks 1–5, fraction 1; peaks 6–12, fraction 2; peak 13, fraction 3). Each fraction was concentrated by evaporating the solvent under nitrogen, weighed, and diluted with paraffin oil to a concentration of 4000 ppm. Such fractions were tested individually, and in all combinations, for attractancy or repellency against *T. ni* adult females, as described above.

Statistical Analysis of Data. All experiments were replicated 5–10 times in a randomized complete-block design, and data were analyzed using the statistical procedures indicated in the footnotes of each table.

RESULTS

Orientation of T. ni *Adults and Larvae.* Diethyl ether extractables from the steam distillates from the susceptible Davis soybean cultivar attracted significantly more ($P < 0.01$, t test) *T. ni* female adults and third-instar larvae as compared to solvent (paraffin oil) control, whereas such odors from resistant PI 227687 plants repelled these insects (Table 1).

Toxicity of Steam Distillates to First-Instar Larvae. Steam distillates from PI 227687 plants, when incorporated in an artificial diet, were more toxic [$P < 0.05$, Duncan's multiple-range test (DMRT)] to first-instar larvae than such extractables from susceptible Davis plants (Table 2, Figure 2A). Larval mortality on Davis extractables-treated artificial diet was low, but significantly ($P < 0.05$, DMRT) more than that on solvent-treated (control) diet. Percentage of *T. ni* larval mortality on PI 227687 extractables-treated diet increased through 72-hr exposure.

Effects of Steam Distillates on Larval Development and Adult Emergence. All third-instar larvae that received a topical application of 5 μg extractables from PI 227687 or Davis plants completed their life cycle. However, adults

TABLE 1. ORIENTATIONAL RESPONSE OF *T. ni* ADULT FEMALES AND THIRD-INSTAR LARVAE TO ODOR OF STEAM DISTILLATES FROM RESISTANT PI 227687 OR SUSCEPTIBLE DAVIS PLANTS[a]

Insect stage	Odor source[b]	Arrivals (%) on nylon-net wall facing filter-paper discs		Difference (A − B)
		Distillates-treated (A)	Paraffin oil-treated (B)	
Adult	PI 227687	26 ± 9.7	74 ± 9.7	−48**[c]
	Davis	70 ± 11.5	30 ± 11.5	40**
Larva	PI 227687	25 ± 8.5	75 ± 8.5	−50**
	Davis	73 ± 11.6	27 ± 11.6	46**

[a] Data are averages of 10 replications; in each replication 10 female adults or third-instar larvae were used.
[b] Odor source for female adults was a 9-cm-diam. filter-paper disc treated with 0.25 ml of 4000 ppm distillates; and for third-instar larvae, was a 2.5-cm-diam. filter-paper disc treated with 100 μl of 4000 ppm distillates.
[c] Differences between means followed by double asterisks are significantly different at $P = 0.01$ level (t test).

TABLE 2. MORTALITY OF FIRST-INSTAR *T. ni* LARVAE IN ARTIFICIAL DIET SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF STEAM DISTILLATES FROM RESISTANT PI 227687 OR SUSCEPTIBLE DAVIS PLANTS[a]

Plant	Distillate conc. (μg/25 g diet)	Mortality (%) hours after release (HAR)[b]		
		24 HAR	48 HAR	72 HAR
PI 227687	100	64 ± 12.6 A	98 ± 4.2 A	100 ± 00 A
	50	25 ± 6.2 B	37 ± 10.1 B	59 ± 11.5 B
	25	13 ± 2.7 C	31 ± 9.9 B	35 ± 9.3 C
Davis	100	13 ± 3.6 C	14 ± 2.9 C	14 ± 2.9 D
	50	16 ± 2.3 C	16 ± 2.3 C	16 ± 2.3 D
	25	14 ± 2.7 C	14 ± 2.7 C	14 ± 2.7 D
Solvent (control)	0	00 ± 00 D	00 ± 00 D	00 ± 00 E

[a] Data are averages of 10 replications; in each replication 10 newly emerged first-instar larvae were used.
[b] Observations were recorded for seven days after release, but there was no increase in mortality after 72 hr.
[c] In a column, means followed by a common letter are not significantly different by Duncan's multiple range test at $P = 0.05$ level.

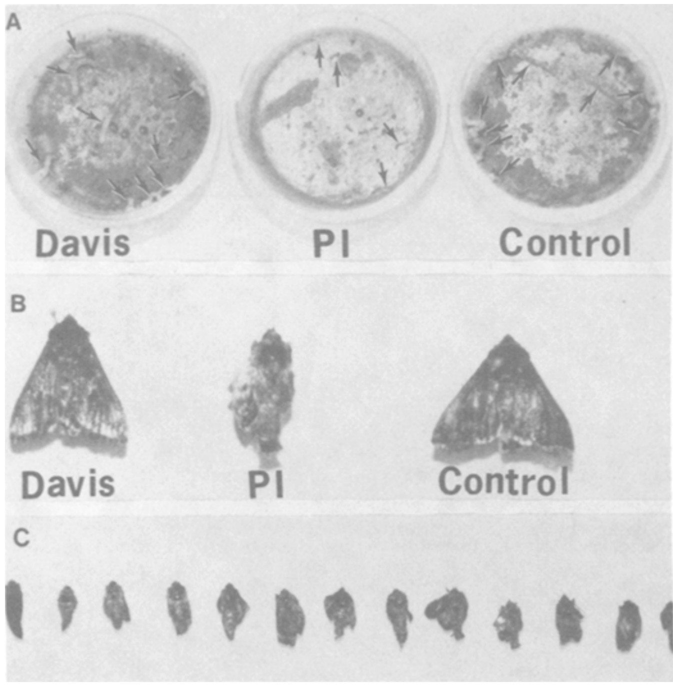


FIG. 2. (A) Surviving (see arrows) *T. ni* larvae on an artificial diet treated with 100 μg steam distillates from susceptible Davis or resistant PI 227687 plants, as compared to control. (B) Adult with deformed wings that emerged from pupa that received 5 μg steam distillates from PI 227687 plants, whereas those that emerged from pupae that received such distillates from Davis plants or acetone (control) were normal. (C) Various forms of wing deformaties in adults that emerged from pupae that received 5 μg of steam distillates from PI 227687 plants.

emerging from pupae that received 5 μg extractables from PI 227687 showed varying degrees of wing deformaties. Such wings were reduced in size and crumpled (Figure 2B,C). Application of 5 μg susceptible Davis extractables did not cause such abnormalities.

Feeding Assays with Steam Distillates. Application of 20 μl of 500, 1000, 2000, or 4000 ppm diethyl ether extractables from the steam distillates from PI 227687 plants to Davis leaf discs significantly reduced ($P < 0.05$, DMRT) feeding by third-instar larvae as compared to that on discs treated with acetone (solvent control) or Davis extractables (Table 3, Figure 3A,C). Such applications of Davis extractables on resistant PI 227687 leaf discs did not increase, or decrease, insect feeding as compared to that on controls (Figure 3B).

TABLE 3. COMPARATIVE FEEDING OF THIRD-INSTAR *T. ni* LARVAE ON LEAF DISCS FROM SUSCEPTIBLE DAVIS OR RESISTANT PI 227687 PLANTS, TREATED WITH DIFFERENT CONCENTRATIONS OF STEAM DISTILLATES FROM PI 227687 OR DAVIS SOYBEAN LEAVES IN CHOICE TEST[a]

	Feeding area (cm^2)[c]		
Extract conc. (ppm)[b]	Davis leaf disc treated with PI 227687 distillates	Davis leaf disc treated with Davis distillates	PI 227687 leaf disc treated with Davis distillates
500	1.52 ± 0.28 B	1.90 ± 0.15 A	0.28 ± 0.06 A
1000	1.16 ± 0.21 C	1.99 ± 0.16 A	0.30 ± 0.09 A
2000	0.44 ± 0.08 D	1.95 ± 0.10 A	0.27 ± 0.07 A
4000	0.22 ± 0.04 D	1.86 ± 0.04 A	0.24 ± 0.04 A
0[d]	2.22 ± 0.13 A	1.93 ± 0.12 A	0.33 ± 0.05 A

[a] Data are averages of five replications.
[b] All concentrations were prepared in acetone.
[c] In a column, means followed by a common letter are not significantly different by Duncan's multiple range test at the $P = 0.05$ level.
[d] Control was the solvent, acetone.

High-Performance Liquid Chromatography. Thirteen HPLC peaks were resolved in the diethyl ether extractables from the steam distillates of both PI 227687 and Davis plants (Figure 4). Three especially prominent peaks in both samples had similar retention times (*RT*), peak 2 ($RT = 3.4$ min), peak 4 ($RT = 4$ min), and peak 13 ($RT = 8$ min). Other peaks also showed similar retention times in both samples, e.g., peaks 1, 3, 5, 6, 7. Peaks 1, 3, and 13 were especially more prominent in PI 227687 than in Davis plants.

Bioassay results with the individual HPLC fractions 1, 2, and 3 from PI 227687 or Davis steam distillates showed that repellent activity was absent in the single fractions (Table 4). All paired combinations of the three Davis fractions (1 + 2, 1 + 3, and 2 + 3) significantly ($P < 0.01$, *t* test) attracted *T. ni* adult females. With PI 227687 steam distillates, one paired combination (1 + 3) of the three HPLC fractions was repellent to *T. ni* adult females.

DISCUSSION

Insects possess many intimate and subtle relationships with plants (Khan and Saxena, 1986). Their distinction between nonhost and host species may be based on several parameters (Norris and Kogan, 1980; Chiang and Norris, 1985). However, minor changes in physical or chemical characteristics among individuals within a host-plant species can cause significant differences in their

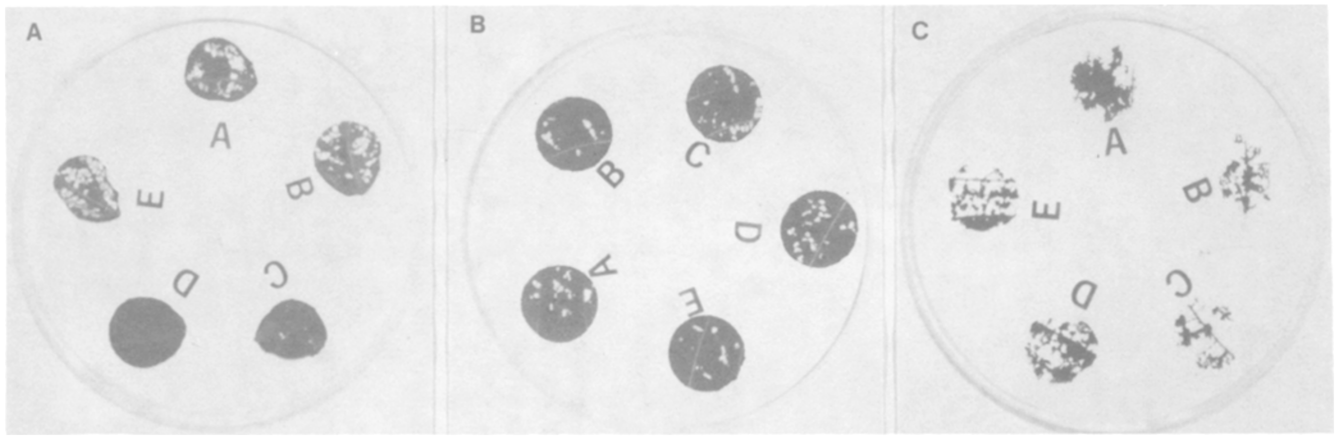


FIG. 3. *T. ni* larval feeding on (A) Davis leaf discs treated with PI 227687 distillates, (B) PI 227687 leaf discs treated with Davis distillates, and (C) Davis leaf discs treated with Davis distillates. Leaf discs were treated with 20 μl of 500 ppm (a), 1000 ppm (b), 2000 ppm (c), or 4000 ppm (d) distillates from PI 227687 or Davis plants, or acetone as control (e).

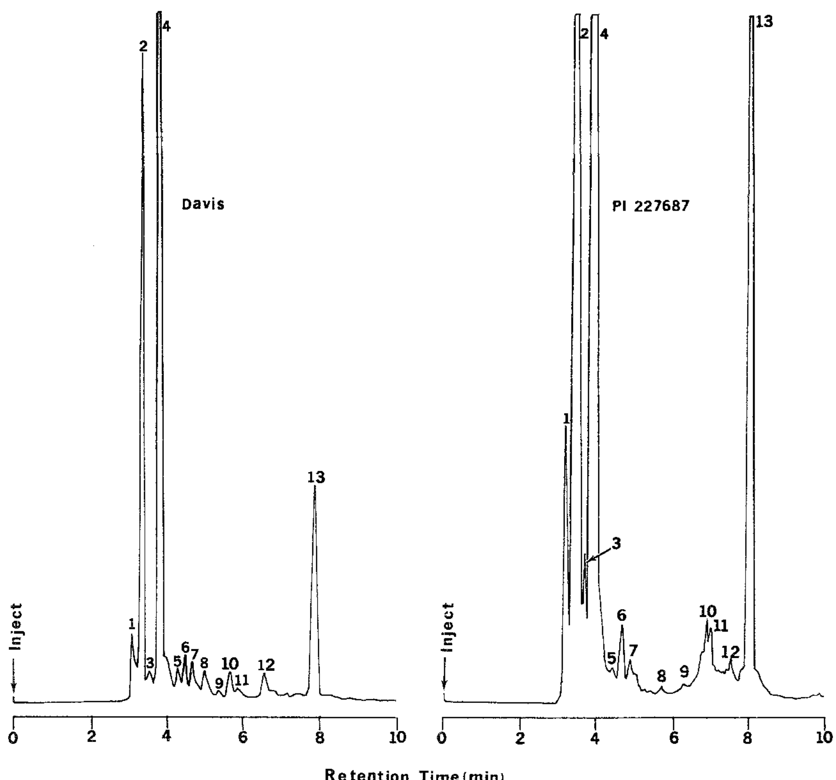


FIG. 4. High-performance liquid chromatograms of steam distillates from susceptible Davis and resistant PI 227687 plants.

relative acceptabilities to an insect species or biotype. Our results in this paper demonstrate specifically how limited chemical differences in soybean plants can alter their relative acceptabilities to *T. ni*.

Recently, we demonstrated significant roles for leaf trichomes (Khan et al., 1986b) and ethyl acetate- and hexane-extractable compounds (Khan et al., 1986a) in making the soybean line, PI 227687, less acceptable than the Davis cultivar for *T. ni* larval feeding. But the primary decisions regarding host plants are usually made by adult female *T. ni* moths in conjunction with selecting ovipositional sites. Because these moths initially approach potential host plants while flying, volatile repellent chemicals emanating from PI 227687 plants might serve a more primary role than leaf trichomes or relatively nonvolatile anti-feedant molecules in the insect's primary rejection of such soybeans as hosts. Diethyl ether extractables from steam distillates from PI 227687 did cause such a repulsion of moths in our currently reported studies. More than one volatile compound is involved in this PI 227687 repellency of *T. ni* adults because a

TABLE 4. ORIENTATIONAL RESPONSES OF *T. ni* FEMALES TO ODORS FROM VARIOUS FRACTIONS OF STEAM DISTILLATES FROM RESISTANT PI 227687 OR SUSCEPTIBLE DAVIS PLANTS[a]

Odor source[b]	Fraction number[c]	Arrivals (%) on nylon-net wall facing filter-paper discs		Difference (A − B)[d]
		Distillate-treated (A)	Paraffin oil-treated (B)	
PI 227687	1	46 ± 11.2	54 ± 11.2	−8 NS
	2	53 ± 8.8	47 ± 8.8	6 NS
	3	51 ± 12.1	49 ± 12.1	2 NS
	1 + 2	32 ± 9.9	68 ± 9.9	−36**
	1 + 3	53 ± 13.7	47 ± 13.7	6 NS
	2 + 3	51 ± 9.5	49 ± 9.5	2 NS
Davis	1	48 ± 11.6	52 ± 11.6	−4 NS
	2	54 ± 7.8	46 ± 7.8	8 NS
	3	52 ± 9.9	48 ± 9.9	4 NS
	1 + 2	68 ± 7.5	32 ± 7.5	36**
	1 + 3	69 ± 8.9	31 ± 8.9	38**
	2 + 3	71 ± 7.3	29 ± 7.3	42**

[a]Data are averages of six replications; each replication involved 10 female adults.
[b]Odor source was 0.25 ml of 4000 ppm of a fraction of steam distillates applied on a 9-cm-diam. filter-paper disc.
[c]Fraction 1, peak numbers 1–5; fraction 2, peak numbers 6–12; and fraction 3, peak number 13.
[d]Differences between means followed by double asterisks are significantly different at $P = 0.01$ level (*t* test); NS, not significant.

positive (repulsive) response was only obtained when two (i.e., fractions 1 and 3) HPLC fractions were combined in the assay.

Our results with Davis plants indicate that *T. ni* moth decisions between two or more soybean cultivars regarding an ovipositional site may involve attractants in addition to repellents. Whereas, the combination of volatile fractions 1 and 3 from PI 227687 repelled the moths, all paired combinations of the three HPLC fractions of comparable volatiles from Davis attracted the moths. In summary, plant volatiles apparently are the first chemical cues which *T. ni* adults contact in making decisions about host preferences among soybean cultivars, and repellent compounds apparently are the dominant volatile chemical messengers in such insect decisions, but attractants may be involved with relatively acceptable soybeans.

The diverse other allelochemical activities of the ether extractables from PI 227687 steam distillates included (1) reduced *T. ni* larval feeding when applied to the surface of otherwise-preferred Davis leaves; (2) mortality of first-instar larvae when added to a standard artificial diet; and (3) abnormal wings in adult *T. ni* emerging from pupae treated topically. Similar morphogenetic effects were reported on the rice stem borer, *Chilo suppressalis* (Walker), for steam distillates from a resistant rice variety (Saxena, 1978).

The range of allelochemical effects revealed now for certain volatiles from soybeans on the various stages of *T. ni* indicates some of the multiple-component chemical defense of this plant species against even an insect that has already adapted to use it as a host.

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TRANSFER OF PYRROLIZIDINE AND QUINOLIZIDINE ALKALOIDS TO *Castilleja* (SCROPHULARIACEAE) HEMIPARASITES FROM COMPOSITE AND LEGUME HOST PLANTS

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Abstract—*Castilleja* (Scrophulariaceae) species of the western United States contain pyrrolizidine and quinolizidine alkaloids. The *Castilleja* obtain the alkaloids by root parasitism on host plants, with *Senecio atratus* and *S. triangularis* (Asteraceae) furnishing the pyrrolizidines, and quinolizidines being obtained from *Lupinus* species and *Thermopsis montana* (Leguminosae). Individual plants within a given *Castilleja* species population may be devoid of alkaloids while others have high alkaloid content. No populations have been found which are made up of both pyrrolizidine- and quinolizidine-containing individuals. These results have important implications for *Castilleja* ecology, for the study of insect herbivores which are *Castilleja* specialists, and in the development of systems for the investigation of the role of alkaloids as plant defenses.

Key Words—*Castilleja*, Scrophulariaceae, *Senecio*, Asteraceae, *Lupinus*, Leguminosae, hemiparasites, alkaloids, plant–insect interactions.

INTRODUCTION

Castilleja (Scrophulariaceae; subfamily Rhinanthoideae) is a large, complex plant genus of about 200 species concentrated in western North America and is difficult from an evolutionary and taxonomic viewpoint (Heckard, 1968). Species and population variations have been ascribed (Heckard and Chuang, 1977) to a high degree of polyploidism and a lack of cross-breeding barriers among

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SCHEME 1.

some species. *Castilleja*, like some members of its subfamily, are hemiparasites, are hosted by a wide range of angiosperms (Heckard, 1962), and in turn are hosts to a number of specialist insects, including checkerspot butterflies [*Euphydryas* and *Chlosyne* (*Thessalia*)] (Ehrlich and Murphy, 1981; Singer, 1983) and plume moths (*Amblyptilia and Platyptilia*) (Lange, 1950).

To elucidate the roles of secondary plant metabolites in the biochemical systematics and plant-insect interactions of *Castilleja*, we undertook plant alkaloid analyses and reported (Roby and Stermitz, 1984a) isolation of unsaturated pyrrolizidine alkaloid senecionine (**1**) from *Castilleja rhexifolia*, ammondendrine and quinolizidine alkaloids from *C. miniata* (McCoy and Stermitz, 1983) and the saturated pyrrolizidines sarracine (**2**) and 7- and 9-angelylplatynecines (**3**, **4**) from an unnamed taxon (Roby and Stermitz, 1984a) variously described as *C. rhexifolia* aff. *miniata* or *C. miniata* aff. *rhexifolia* (Heckard and Chuang, 1977) (Scheme 1). More recently (Stermitz et al., 1986), we found populations of the latter two taxa with the quinolizidine *N*-methylcytisine (**5**) as a major alkaloid and, importantly, found populations of *C. sulphurea* which had no alkaloids, only saturated pyrrolizidines, only unsaturated pyrrolizidines, or only quinolizidines. Although we have not yet studied all species and populations where alkaloids were encountered, the results reported here establish alkaloid uptake from a host plant as the cause of the variable *Castilleja* alkaloid content. Most of the *Castilleja* taxa also contain pyridine monoterpene alkaloids, but these iridoid-derived alkaloids are inherent to the *Castilleja*.

METHODS AND MATERIALS

Plant collections are listed in Table 1 and exact locations given on the voucher specimens. D. Wilken (Colorado State University), L. Heckard (University of California, Berkeley), and D. Dunn (University of Missouri) assisted with species determinations.

Alkaloid isolation and other general procedures were as previously given (Roby and Stermitz, 1984a; McCoy and Stermitz, 1983; Stermitz et al., 1986). Briefly, above-ground plant material was wetted with dilute $NaHCO_3$ solution, extracted with 1 : 1 butanol–toluene, and this solution extracted with 1 M H_2SO_4. The acidic solution was made basic to pH 9 and extracted with $CHCl_3$. The $CHCl_3$ layers were evaporated to yield a purified total alkaloid mixture which could be analyzed by TLC, [^{1}H]NMR, and/or NH_3 CI-MS (McCoy et al., 1983). Individual alkaloids were then separated and purified by chromatogra-

TABLE 1. *Castilleja* AND HOST-PLANT COLLECTIONS

Species	Population	Voucher number
C. linariifolia Benth. in D.C.	Taylor Reservoir	FRS292
C. miniata Dougl.	Michigan Hill	FRS271
	Emerald Lake	GH167
C. rhexifolia aff. *miniata* (Heckard)	Cameron Pass	CSU65241
	Old Monarch Pass	FRS266
C. rhexifolia Rydb.	Cameron Pass	CSU65242
	Kite Lake	GH116
	Cumberland Pass	FRS259
	Monarch Pass Campground	FRS269
	Schofield Pass	FRS262
C. sulphurea Rydb.	Emerald Lake	GH168
	Gould	GH144
	Winfield	FRS309
	Cameron Pass	GH123
S. atratus Greene	Cameron Pass	GH176
	Winfield	FRS312
	Emerald Lake	FRS313
S. triangularis Hook.	Cameron Pass	GH177
L. bakeri (Greene) subsp. *amplus* Fleak and Dunn	Taylor Reservoir	FRS293
L. argenteus Pursh. subsp. *rubricaulis* (Greene) Hess and Dunn	Gould	GH171

phy. Identifications were by TLC, high-field (270- or 360-MHz) [^{1}H]NMR, and mass spectra in comparison with compounds we had previously reported, with obtained standards, or with literature values.

RESULTS

Key preliminary results came from qualitative alkaloid analysis of plants within alkaloid-containing populations. Individual plants were encountered that did not contain alkaloids and were hosted by plant species that also contained no alkaloids. In a given population, individual *Castilleja* plants that contained alkaloids had the same qualitative spectrum of alkaloids as did a host plant. Finally, entire alkaloid-free populations of each *Castilleja* taxon were found.

For example, thin-layer chromatography (Figure 1) showed that *C. linariifolia*, hosted in eastern Gunnison County, near Taylor Reservoir by sage-

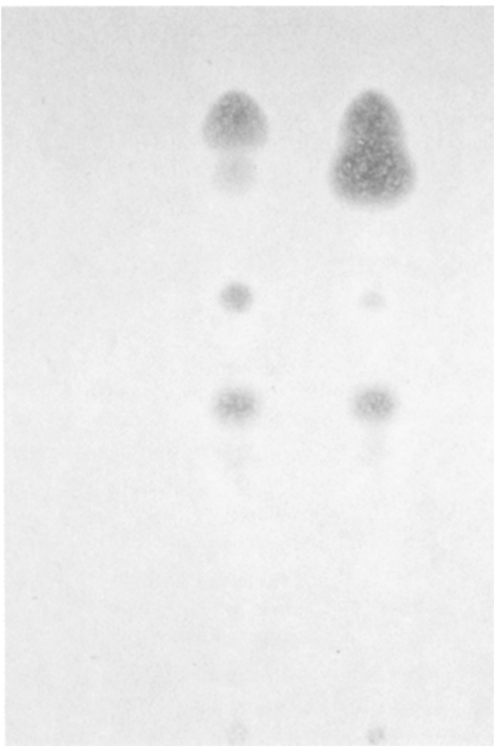

FIG. 1. TLC of total alkaloid isolate from: (A) *Castilleja linariifolia* on *Artemisia* and (B) *C. linariifolia* on *Lupinus bakeri* subsp. *amplus*; (C) *L. bakeri* subsp. *amplus*. (silica gel; 7:3 cyclohexane–diethylamine; iodoplatinate visualization).

brush (*Artemisia* sp.), was devoid of alkaloids, while *C. linariifolia* hosted by *Lupinus bakeri* subsp. *amplus* and the *Lupinus* host plant itself contained the same five quinolizidine alkaloids, although in different concentration ratios. Similarly, a central Colorado *C. miniata* population (Michigan Hill) growing with *Thermopsis montana* contained exactly the same five quinolizidine alkaloids (*N*-methylcytisine major) as did the host plant. These two parasite–host pairs have only been qualitatively studied so far, but represent clear-cut cases of a complex spectrum of alkaloids being identical in the host and parasite. Several parasite-host pairs were studied in more detail as described in the following.

C. rhexifolia *aff.* miniata *and* C. sulphurea *on* Senecio. Throughout Colorado, the *C. rhexifolia* aff. *miniata* taxon occurs mainly in the 2900 to 3200-m altitude region. Its host plant has been found to be *Senecio atratus*, which is considered (Pesman, 1959) to be an indicator of the same subalpine region. Both taxa are most often found in disturbed areas such as sandy roadbanks. Sarracine, 7- and 9-angelylplatynecines and their *N*-oxides were previously reported (Roby and Stermitz, 1984a) from the *Castilleja*. From one population (Cameron Pass), *S. atratus* (a previously uninvestigated species) was analyzed and found to contain the same sarracine and angelylpatynecine derivatives. Although sarracine has been reported from other *Senecio* species, the angelylplatynecines are only known from *C. rhexifolia* aff. *miniata*, and they are now shown to also be in *S. atratus*.

Individual *Castilleja* plants were found which did not contain alkaloids. For example, several were growing in association with *Antennaria anaphaloides* (Asteraceae), and neither these *Castilleja* nor the host plant contained alkaloids. Hemiparasites in the Rhinanthoideae of the Scrophulariaceae, such as *Castilleja*, produce specialized root structures, haustoria, which form the connection between parasite and host plant (Kuijt, 1969; Riopel and Musselman, 1979; Heckard, 1962). We found haustoria on the *Castilleja* root connections to *S. atratus*. Other *C. rhexifolia* aff. *miniata* populations on *S. atratus* were found at Winfield and Monarch Pass, with qualitative alkaloid TLC analyses confirming the parasite–host relationship.

C. sulphurea is also hosted by *S. atratus* (Winfield and Emerald Lake populations) as indicated by comparative alkaloid TLC analyses. In the latter population, the *C. sulphurea* is growing intermixed with *C. miniata*. The *C. miniata* from this location contained only quinolizidine alkaloids (α-isolupanine, 5,6-dehydro-α-isolupanine, and thermopsine) and no pyrrolizidines, but *C. sulphurea* did not contain quinolizidines. A lupine species (unflowered and unidentified) also grows within these populations and is likely to be the source of the quinolizidines in *C. miniata*.

C. sulphurea *on* Lupinus argenteus *subsp.* rubricaulis. Alkaloid isolation from a *C. sulphurea* population in eastern Jackson County near Gould yielded several quinolizidine alkaloids of which the major one ($C_{15}H_{22}N_2O_3$ by high-

resolution mass spectrometry) is new. Its structure will be described elsewhere. The alkaloid content was very low in the total *Castilleja* population sample, but high or completely absent in individual plants. The alkaloid-containing plants were in close association with *L. argenteus* and haustorial attachments could be identified. The alkaloid content of the *Lupinus* was essentially identical with that of the *C. sulphurea* which it hosted.

C. rhexifolia *and* C. sulphurea *on* S. triangularis. *C. rhexifolia* generally occurs at higher altitudes (3200–3500 m) than *C. miniata* or *C. rhexifolia* aff. *miniata*, although its range occasionally overlaps with these. It can be found with *C. sulphurea* and close to *C. occidentalis* at the upper altitude range. Four populations of *C. rhexifolia* (Cameron Pass, Kite Lake, Cumberland Pass, Schofield Pass) were found to be dominated in alkaloid content by senecionine and its *N*-oxide. No populations contained quinolizidine alkaloids or sarracine, and one population (Monarch Pass Campground) contained no alkaloids. Some populations close to Cameron Pass contain virtually no alkaloids as well. Absolute identification of a host plant has been difficult since the *Castilleja* roots in the swampy habitat are entwined with those of many other plants in a heavy, wet root ball. Nevertheless, the common denominator of all populations appears to be *S. triangularis*, with which the *Castilleja* is growing in close proximity wherever it contains alkaloids. Analysis of *S. triangularis* indeed showed it to have a high level of senecionine, as was previously reported (Roitman, 1983) from a California collection.[2] At Cameron Pass, *C. sulphurea* populations also occur and individual plants are occasionally found in close proximity to *C. rhexifolia*. We previously (Stermitz, et al., 1986) reported high levels of senecionine in these *C. sulphurea*. Close examination of the populations has now shown that these, too, are growing with *S. triangularis*. Table 2 summarizes the *Castillegia*-hostplant-alkaloid relationships found.

TABLE 2. *Castilleja* TAXA, HOST PLANTS, AND ALKALOIDS TRANSFERRED

Castilleja sp	Host plant(s)	Alkaloid(s)
rhexifolia	*Senecio triagularis*	senecionine, **1**
rhexifolia aff. *miniata*	*S. atratus*	sarracine **2**, platynecines **3** and **4**
linariifolia	*Lupinus bakeri*	quinolizidines
miniata	*Thermopsis montana*	N-methylcytisine **5** (and other quinolizidines)
sulphurea	*S. triagularis*	senecionine, **1**
sulphurea	*S. atratus*	sarracine **2**, platynecine **3**, **4**
sulphurea	*Lupinus argenteus*	quniolizidines

[2]Rueger and Benn (1983) failed to find senecionine in a Canadian collection.

DISCUSSION

There is now little doubt that the high variability in alkaloid content previously observed among *Castilleja* taxa has its genesis in variability of host plant use by this hemiparasitic genus. Although alkaloid transfer from host plant into obligate, chlorophyll-lacking parasites such as *Orobanche*, *Cuscuta*, and species in the mistletoe (Loranthaceae) family have been demonstrated (Wink et al., 1981; references in Atsatt, 1977; Kuijt, 1969), this appears to be the first unequivocal demonstration of such transfer into a hemiparasite. Some reviews quote the finding (Ubaev, et al., 1963) of two alkaloids typical of *Plantago* in *Pedicularis* as evidence for such a transfer. However, these were pyridine monoterpene alkaloids common to many Scrophulariaceae, and there was no evidence for transfer. The same workers, however, reported the presence of *N*-methylcytisine in *P. olgae*. This alkaloid is typical of *Lupinus* and does suggest that alkaloid transfer from *Lupinus* to *Pedicularis* has occurred.

The implications of these results for our studies on *Castilleja* ecology are far reaching and can only be adumbrated here until such time as detailed testing of new hypotheses can be accomplished. Several specific observations can, however, be made.

The particularly high alkaloid variability of *C. sulphurea* as compared to the other *Castilleja* taxa (Stermitz et al., 1986) is now explicable since *C. sulphurea* is far less restricted in habitat than the other taxa and can be found where it has exploited each of the three plant hosts containing either unsaturated pyrrolizidines (senecionine-type), saturated pyrrolizidines (sarracine-type), or quinolizidines. Are the *c. rhexifolia* and *C. rhexifolia* aff. *miniata* more restricted in habitat because their preferred alkaloid-containing hosts are also restricted to those habitats? This seems less likely since populations and individuals that are hosted by plant species which do not contain alkaloids seem to be sustainable.

The question of specificity in host-plant choice is a particularly difficult one to answer at this time. In a short and clearly incomplete listing of *Castilleja* host plants, 10 separate genera from six families were noted (Heckard, 1962). There seems to be no reason why the list could not approach that of *Pedicularis*, also of the Scrophulariaceae, where 80 species from 35 families were identified as hosts for *P. canadensis* (Piehl, 1963). It has been suggested (Kuijt, 1969) that all hemiparasites are nonspecific. Yet some specificity must occur since we found no cases where populations of a single taxon contain both pyrrolizidine- and quinolizidine alkaloids. In several habitats there is clear opportunity for populations to choose among several different alkaloid-containing host-plant species yet only one appears to be utilized. Particularly striking were the results near Emerald Lake where *C. sulphurea* and *C. miniata* grow intermixed. The former contains only sarracine and platynecines, while the latter contains only quinolizidines. The presumed host plants, *S. atratus* and a *Lupinus* species are

part of the same habitat as are *Delphinium* and other alkaloid-containing genera of the Ranunculaceae and Boraginaceae whose particular types of alkaloid structures have not as yet been encountered in *Castilleja*. Whether or not this specificity can be due to recognition of an individual alkaloid, perhaps as an inducer of haustoria formation (Steffens et al., 1982), is yet to be determined.

A seminal paper (Atsatt, 1977) proposed parallelisms in ecological and evolutionary patterns between herbivorous insects on the one hand and parasitic seed plants on the other. Many of the concepts and terminologies of herbivory theory were suggested to be applicable to the parasitic seed plant system and a number of suggestions were made for specific exploitation of these ideas. Of particular importance was a discussion of "secondary utilization of host defensive compounds," focusing on the possible chemical differences among *Orthocarpus* and *Plantago* hosts for the checkerspot butterfly *Euphydryas editha*, depending upon the inclination for *Orthocarpus* to be parasitic on *Plantago*. Clearly, if alkaloids are important as plant defensive substances (Wink, 1985), their presence or absence in individual plants of a given *Castilleja* population which is hosting an insect herbivore should profoundly affect individual plant choice and utilization. We are currently investigating this hypothesis in the cases of *Euphydryas*, *Chlosyne* (*Thessalia*), and *Amblyptilia* hosted by *Castilleja*. In addition to an exploration of the effect of alkaloid presence on insect utilization in natural populations, one can also now design field experiments for the study of alkaloids *per se* as defensive substances. Identification of alkaloid-containing and alkaloid-free individual plants in an otherwise uniform species population would allow for relatively natural control and test samples for comparative growth and survival studies.

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PREPARATION OF 8-METHYL-2-DECANOL: General Synthesis of Diastereomeric Mixtures of Alkyl Branched Insect Pheromones[1]

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Abstract—A general method for synthesis of insect pheromones having alkyl branched carbon skeletons is demonstrated with the preparation of a diastereomeric mixture of 8-methyl-2-decanol, whose propionate is an attractant of some *Diabrotica* species. The procedure involves reaction of a ketone with lithium acetylide ethylenediamine complex to afford a propargylic alcohol containing the branch of the target molecule. Copper (1) mediated alkylation of the derived propargylic acetate with a primary alkyl halide yields a trisubstituted allene having the desired chain length, and isomerization with an alkali metal amide of either ethylenediamine or 1,3-diaminopropane, affords the alkyl branched terminal acetylene. The triple bond is converted to the methyl ketone and reduced to the methyl carbinol. The reactions proceed in good yield, and can be conveniently carried out on large scale. The method should prove useful for production of pheromone components in cases where diastereomeric mixtures can be employed.

Key Words—Alkyl branched, pheromone, triple bond, alkyne, allene, isomerization, 8-methyl-2-decanol, 8-methyl-1-decyne.

INTRODUCTION

The sex pheromone of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte has been identified as the propionate of 8-methyl-2-decanol, and the synthetic racemic mixture is reported to attract western corn rootworms at all concentrations tested (Guss et al., 1982). Males of the Mexican corn rootworm, *D. virgifera zeae* Krysan and Smith, and *D. porracea* Harold are also attracted by the racemic mixture (Guss et al., 1984). Males of the northern corn

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rootworm, *D. barberi* Smith and Lawrence, are attracted to the racemic mixture in low doses, but at higher doses the 2*S*,8*R* and 2*S*,8*S* isomers inhibit attraction (Guss et al., 1985). Syntheses of both the racemic mixture (Guss et al., 1982) and the four stereoisomers of 8-methyl-2-decanol have recently been published (Sonnet and Heath, 1982; Sonnet et al., 1985; Mori and Watanabe, 1984).

Many other insect pheromones have been found to have alkyl branched carbon skeletons (Rossi, 1978). In some cases the racemic mixtures have been found to be biologically active. Among these is the pheromone of the southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber, which has been identified as 10-methyl-2-tridecanone; the *R* enantiomer has been found to be active while the *S* enantiomer is inactive and not inhibitory (Guss et al., 1983). The sex pheromone of the peach leafminer moth, *Lyonetia clerkella* Linne, 14-methyl-1-octadecene has been shown to have the *S* configuration; the *R* enantiomer was found to be inactive (Manabe et al., 1985). The terminal alcohol, aldehyde, and ester of 14-methyl-8-hexadecenyl skeleton, with both *cis* and *trans* double bonds have all been identified as pheromones (Rossi, 1978). Synthesis and testing of the optically active forms of (Z)-14-methylhexadec-8-enal, the pheromone of the female dermestid beetle, *Trogoderma inclusum* LeConte revealed that the (*R*)-(−)-enantiomer was the active compound, and the *S* isomer was biologically inactive (Mori et al., 1978).

We have developed a general synthesis of this class of alkyl branched insect pheromones based upon an extension of our modifications (Abrams and Shaw, 1986; Abrams, 1982) to known acetylene isomerization chemistry (Brown and Yamashita, 1975). The method is illustrated with the preparation of 8-methyl-2-decanol, an intermediate in the synthesis of the western corn rootworm pheromone. The advantages of the present synthesis over those in the literature are that the procedure is brief and the starting materials are readily available. The yields are good, and the process should be amenable to production of large quantities of pheromone.

METHODS AND MATERIALS

The reactions that required dry conditions were performed in oven-dried (110°C for more than 2 hr) glassware, under a positive pressure of argon. 1,3-Diaminopropane was distilled from barium oxide and stored over activated 4A sieves.

[^{1}H]NMR spectra were recorded on a Bruker AM-360-WB (360 MHz) spectrometer, employing $CDCl_3$ as solvent with $CHCl_3$ as reference. IR spectra were obtained with a Perkin Elmer 237B instrument. Gas chromatographic separations were carried out with a Varian 3700 instrument equipped with a DB-5 capillary column (J and W Scientific, 30 m) and a flame ionization detector. GC-MS spectra were obtained by using a DB-5 column (60 m) in a Finnigan

4000 E instrument in the electron impact mode with an Incos 2300 data system. Mass spectra are reported in mass to charge units (m/z) with the relative intensities as percentages of the base peak given in parentheses.

3-Methyl-3,4-decadiene. To a suspension of CuI (10.47 g, 55.0 mmol) in (1964), to 3-methyl-1-pentyn-3-ol (Aldrich, 98 g, 1.00 mmol) was added a solution of H_3PO_4 (1.5 g) in acetic anhydride (122.5 g, 1.20 mol) at such a rate that the internal temperature did not exceed 60°C. The reaction mixture was allowed to stand overnight at ambient temperature and then was poured into ice water and extracted with ether (3×). The combined ethereal extracts were washed with saturated Na_2CO_3 solution until the aqueous phase was basic, and then further washed with water (1×) and NaCl solution (1×), then dried over Na_2SO_4, filtered, and distilled to afford 3-acetoxy-3-methyl-1-pentyne (126 g, 90%) which had bp 46–49°C at 11 mm Hg, and which gave N_D^{25} 1.4232 [lit. N_D^{25} 1.4212, Julia et al., (1964)].

3-Methyl-3,4-decadiene. To a suspension of CuI (10.47 g, 55.0 mmol) in diethyl ether (anhydrous, 100 ml) at −78°C, was added a solution of pentyl magnesium bromide in ether [prepared from bromopentane (18.12 g, 120 mmol), magnesium turnings (2.92 g, 120 mmol), and ether (90 mL)] at such a rate that the internal temperature was maintained at approximately −45°C. After stirring for 10 min, a solution of 3-acetoxy-3-methyl-1-pentyne (7.5 g, 50 mmol) in ether (50 ml) was added dropwise with the internal temperature again maintained at −45°C. After continued cooling for 1 hr, the temperature of the mixture was allowed to rise to ambient, was stirred for a further 2 hr, and then was poured into saturated NH_4Cl solution. The organic phase was separated and washed with H_2O (3×) and then with saturated NaCl solution (1×), and dried briefly over Na_2SO_4. Distillation afforded 3-methyl-3,4-decadiene (bp 62–68°C at 10 mm Hg, 5.0 g, 33 mmol, 66%) contaminated with decane (2.4 g, estimated by [^{1}H]NMR).

From another run the allene was obtained in pure form by careful distillation (bp 100°C at 90 mm Hg) and gave [^{1}H]NMR ($CDCl_3$, 360 MHz, rel. to TMS) δ: 5.02 (m, 10 lines, C=C=CH, 1H), 1.9 (m, $H_2CC{=}C{=}C$, 4H), 1.66 (d, J = 2.8 Hz, $H_3CC{=}C{=}C$, 3H), 1.2–1.4 (m, CH_2, 6H), 0.97 (t, J = 7.4 Hz, $\underline{H_3}CCH_2C{=}C{=}C$, 3H), and 0.88 (t, J = 7 Hz, $\underline{H_3}CCH_2CH_2$, 3H); IR (film) ν_{max} 1960 cm^{-1} (w); MS (CI) 153 (13, $M^+ + 1$), 151 (42), 96 (51), 95 (53), and 83 (100); MS (EI) 152 (M^+, absent), 96 (57), and 81 (100).

8-Methyl-2-decanol. To the isomerization reagent (prepared from lithium (420 mg, 60 mmol), 1,3-diaminopropane (20 ml, dry, previously distilled from BaO and stored over 4A sieves) and potassium *t*-butoxide (4.95 g, 45 mmol), according to Abrams and Shaw (1986), at room temperature was added a solution of 3-methyl-3,4-decadiene (1.3 g, 8.6 mmol) in 1,3-diaminopropane (5 ml). The mixture was stirred for 1.5 hr, then poured into ice water and extracted with pentane (4× 50 ml). The pooled pentane extracts were washed succes-

sively with water, 10% HCl solution, and saturated NaCl solution, then dried over Na_2SO_4, and filtered; the solvent was evaporated at atmospheric pressure.

To the residue was added water (0.9 ml, 50 mmol), ethanol (10 ml), and mercuric-ion-impregnated ion-exchange resin (500 mg; Olah and Meidar, 1978). The mixture was stirred for 6.5 hr, then was filtered through a fine sintered glass funnel, and the catalyst was washed with ethanol (50 ml). $NaBH_4$ (1.14 g, 30 mmol) was added to the filtrate, and the mixture was stirred for 0.75 hr, then diluted with water, and extracted with hexane (3×). The combined organic extracts were dried over Na_2SO_4, and the solvent was removed at reduced pressure. The product was purified by silica gel chromatography eluting first with hexane to remove nonpolar impurities, and then with 50% ether–50% hexane to obtain the chromatographically pure product 8-methyl-2-decanol [570 mg, 3.3 mmol, 38% from the allene which gave NMR and IR data identical with those reported by Mori and Watanabe (1984)].

RESULTS AND DISCUSSION

The principle upon which the synthesis was based is outlined in Figure 1. The alkyl branch is constructed first by the well-known reaction of a ketone with lithium acetylide ethylenediamine complex (for example, Schmidt, 1976) affording the propargylic alcohol. The carbon skeleton is completed by alkylation of the derived propargylic acetate with a Grignard or organolithium reagent of a straight chain primary halide in the presence of cuprous salts. This reaction is known to proceed cleanly and in good yield to give trisubstituted allenes (Rona and Crabbe, 1969).

FIG. 1. General synthesis of alkyl branched pheromone components.

The key step of the synthesis is the isomerization of the allene to the triple bond at the terminus of the chain. The rearrangement of triple bonds of hydrocarbons (Brown and Yamashita, 1975) and alkynols (Abrams and Shaw, 1986) mediated by the very strongly basic isomerization reagents, alkali metal amides of ethylenediamine or 1,3-diaminopropane, has been employed to prepare terminal alkynes in high yields. Disubstituted allenes have been observed as intermediates in such rearrangements (Abrams and Shaw, 1985). Allenes, particularly trisubstituted ones, have not before been used as starting materials.

The terminal acetylene thus produced is a versatile intermediate that can be transformed as desired using well-established chemical procedures. Thus, either carbon can be oxygenated or the triple bond alkylated affording the disubstituted alkyne, or *cis* or *trans* olefin.

Based upon this scheme, the synthesis of 8-methyl-2-decanol has been undertaken (Figure 2), employing as the starting material commercially available 3-methyl-1-pentyn-3-ol, the product of reaction of butanone and lithium acetylide ethylenediamine complex. The acetate of 3-methyl-1-pentyn-3-ol was constructed according to the method of Julia et al. (1964) in 90% yield. The desired carbon skeleton was prepared by alkylation of the acetate with pentyl magnesium bromide and cuprous iodide in either to afford the allene, 3-methyl-3,4-decadiene. In our hands the Grignard reagent gave slightly higher yields than the corresponding alkyllithium reagent. A by-product of the reaction, decane, due to coupling of the Grignard reagent with itself, codistilled with the allene, but did not interfere with subsequent steps and was removed at the end of the sequence. The yield of the allene after distillation was determined to be 66% by [^{1}H]NMR.

Remote functionalization of the hydrocarbon chain and manipulation of the triple bond to the secondary alcohol was carried out in a three-step sequence

OAc

$C_5H_{11}MgBr/CuI$

66%

I

$LiHN(CH_2)_3NH_2/KOtBu$

$H_2N(CH_2)_3NH_2$

II

Hg^{++} Ion exchange resin

O

III

$NaBH_4$

OH

IV 39% from I

FIG. 2. Synthesis of 8-methyl-2-decanol.

that required no purification of intermediates. The allene, on being subjected to the strongly basic reagent, the mixed lithium–potassium salt of 1,3-diaminopropane, gave the terminal alkyne, 8-methyl-1-decyne, contaminated with decane and approximately 10% of by-products that appeared by NMR to be mainly isomeric conjugated dienes. These by-products were not investigated further as they did not interfere with the subsequent steps and could be removed in the final chromatography.

The isomerization reaction has also been carried out with ethylenediamine, and the same distribution of products was observed. In general, the yields in such rearrangement reactions have been observed to be slightly higher with 1,3-diaminopropane, but the lower cost of ethylenediamine may make it more attractive in some applications.

The crude isomerization product was conveniently hydrated to the methyl ketone in ethanol employing an ion-exchange resin to which mercuric ion was bound (Olah and Meidar, 1978). Other standard hydration techniques were also effective (Abrams, 1981). Filtration of the catalyst and reduction of the product with sodium borohydride gave the alcohol. The 8-methyl-2-decanol was purified by chromatography to separate the hydrocarbon by-products from the more polar alcohol. The yield of pure 8-methyl-2-decanol obtained from 3-methyl-3,4-decadiene was 39%.

The short sequence demonstrates the general method for building up alkyl branched hydrocarbons using triple-bond isomerization chemistry. The procedure is limited to the synthesis of racemic mixtures. Other work in our laboratory (Abrams and Shaw, submitted for publication) has shown that stereochemistry at the alkyl branch is lost on subjection of allenes and alkynes to the isomerization conditions.

It is hoped that the procedure described here will find use in syntheses of other alkyl branched insect sex pheromones.

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AN EXAMINATION OF THE PHYTOTOXICITY OF THE WATER SHIELD, *Brasenia schreberi*

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Abstract—Antialgal, antibacterial, and allelopathic properties of the submersed, aquatic, dicotyledonous water shield, *Brasenia schreberi* Gmelin (Cabombaceae), were examined. Extracts of *B. schreberi* were found to inhibit the eukaryotic alga, *Chlorella pyrenoidosa*, and the prokaryotic alga, *Anabena flosaqua*, as well as nine different bacteria. Lettuce seedling bioassay showed *B. schreberi* to be allelopathic. These phytotoxic properties probably contribute to the observed dominance of *B. schreberi* in aquatic environments and also make it a promising candidate for allelopathic plant management of aquatic weeds.

Key Words—Phytotoxicity, allelopathy, *Brasenia schreberi*, Cabombaceae, water shield, antibiosis, bacteria, algae.

INTRODUCTION

Brasenia schreberi Gmelin (Cabombaceae), common name water shield, is a submersed, aquatic dicotyledonous plant widely, sporadically distributed in North and Central America, West Indies, East Asia, and Australia. Although the plant was first described in 1789, studies of this unique species have been largely restricted to anatomy, morphology, and systematics. Submersed organs of *B. schreberi* are remarkable in that they are covered by a thick, clear mucilagenous substance. This mucilage originates from secreting glands which project into the air spaces of organs (Schrenk, 1888). Interest in this mucilage has led to its characterization as a polysaccharide with an extremely complicated structure (Misaki and Smith, 1962; Kakuta and Misaki, 1979a,b).

Brasenia schreberi is not an exotic or imported plant species, yet its pop-

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ulations appear to be the sole occupants within any area in which they occur in large numbers. Occasionally very dense populations impair boat travel (Burkhalter, 1978). Once *B. schreberi* is established in an area, growth of other plants may well be inhibited by shading effects of its densely packed floating leaves. Observations of sequences of events over a three-year period within dense populations of fragrant white waterlilies (*Nymphaea odorata* Ait.) indicate that once a single water shield propagule is established within the area, the species rapidly becomes dominant, leading to the exclusion of other species. The small size of the *B. schreberi* leaves relative to the waterlilies suggests that shading is not the principle competitive factor involved in water shield's rapid dominance. Both species are perennials, exhibit partial leaf retention throughout the winter, and resume spring growth at about the same time. Other factors must be involved; this paper examines some possible factors for the observed dominance of *B. schreberi*.

METHODS AND MATERIALS

Plant Collection and Processing. Brasenia schreberi was collected during the late spring and throughout the summer from a population along US 98, a highway five miles west of Hattiesburg, Mississippi, in Lake Serene. The plants were washed free of debris, dried at 55°C, coarsely ground in a commercial meat grinder, and stored at room temperature in plastic bags until used.

Extraction Solvents. Reagent grade solvents were glass distilled in our laboratory prior to use.

Extraction and Fractionation. A 20-g portion of the dried plant material was extracted with 400 ml of methanol for 24 h in a Soxhlet apparatus. The extract was concentrated under reduced pressure, and the concentrate was subjected to antialgal and antibacterial bioassay. The concentrate was fractionated according to the following scheme, and each fraction (A–E) was subjected to the same antialgal and antibacterial bioassays as the unfractionated concentrate. The concentrate was extracted with 5% aqueous HCl. The acid-insoluble portion was taken up in chloroform and filtered to give a chloroform-insoluble fraction (C) and a chloroform-soluble fraction which was concentrated and partitioned between 90% CH_3OH and hexane to give, after concentration, fractions A and B, respectively. The acid-soluble fraction was neutralized to pH 7 with sodium hydroxide, and the now neutral fraction was extracted with three portions of ethyl ether. The combined ether extracts were concentrated and labeled fraction D. The neutral fraction remaining was made strongly basic with sodium hydroxide and reextracted with ether. This ether extract was concentrated and labeled fraction E.

A second portion of dried plant material was successively extracted for four days each with petroleum ether, ethyl ether, acetone, and methanol in a

Soxhlet apparatus with about 1000 ml solvent per 250 g dried plant material used. After concentration, the petroleum ether, ethyl ether, and acetone extracts were subjected to lettuce seedling bioassay.

Antialgal Bioassay. Axenic cultures of *Anabena flosaqua* UTEX 1444 and *Chlorella pyrenoidosa* UTEX 26 were obtained from Dr. Richard Star, University of Texas algae collection, kept in culture in Bold's modification of Bristol's medium (Bold, 1949), and subcultured onto slants of this same medium solidified with agar. For assays, 9-cm Petri dishes containing Bold's basic agar were inoculated either with 0.1–0.2 ml of settled cells removed from the liquid culture after turning off the aerator or with a portion of an inoculum prepared by vortex mixing of that number of algal cells which could be removed from the agar slants with a sterile loop with a 0.3-ml to 1.0-ml portion of sterile water. The sample being tested was dissolved in an appropriate solvent, and a small sterile filter paper disk was saturated in the solution. The solvent was allowed to evaporate, and the sample paper disk was placed on the algal inoculated test plate. Control dishes included plain paper disks and disks which had been saturated in the solvent(s) used to dissolve samples and allowed to evaporate for an equivalent period of time. The test was read when a control plate (no sample or control disks) first showed good growth. Tests were scored as $-$ (no inhibition) and $+$, $++$, or $+++$ for degrees of inhibition. Since neither the algal concentration nor the sample concentration is known, the results are qualitative.

Antibacterial Bioassay. Antibacterial activity was determined by a modified procedure of the Bauer-Kirby single disk method (Bauer et al, 1966). Standard bacterial cultures of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella gallinarium*, *Klebsiella pneumoniae*, and *Mycobacterium smegmatis* were obtained from the University of Southern Mississippi Biological Sciences Department. Three bacterial strains were cultured from water taken from Lake Serene where the *B. schreberi* was collected but from an area where it was not growing. One of these latter three was subsequently identified as *Chromobacterium violacens*, a gram-negative bacterium. The other two remain unidentified. The bacteria were kept in culture on nutrient agar slants. For assays, a sterile loop was used to remove cells which were mixed with 10 ml of water; 9-cm Petri dishes containing nutrient agar were inoculated with 0.1 ml of this mixture. Sample-saturated paper disks were prepared in the same manner as for antialgal bioassay; the plates were scored in a similar manner.

Lettuce Seedling Growth Bioassay. Radical growth inhibition assay of lettuce (black-seeded Simpson) was carried out as previously reported (Elakovich and Stevens, 1985).

Data Analyses. The results of the lettuce seedling growth bioassay were analyzed separately using a Honeywell DPS-8 mainframe computer and a Statistical Package for the Social Sciences, version 9 (Hull and Nie, 1981). Statis-

tical analyses included one-way analysis of variance and the Duncan multiple-range test for differences among all treatment means.

RESULTS

Antialgal Bioassays. The methanol extract of dried, ground *B. schreberi* inhibited the growth of the eukaryotic alga, *Chlorella pyrenoidosa*, and, to a lesser extent, also inhibited the prokaryotic alga, *Anabena flosaqua* (Table 1). All fractions (A–E) obtained from *B. schreberi* also inhibited *C. pyrenoidosa*. Only fractions C and D inhibited the growth of *A. flosaqua*; C was the more active fraction.

Antibacterial Bioassays. The methanol whole-plant extract as well as all fractions (A–E) were tested against a standard culture of *Salmonella gallinarium* and against the three bacteria cultured from the lake where *B. schreberi* was collected. All fractions were inhibitory except for fraction C, which was inactive toward *S. gallinarium* and whose activity toward the unknown gram-negative bacterium was in question (Table 2). Fractions A–E were also tested against five additional bacterial strains, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Mycobacterium smegmatis*, and *Staphylococcus aureus*. Activity was least toward *K. pneumoniae* and greatest toward *S. aureus* and *M. smegmatis*. Both gram-negative and gram-positive bacteria were inhibited, but no pattern of inhibition related to Gram stain characteristic could be detected.

Lettuce Seedling Growth Bioassay. The three Soxhlet extracts tested, Skelly F, ethyl ether, and acetone, all caused reduction in lettuce seedling root growth (Table 3). The Skelly F extract was only slightly water soluble and oiled out of the aqueous agar solution in concentrations higher than 375 ppm. At this concentration, it reduced lettuce radical growth by 24% as compared to the control, a result which was not significantly different from the 28% reduction shown by

TABLE 1. EFFECT OF *Brasenia schreberi* ON ALGAL GROWTH[a]

Fraction assayed	*Anabena flosaqua*	*Chlorella pyrenoidosa*
CH_3OH whole plant extract	+	++
A	−	+
B	−	+
C	++	+
D	+	++
E	−	++

[a] A minus sign indicates no inhibition. Inhibition was scored as +, ++, or +++.

TABLE 2. EFFECT OF *Brasenia schreberi* ON BACTERIAL GROWTH

Challenge organism	Fraction assayed[a]					
	Whole plant	A	B	C	D	E
Chromobacterium violacens	++	++	+	++	++	+++
Unknown gram-positive bacterium	++	+	+	+	++	++
Unknown gram-negative bacterium	++	+	+	(+)[b]	++	++
Staphylococcus aureus		++	++	++	+++	+++
Bacillus subtilis		+	+	+	++	++
Escherichia coli		+	++	+	+++	+++
Salmonella gallinarium	++	+	+	−	++	++
Klebsiella pneumoniae		(+)[b]	+	−	+	+
Mycobacterium smegmatis		++	++	++	+++	+++

[a] See footnote "*a*" of Table 1.
[b] Results were not definitive.

the 100-ppm sample level. This would suggest that 25% is close to the maximum inhibition level achievable by the Skelly F extract. At 100 ppm, the ethyl ether extract reduced radical growth by 14%, but this reduction was not significantly different from the control. At 500 ppm, this extract reduced radical growth by 65%. The acetone extract was the most active, reducing radical growth by 39% at 100 ppm and by a dramatic 78% at 500 ppm. Neither the

TABLE 3. EFFECT OF SOXHLET EXTRACTS OF *Brasenia schreberi* ON RADICAL LENGTH OF LETTUCE SEEDLINGS

Extract	Concentration (ppm)	Mean root length (mm)[a]	% of control	N[b]
Skelly F	0	23.6a		33
	100	16.9b	72	10
	375	18.0b	76	10
Ethyl ether	0	23.6a		33
	100	20.4a	86	10
	500	8.3b	35	10
Acetone	0	23.6a		33
	100	14.5b	61	10
	500	5.2c	22	10

[a] Means associated with a given extract with different letters are significantly different at $P \leq 0.05$ determined by the Duncan multiple-range test. The same control served for all three extracts.
[b] Number of roots measured.

ethyl ether nor the acetone extract were tested at concentrations greater than 500 ppm. It is possible both extracts would have demonstrated even greater radical growth inhibition at higher concentrations.

DISCUSSION

Integrated approaches to aquatic plant management are increasingly considering use of allelopathic plant species. A search of the literature shows that most aquatic plants thus far reported to have allelopathic potential are *not* deep water plants. Thus *B. schreberi* with its antibacterial activity, its antialgal activity, and its ability to inhibit lettuce seedling radical growth is a promising candidate for allelopathic plant management of aquatic weeds. The ability of *B. schreberi* to inhibit the three bacterial strains cultured from lake water where the plant was collected, but not growing, suggest that its antibacterial activity may contribute to its rapid dominance. Growth inhibition of the six additional standard bacterial strains suggests the antibacterial activity of *B. schreberi* is broad-based rather than focused. This is underscored by the fact that both gram-negative and gram-positive bacteria and both eukaryotic and prokaryotic algae were inhibited. The fact that inhibitory activity is displayed by the different fractions A–E and by the Skelly F, ether, and acetone Soxhlet extracts suggests that *B. schreberi* produces a series of allelochemicals. Studies are curently underway to determine the chemical nature of the allelochemicals of *B. schreberi*.

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VARIATIONS IN MOUSE (*Mus musculus*) URINARY VOLATILES DURING DIFFERENT PERIODS OF PREGNANCY AND LACTATION

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Abstract—Mouse urine samples from different pregnancy and lactation periods were examined by capillary gas chromatography to assess variations in the volatile signals that may affect the endocrine function of other females. Statistically significant changes in the excretion of certain urinary volatiles were observed; from 26 readily quantifiable constituents, 14 appear to be under the endocrine control. These selected components, positively identified through mass spectrometry and retention data, and the synthetic standards are ketones, unsaturated alcohols, esters, and cyclic vinyl ethers.

Key Words—Female mouse urine, pregnancy, lactation, urinary volatiles, mouse, *Mus musculus*.

INTRODUCTION

In the house mouse (*Mus musculus*), there are at least three female urinary chemosignals that can affect the reproduction status of other females: (1) the delay of puberty occasioned by treatment with urine from grouped females (Drickamer, 1974, 1977, 1983a); (2) the acceleration of puberty by the urine from estrus females (Drickamer 1982a); and, (3) the acceleration of puberty (Drickamer and Hoover, 1979) and prolongation of the estrous period (Hoover and Drickamer, 1979) through exposure to the urine from pregnant and lactating females. It has been suggested (Drickamer, 1982b) that some chemical substances in the urine of females may act as the general signals to other conspecifics regarding the adequacy of social and environmental conditions which are important for successful reproduction.

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Urine samples collected at different time periods during pregnancy and lactation are differentially effective in terms of the acceleration of the first estrus relative to control females treated with water (Drickamer, 1983b, 1984). The chemosignals from pregnant/lactating females seem to be present in the urine only during the last two thirds of pregnancy or lactation (Drickamer, 1983b). In addition, Cowley and Wise (1972) reported that the urine from pseudopregnant females applied to the nasal region of neonatal females has an accelerating effect of their growth, while the application of urine from females in late pregnancy is associated with a slow rate of growth. It is not yet known what set of hormonal and related physiological conditions within the female mice may reflect these changes in the chemical signals released by donor females.

Since no chemical investigations on these signals have been conducted to date, their nature remains a subject of speculation. Earlier study by Drickamer and Hoover (1979) has shown that the urine from pregnant or lactating females, either applied daily on the external nares of the young animals or provided to them in a plastic (inaccessible) capsule, can accelerate their sexual maturation. In addition, the urine from pregnant or lactating females that is exposed to the ambient air remains effective for only one to three days (Drickamer, 1986). These findings suggest that the pregnancy/lactation chemosignal(s) is either an air-sensitive or a volatile substance.

The chemosignals from pregnancy urine and that of lactating animals may or may not contain similar substances. Both chemical characterization and biological experiments are needed to elucidate this problem.

Our previous successful uses of a headspace sampling technique (Novotny et al., 1974) and capillary gas chromatography–mass spectrometry to identify the male chemosignals responsible for aggression (Novotny et al., 1985), estrus synchronization (Jemiolo et al., 1986a), and the female signals causing the delay of puberty (Novotny et al., 1986) have prompted us to apply similar techniques to the identification of additional female chemosignals in this study. As the initial approach to this complex problem, the urinary profiles of volatile substances were quantitatively followed throughout the pregnancy and lactation periods. Statistically significant alterations in quantities of certain volatile components are reported here and correlated with the animals' endocrine status. The structures of these components were verified through their mass spectra and chromatographic behavior of authentic substances.

METHODS AND MATERIALS

All mice used as urine donors were from a randomly bred, closed colony of ICR/Alb (*Mus musculus*) purchased from Ward's Natural Science Establishment, Inc., Rochester, New York. Females were housed in plastic cages (12 × 28 × 27 cm), maintained at 21°C ± 0.2°C, 50–70% humidity, and a 12-

hr light–12-hr dark daily regime. Unlimited amounts of Purina Mouse Chow (Ralston Purina Corp., St. Louis, Missouri) and water were supplied throughout the whole experimental period. Bedding was changed weekly.

For use as urine donors, 120 virgin and 120 multiparous females, in total, were bred with fertile males. The occurrence of pregnancy was established by a daily examination for vaginal plugs (day 0). After insemination, each female was caged alone over the whole period of pregnancy. The urine from pregnant females was collected from days 2 to 7 (period 1), 8 to 12 (period 2), 13 to 15 (period 3), and from day 16 postplug to the last day before parturition (period 4). An additional 120 multiparous pregnant female mice were isolated into individual cages and checked daily for newborns. On the day of delivery, the litters were counted and reduced to 10 pups. Collection of urine from lactating females was done at the same periods as during pregnancy: days 2–7 (period 1), 8–12 (period 2), 13–15 (period 3), and from day 16 postpartum until the young were 21 days old.

All females used as urine donors were 80–130 days of age. Fresh urine was collected by holding a mouse over a glass vial and gently squeezing the abdomen and flanks. Immediately after collection, samples were stored at −20°C and analyzed within two to four days. All types of urine were analyzed three to six times by using each time a 1-ml sample obtained from different collections within the same experimental period. For each sample, the urine was collected and pooled from 8 to 10 pregnant or lactating females. As a standard sample, we used urine collected from virgin, nonreproducing females at 90–120 days of age, that were housed singly. Collection of the urine was carried out throughout all stages of the estrous cycle.

The urinary volatiles were analyzed using the headspace technique, employing Tenax GC (a porous polymer) as the adsorption medium (Novotny et al., 1974). The volatiles were sparged from 1-ml urinary samples at room temperature with purified helium gas at a flow rate of 100 ml/min and adsorbed onto a precolumn packed with 4 mg of Tenax GC. The sample was subsequently desorbed in the heated injection port (220–240°C) of a gas chromatograph (Perkin-Elmer, 3920 instrument, Norwalk, Connecticut) equipped with a flame ionization detector, and retrapped into a cooled section of a glass capillary column. The analytical column was a glass capillary (60 m × 0.25 mm, ID) of a soda-lime type, coated statically with UCON 50-HB-2000 (Schwende et al., 1984a,b).

While quantitative comparisons were obtained through peak integration routines (Sigma 10, Perkin-Elmer), identification of the individual profile constituents was established through a combined GC-MS (Hewlett-Packard, 5981 dodecapole mass spectrometer, Palo Alto, California) using electron impact ionization at 70 eV. Wherever feasible, authentic samples were either purchased or synthesized in the laboratory to verify agreement of spectral information and gas-chromatographic retention time.

Statistical Calculations. Statistical comparisons of the levels of excreted volatiles were made using one-way analysis of the variance (ANOVA) with Duncan's new multiple-range test (Zar, 1974). The probability level for statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSIONS

The complexity of mouse urinary volatile profiles has been demonstrated in our previous studies (Novotny et al., 1980, 1984, 1986; Schwende et al., 1984a,b, 1986; Jemiolo et al., 1986a,b); genetic background, sex, endocrine status, etc., all seem to influence the quantitative proportions of various urinary constituents. The capillary chromatogram shown in Figure 1 is representative of a profile obtained from pregnant female ICR/Alb mice. From a large number of components, 26 substances are numbered; most have been positively identified by combined capillary gas chromatography–mass spectrometry and the retention measurements of authentic compounds (Table 1).

Through careful visual inspection of the profiles obtained from different stages of pregnancy and lactation, as well as from nonreproducing females, we first established that 14 constituents showed obvious variations in their chro-

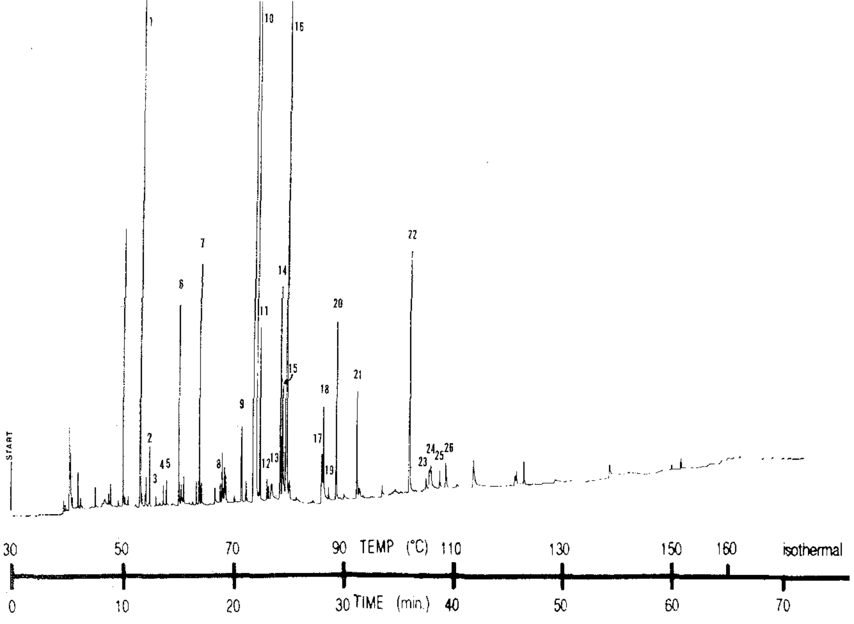

FIG. 1. Gas chromatogram of urine volatiles from ICR/Alb primiparous females at the 13th to 15th day of pregnancy (period 3).

TABLE 1. VOLATILE COMPOUNDS IN URINE OF PREGNANT PRIMIPAROUS (A); PREGNANT MULTIPAROUS (B); AND LACTATING (C) FEMALE MICE

Peak number	Structure	Changes in concentration of compounds during pregnancy and lactation		
		A	B	C
1	mol wt 126[a]	–	+[b]	–[c]
2	4-ethylcyclohexene	–	–	–
3	3-methyl-1-buten-3-ol	–	–	–
4	2-ethyl-5-methyl-furan	–	–	–
5	3-hexanone	–	–	–
6	2-hexanone	–	+	–
7	m.w 126[a]	–	+	–
8	4-heptanone	+	+	+
9	m.w 126[a]	–	–	–
10	2-heptanone	+	–	+
11	n-pentyl acetate	+	+	+
12	methyl hexanoate	–	–	–
13	6-hepten-2-one	–	–	–
14	cis-2-penten-1-yl acetate	+	–	+
15	trans-5-hepten-2-one	+	–	+
16	trans-4-hepten-2-one	–	–	+
17	4-penten-1-ol	+	+	+
18	unidentified ester of mol wt 142	+	–	+
19	2,5-dimethylpyrazine	–	–	–
20	6-methyl-6-hepten-3-one	+	+	–
21	6-methyl-5-hepten-3-one	+	+	–
22	dehydrobrevicomin	–	–	–
23	2-nonanone	–	–	–
24	benzaldehyde	–	–	–
25	unidentified	–	–	–
26	1-octen-3-ol	+	+	+

[a] Presumed isomeric cyclic vinyl ethers unique to the mouse (Schwende et al., 1986); dehydration products of known 5,5-dimethyl-2-ethyltetrahydrofuran-2-ol.
[b] Concentration of the compound dependent on a period of pregnancy or lactation.
[c] Concentration of the compound does not seem dependent on a period of pregnancy or lactation.

matographic peak areas (Table 1, see peaks numbered in bold type). The remaining substances listed in this table demonstrated relatively constant concentrations throughout all periods of pregnancy and lactation. The additional peaks in the chromatogram (Figure 1) have not been quantified for two reasons: (1) their small peak areas would not permit reliable measurements; and (2) for the early eluting constituents (substances with high volatility), peak areas are greatly affected by the sampiing procedure.

TABLE 2. MEAN VALUE (±SEM) OF PEAK AREAS (ARBITRARY UNITS) FOR VOLATILE COMPOUNDS FOUND IN URINE OF PREGNANT PRIMIPAROUS (A), MULTIPAROUS (B); AND LACTATING (C) FEMALES DURING DIFFERENT PERIODS OF PREGNANCY AND LACTATION[a]

Peak designation	Type of sample	Periods of pregnancy and lactation			
		1	2	3	4
Ketones					
6	A	3.9 ± 1.2	5.6 ± 0.3	4.6 ± 0.6	3.2 ± 0.8
	B	[a]2.4 ± 0.5	[b]4.9 ± 0.6	[a,b]3.2 ± 0.5	[a]2.4 ± 0.6
	C	1.4 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
8	A	[a]3.3 ± 0.6	[a]2.3 ± 0.2	1.0 ± 0.0	1.0 ± 0.1
	B	[a]2.6 ± 0.3	[b,a]1.5 ± 0.4	[a]2.7 ± 1.1	[b]1.0 ± 0.1
	C	1.0 ± 0.0	[a]4.2 ± 1.8	1.6 ± 0.6	0.4 ± 0.1
10	A	[a]150.3 ± 20.4	[b]77.0 ± 16.1	[b,a]140.9 ± 25.1	[c]211.1 ± 30.7
	B	98.7 ± 26.1	58.9 ± 3.7	56.9 ± 1.8	109.1 ± 20.4
	C	[a]91.1 ± 18.4	27.8 ± 2.8	32.5 ± 10.7	13.9 ± 1.3
15	A	[a]9.8 ± 1.5	[b]4.5 ± 0.9	[b,a]6.7 ± 0.8	[b,a]7.2 ± 1.0
	B	6.8 ± 1.5	4.2 ± 1.0	4.8 ± 0.7	4.5 ± 1.0
	C	[a]3.6 ± 1.0	0.5 ± 0.2	0.6 ± 0.2	0.4 ± 0.1
16	A	25.9 ± 2.4	18.8 ± 1.6	24.5 ± 5.5	21.9 ± 6.5
	B	17.6 ± 2.2	16.6 ± 1.0	19.2 ± 2.4	12.4 ± 1.1
	C	[a]14.3 ± 3.6	3.0 ± 1.1	3.4 ± 1.1	2.4 ± 0.4
20	A	[b]16.6 ± 2.5	[a]7.9 ± 1.3	[a]9.5 ± 2.4	[b,a]10.9 ± 1.3
	B	[b,a]9.9 ± 1.3	[b]6.5 ± 0.6	[b,a]10.6 ± 1.8	[a]13.0 ± 1.0
	C	5.2 ± 0.6	4.1 ± 0.6	5.7 ± 0.6	5.9 ± 0.7
21	A	[b]10.7 ± 1.9	[a]4.6 ± 0.9	[a]6.3 ± 2.2	[b,a]8.7 ± 0.2
	B	[a]5.4 ± 0.9	[a]3.4 ± 0.2	[b]8.4 ± 2.1	[b]9.5 ± 0.6
	C	4.5 ± 0.6	3.2 ± 0.4	4.6 ± 0.6	5.0 ± 0.3

Esters					
11	A	^{a}9.9 ± 3.2	^{b}1.9 ± 0.3	^{b,a}7.5 ± 3.0	^{c}30.7 ± 6.8
	B	2.1 ± 0.3	1.5 ± 0.1	3.4 ± 0.5	^{a}10.3 ± 1.4
	C	^{a}7.3 ± 2.8	0.7 ± 0.1	0.6 ± 0.1	0.2 ± 0.1
14	A	^{a}9.6 ± 2.0	^{b}4.9 ± 1.2	^{b,a}6.4 ± 1.1	^{a}8.3 ± 1.9
	B	2.7 ± 0.2	2.7 ± 0.2	4.4 ± 0.2	4.3 ± 0.8
	C	^{a}3.7 ± 1.2	0.3 ± 0.1	0.5 ± 0.1	0.2 ± 0.1
18	A	^{a}5.3 ± 0.8	^{b}2.1 ± 0.2	^{b,a}3.4 ± 0.7	^{a}7.2 ± 1.2
	B	4.3 ± 1.0	^{a}2.4 ± 0.2	3.5 ± 0.8	3.3 ± 0.6
	C	^{a}3.4 ± 0.2	0.9 ± 0.2	0.8 ± 0.01	0.5 ± 0.0
Alcohols					
17	A	2.6 + 0.6	1.2 ± 0.5	2.9 ± 1.0	^{a}10.6 ± 3.1
	B	^{a}1.5 ± 0.4	^{a}1.4 ± 0.3	^{b}5.7 ± 1.0	^{b}7.6 ± 1.7
	C	^{a}3.3 ± 0.9	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
26	A	^{b,a}2.9 ± 0.9	^{c}0.9 ± 0.0	^{c,a}1.8 ± 0.5	^{b}4.7 ± 0.0
	B	^{a}1.1 ± 0.0	^{a}1.2 ± 0.4	^{b}2.4 ± 0.4	^{b}2.3 ± 0.0
	C	^{a}3.1 ± 0.3	^{a}2.9 ± 0.7	^{b}1.4 ± 0.2	^{b}0.5 ± 0.0
Dihydrofurans					
1	A	35.5 ± 7.4	23.9 ± 5.0	29.3 ± 7.9	43.4 ± 7.3
	B	^{a}18.8 ± 3.1	^{a}17.9 ± 1.5	^{b,a}28.8 ± 2.2	^{b}34.8 ± 1.1
	C	20.1 ± 5.1	13.9 ± 1.5	11.4 ± 2.2	7.8 ± 4.0
7	A	10.3 ± 2.4	6.8 ± 1.6	7.7 ± 3.4	9.8 ± 0.7
	B	^{b,a}6.1 ± 1.4	^{a}3.9 ± 1.5	^{b,a}7.7 ± 1.7	^{b}9.8 ± 0.9
	C	6.8 ± 1.5	4.4 ± 0.4	3.4 ± 0.3	2.4 ± 0.9

[a] N = 3–6 runs for each average peak area value; 1 ml of urine collected from 8–10 females was used in one run. Those means not connected by the same vertical lines are significantly different at the 0.05 level. Those means in rows not marked by the same superscript letter (a, b, c) are significantly different at the 0.05 level. If there are no superscript letters in a row, there are no significant differences among the means.

The 14 components exhibiting some dependence on the animals' endocrine status can be readily classified into one of four structurally distinct categories: ketones, esters, alcohols, and dihydrofurans (cyclic vinyl ethers). The components selected for a careful quantitative evaluation in the urine of pregnant primiparous (A), pregnant multiparous (B), and lactating (C) females are listed in Table 2. A majority of the investigated urinary components tended to be more concentrated in pregnant primiparous than in either pregnant multiparous or lactating females. This trend was observed for all distinct periods; however, only in a few cases (Table 2) are the differences between the pregnant primiparous and pregnant multiparous animals statistically significant. On the other hand, statistical significance ($P < 0.05$) is obvious when comparing the levels of ketones, esters, alcohols, and dihydrofurans of lactating females with the pregnant animals. Substantial decreases were generally observed.

The levels of the 14 investigated compounds changed clearly during the different periods of pregnancy and lactation. Most of these appeared at a higher average level during the first period of pregnancy or lactation than in the second. Some of the ketones and esters that decreased in concentration at the second period of pregnancy have shown a tendency toward elevation in their levels during the consecutive periods (Table 2). In the urine of lactating females, most ketones and all esters also decreased initially during the second period, but maintained the same levels throughout the rest of the experiment. The concentrations of alcohols in female urine have a tendency to increase during the last periods of pregnancy. Yet, the same substances drop after the first period of lactation and remain at low levels until parturition.

In order to provide "reference levels" of the investigated urinary volatile constituents, samples from singly caged, nonreproducing females were analyzed under identical experimental conditions (Table 3). The average levels of the 14 volatile compounds found in the urine of pregnant and lactating females were then tabulated and compared to the same 14 peaks from urine of the nonreproducing females (control data). The percentage of the difference in the peak area of ketones, esters, alcohols, and dihydrofurans is shown in Figures 2–7. On the basis of our earlier work (Novotny et al., 1986), we have learned that the urine of group-caged females contains a similar set of volatile compounds, but the concentrations of such compounds were clearly different as compared to singly caged, nonreproducing animals. The data reflecting these percentage differences have been placed at the right side of each figure (Figures 2–7) to provide an additional point of comparison. Seven ketones (peaks 6, 8, 10, 15, 16, 20, and 21 of Figure 1) varied in concentration in the urine of pregnant primiparous females (Figure 2). 2-Hexanone (6), 4-heptanone (8), and 2-heptanone (10) exhibited concentrations that were significantly ($P < 0.05$) higher, as compared to those in nonreproducing animals, throughout all distinct periods.

TABLE 3. MEAN VALUE (±SEM) OF PEAK AREAS (ARBITRARY UNITS) FOR SELECTED VOLATILE COMPOUNDS FOUND IN URINE OF SINGLY CAGED, NONREPRODUCING FEMALES[a]

Peak designation	Structure	Average levels
1	mol wt 126	43.2 ± 2.7
6	2-hexanone	2.5 ± 0.4
7	mol wt 126	12.7 ± 0.8
8	4-heptanone	1.3 ± 0.1
10	2-heptanone	52.1 ± 4.3
11	*n*-pentyl acetate	4.1 ± 0.6
14	*cis*-2-penten-1-yl acetate	4.9 ± 0.5
15	*trans*-5-hepten-2-one	4.8 ± 0.3
16	*trans*-4-hepten-2-one	18.3 ± 1.2
17	4-penten-1-ol	1.5 ± 0.3
18	mol wt 142 ester	3.0 ± 0.2
20	6-methyl-6-hepten-3-one	12.3 ± 0.7
21	6-methyl-5-hepten-3-one	7.5 ± 0.5
26	1-octen-3-ol	1.4 ± 0.7

[a] N = 13–17 runs for each peak area value; 1 ml of urine collected from 10–20 females was used in one run.

The remaining ketones, *trans*-5-hepten-2-one (15), *trans*-4-hepten-2-one (16), 6-methyl-6-hepten-3-one (20), and 6-methyl-5-hepten-3-one (21) exhibited either an increase or decrease in concentration compared to nonreproducing females, but the range of changes was not significant.

In the urine of group-caged females, the concentrations of 2-hexanone (6) and 4-heptanone (8) were similar to those appearing in nonreproducing animals. However, this type of urine contained additional ketones, such as 2-heptanone (10), *trans*-5-hepten-2-one (15), and *trans*-4-hepten-2-one (16) in higher concentration than did the urine from nonreproducing females (Figure 2).

The increases observed for ketones in the urine of pregnant multiparous animals were less than in pregnant primiparous females. However, a trend toward more elevated levels of 2-hexanone (6), 4-heptanone (8), and 2-heptanone (10) as compared to the control group of females was still noticeable (Figure 3). Two compounds, 6-methyl-6-hepten-3-one (20) and 6-methyl-5-hepten-3-one (21), have become significantly suppressed in the pregnant multiparous animals as compared to nonreproducing females (Figure 3).

For all the ketones, the most dramatic changes were seen in the urine of the lactating females (Figure 4). There was a clear suppression of these compounds, from 45–95%, compared to nonreproducing females. That suppression

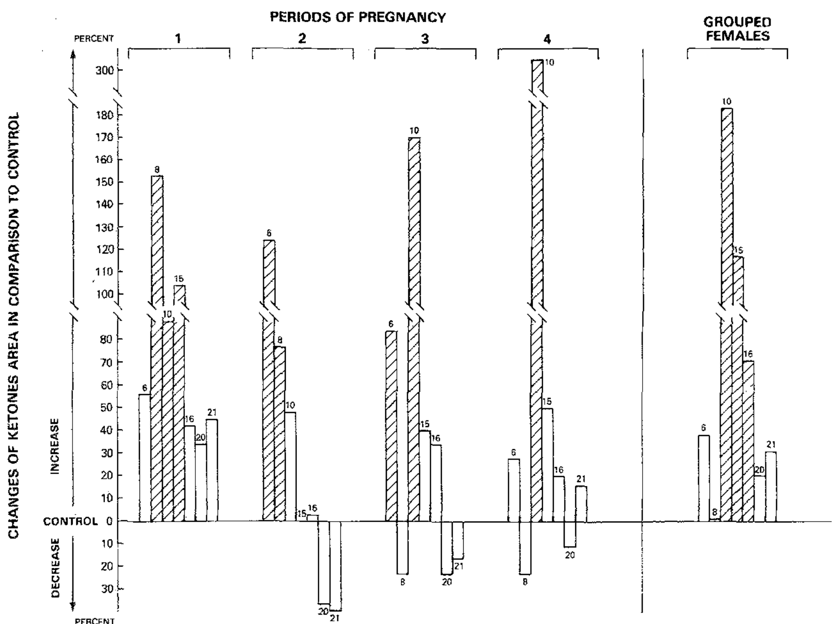

FIG. 2. Percentage difference in the peak areas of urinary ketones between pregnant primiparous and nonreproducing (control) females of ICR/Alb strain. Diagonally striped bars represent significant increases of ketones in pregnant and grouped females as compared to controls.

was observed throughout all lactation periods. Two exceptions were noted: for 2-heptanone during the first period, and for 4-heptanone during the second period. The latter substance attained a level never noted before with any urine sample.

The levels of esters are dealt with in Figure 5; *n*-pentyl acetate (11), 2-penten-1-yl acetate (14), and an unidentified ester (18) showed both increases and decreases in urines of pregnant, lactating, and group-caged females as compared with the control. Here, the concentration of esters is variable and dependent on a type of urine and period of collection. Grouped and pregnant females produced these compounds in very high concentrations only during the first and last periods of pregnancy. In the mid-pregnancy intervals, these esters were found at a low level (similar to the control group). The urine collected at the first period of lactation (up to seven days) contained 80% more 2-pentyl acetate (11) than did the urine from nonreproducing females. During the next three periods, the concentration of esters in the urine of females dropped significantly (Figure 5).

The same type of comparison used for ketones and esters was also em-

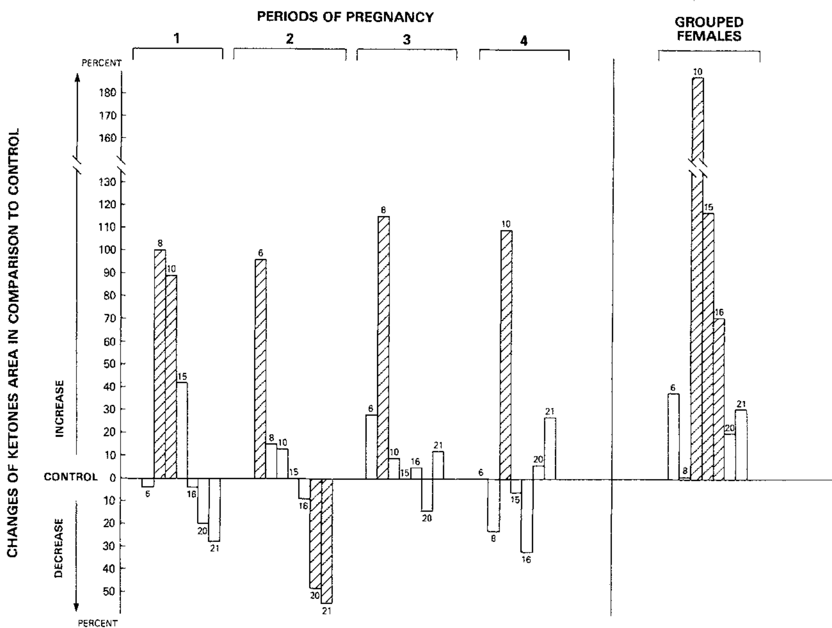

FIG. 3. Percentage difference in the peak areas of urinary ketones between pregnant multiparous and nonreproducing (control) females of ICR/Alb strain. Diagonally striped bars represent significant increases and decreases of ketones in pregnant and grouped females as compared to controls.

ployed for alcohols and dihydrofurans (see Figures 6 and 7). Extremely high levels of alcohols were found in the urine of pregnant females, both primiparous and multiparous (Figure 6). In both types of urine, an elevation of 4-penten-1-ol (17) and 1-octen-3-ol (26) appeared during the last period of pregnancy rather than at the beginning. The urine of grouped females also contained alcohols in very high concentration. Only the urine from lactating females showed no changes in the concentration of alcohols, as compared to controls, during the last periods. However, in the first period of lactation, the levels of alcohols were significantly different from those of control females (Figure 6).

The last group of investigated compounds, the dihydrofurans of molecular weight 126, were structurally postulated in a previous publication (Schwende et al., 1986). For these "mouse-specific" compounds, significant variations were found as well (Figure 7). Concentration of the dihydrofurans was decreased 20–45% in the urine of pregnant primiparous females, while the samples from pregnant multiparous and lactating animals exhibited levels 35–80% lower.

Although a wealth of statistically significant data has been acquired here

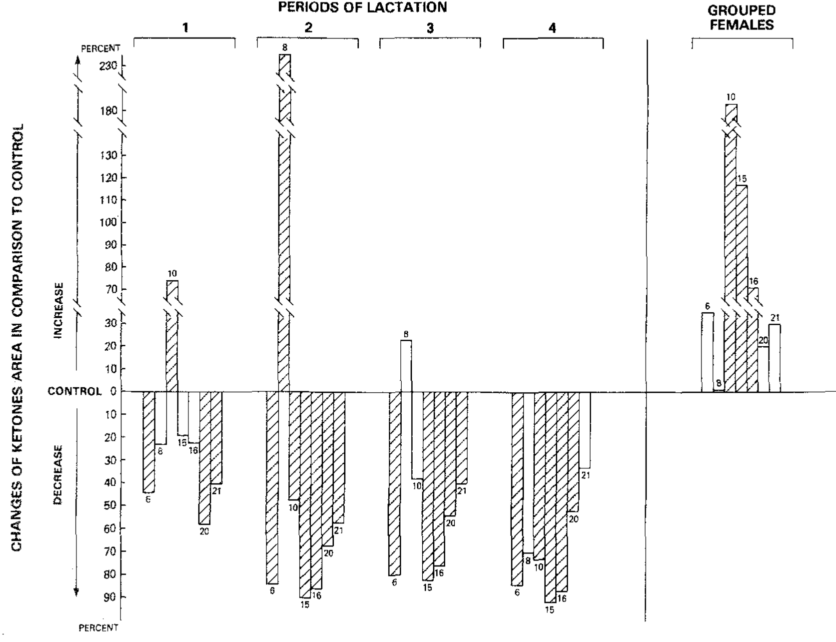

FIG. 4. Percentage difference in the peak areas of urinary ketones between lactating and nonreproducing (control) females of the ICR/Alb strain. Diagonally striped bars represent significant increases and decreases of ketones in lactating and grouped females as compared to controls.

regarding the occurrence and levels of structurally interesting compounds in different stages of pregnancy and lactation, the ultimate interpretation of their roles in the previously observed biological phenomena remains distant. The volatile substance profiles from urines collected at various reproductive stages of females appear to be distinguished more by the differences in their relative proportions than by a presence or absence of a specific substance. Interestingly, all four investigated classes of compounds are biochemically related to each other.

Comparison of these profiles provides a basis for the following general conclusions regarding the urinary chemosignals from pregnant and lactating females: (1) there are obvious differences in the concentration of urinary volatiles between pregnancy (both cases) and lactation; (2) production of urinary volatiles in lactating females is at a considerably lower level in comparison to either pregnant or singly caged, nonreproducing females. On the other hand, Drickamer (1983b, 1984, 1986) has clearly demonstrated that the urine from both sources has the same biological activity. The urine of both pregnant and lactating females accelerates the sexual development of young mice (Drickamer and

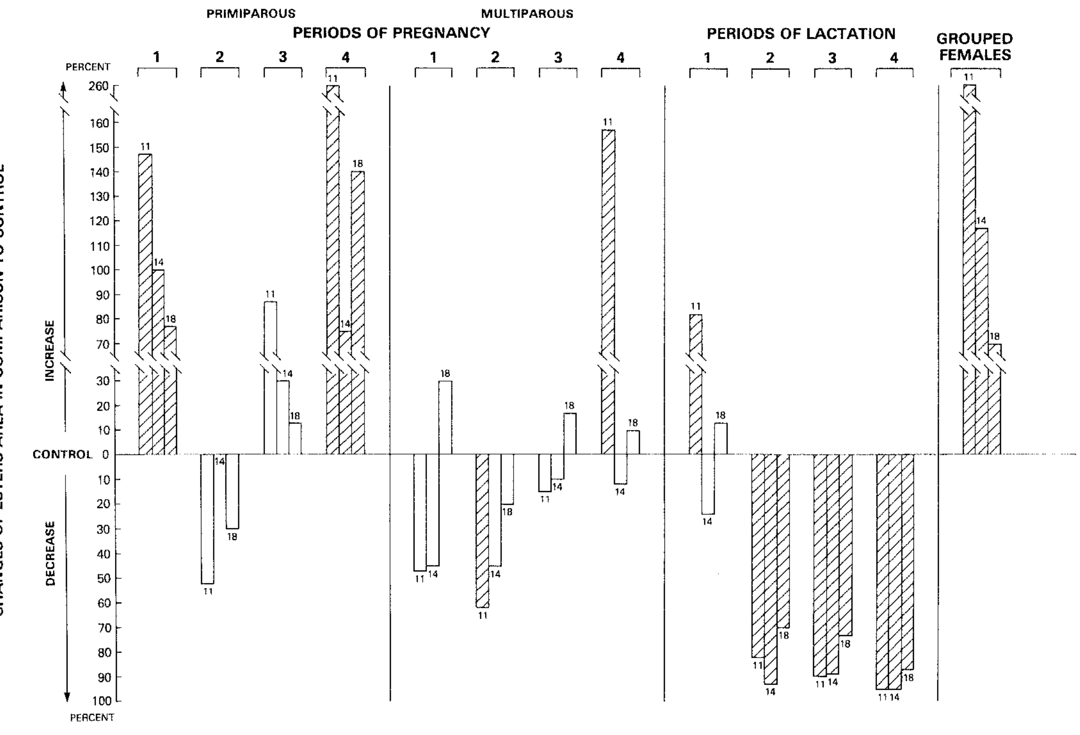

FIG. 5. Percentage difference in the peak areas of urinary esters between either pregnant or lactating and nonreproducing (control) females of ICR/Alb strain. Diagonally striped bars represent significant increases or decreases of esters in pregnant, lactating, and grouped females as compared to control.

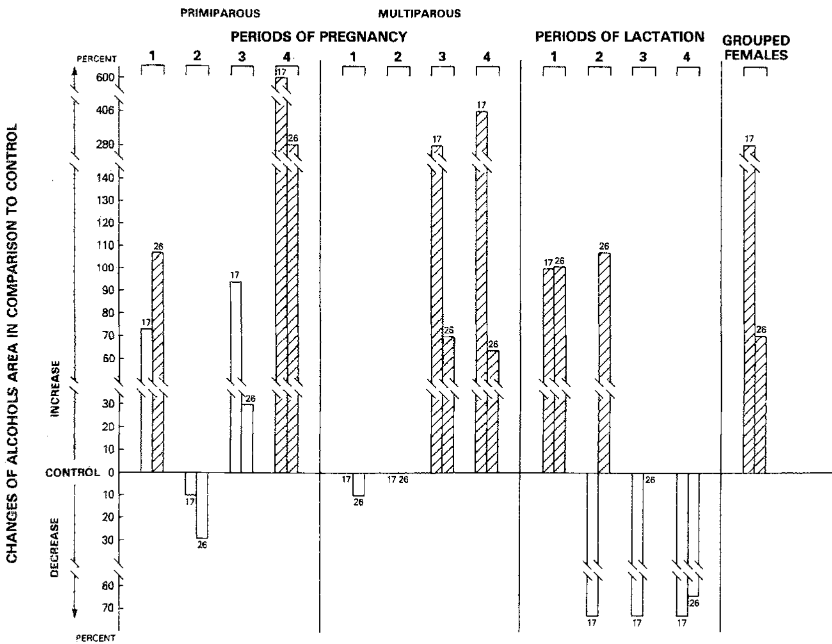

FIG. 6. Percentage difference in the peak areas of urinary alcohols between either pregnant or lactating and nonreproducing (control) females of ICR/Alb strain. Diagonally striped bars represent significant increases of alcohols in pregnant, lactating, and grouped females as compared to controls.

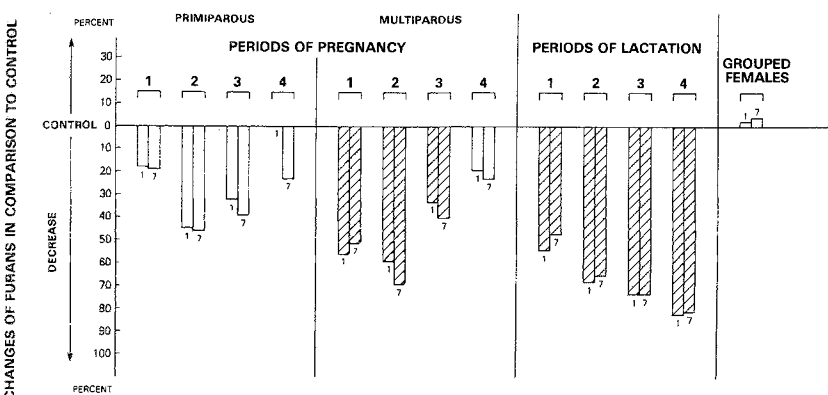

FIG. 7. Percentage difference in the peak areas of urinary dihydrofurans between either pregnant or lactating and nonreproducing (control) females of ICR/Alb strain. Diagonally striped bars represent significant decreases of dihydrofurans in pregnant, lactating, and grouped females as compared to controls.

Hoover, 1979). Possibly, the acceleratory chemosignals from pregnant/lactating urines have not formed specifically as a communication signal but may be secondary metabolic products of physiological processes within the female. Only under specific social and environmental conditions could their total levels or quantitative proportions trigger biological activity of the whole urine.

According to Drickamer (1984), the unidentified urinary factors that accelerate maturation in young females are present in pregnancy or lactation urines regardless of the sample collection time during the day. However, the substance(s) is present only during the last two thirds of pregnancy or lactation (Drickamer, 1983b). Our observations here indicate a different quantitative composition for the urinary ketones in the second part of pregnancy and lactation periods as compared to the initial period. The urinary esters, which had previously been implicated as puberty-delaying factors (Novotny et al., 1986), were found in mid-pregnancy and lactation at levels comparable to nonreproducing, singly caged females, but significantly lower than in grouped females. 2,5-Dimethylpyrazine, another puberty-delaying substance (Novotny et al., 1986), was found at a trace, constant level during the entire pregnancy and lactation period.

It remains unclear whether a single volatile substance or a set of volatiles act in concert in the puberty-acceleration phenomenon. It is even conceivable that a lack of puberty-delaying factors in sufficient amounts could be a contributory factor. Chemical syntheses are currently underway in our laboratory to enable us to test the 14 substances separately or in groups for any possible puberty acceleration effects.

It is known that, under environmental conditions adequate for successful reproduction, pregnant and lactating females release the puberty-acceleration factor throughout the year (Drickamer, 1986). It is possible that some reduction of the urinary volatiles will be observed when pregnant or lactating females are housed under conditions of shortened photoperiods, limited food supply, or high densities.

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RESPONSES OF FRESHWATER PRAWN, *Macrobrachium rosenbergii*, TO CHEMICAL ATTRACTANTS

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Abstract—Chemoreception in the adults of the freshwater prawn *Macrobrachium rosenbergii* was investigated under controlled laboratory conditions, using behavioral assays. Tests were carried out on groups, as well as on individuals, all at their intermolt stages of the molt cycle, and prestarved for three to four days. Of 28 different substances tested, the amino acids taurine, glycine, arginine, and betaine, as well as trimethylamine, elicited a positive behavioral response in at least 50% of the test animals when applied at a threshold concentration of 10^{-5}–10^{-8} M. A positive response comprises enhanced antennular flicking and food search motion. Of the various nucleotides tested for chemoattraction, only adenosine monophosphate elicited a response similar to that of the above amino acids, although at a concentration of 10^{-4} M, whereas adenosine diphosphate required a dosage of 10^{-1} M.

Key Words—*Macrobrachium rosenbergii*, Crustacea, chemoreception, taurine, glycine, trimethylamine, adenosine monophosphate, feeding stimulants.

INTRODUCTION

Chemoreception is an important mechanism governing the process of food searching in many aquatic animals. Earlier work on the attraction of *Macrobrachium rosenbergii* to foodstuffs has shown that while larvae of this prawn do not seem to respond to waterborne stimuli, postlarvae actively swim towards the apparent source of chemical attractants (Moller, 1978). Moller's work,

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however, was of a preliminary and qualitative nature, performed mainly in Petri dishes on crude materials, and did not include the adult stage.

In a recent preliminary study by Holland (1985), in which *M. rosenbergii* prawns were individually tested, a fish extract and some chemically defined materials were identified as inducing two grades of reaction: food detection and search. A similar characterization of the behavioral response pattern of the prawn has been observed by Harpaz et al. (1987), who also demonstrated the importance of the prawn's stage in the molt cycle during chemotactic examinations.

The present work reports the results of quantitative studies on a number of chemically identified crustacean attractants, including certain nucleotides. The latter were included because they serve as potent attractants for some marine decapods (Carr and Thompson, 1983; Derby et al., 1984).

METHODS AND MATERIALS

The effects of chemoattractants on *M. rosenbergii* were tested on groups of prawns and on individuals. Tests on groups served only as a preliminary screening of various chemicals. Those found attractive were then tried on individuals to obtain dose–response data.

Animals were kept in glass aquaria at a constant temperature of $27 \pm 2°C$. They were fed commercial pelleted fish feed containing 25% protein supplemented occasionally with 1-g pieces of frozen fish and fresh leaves (see Harpaz and Schmalbach, 1986).

Prior to every test, the animals were starved for three to four days. The weight range of both sexes of adult prawns employed in the tests was 6–20 g, although within each test group prawns were selected for more uniform weight.

M. rosenbergii are nocturnal, thus the tests were conducted under dimmed light conditions; when necessary, only red light illumination (brightness of 0.9 lamberts) was used, since crustaceans are not sensitive to it (Kennedy and Bruno, 1961). These conditions minimized the interfering effects of shyness due to the presence and movements of the human experimenter.

The test attractants were introduced into the aquaria by means of a 10-ml pipet containing 7 ml of the stimulant, with an enlarged tip aperture. The pH of aquarium water fluctuated between 6.5 and 7.5 and, when necessary, the stimulant's pH was adjusted accordingly. A similar pipet, containing aquarium water only, was introduced simultaneously at another corner of the aquarium in order to distinguish between chemical, as opposed to visual and/or mechanical response. For this particular purpose, water circulation (see below) had to be stopped, and time allowed for the animals to readjust. Under such almost still conditions, diffusion of the attractants from the pipet would be very slow were it not for the differences between the level of liquid in the pipet and that of the water in the aquarium. This provided an initial hydrostatic pressure for pushing

the solution out of the pipet so as to elicit a response within 60 sec. The respective concentration reaching the prawn's chemoreceptors might be up to 1000-fold lower depending on the position of the prawn and the slight water turbulence caused by the gill bailers, as well as prawn's movements. In dye simulation, the cloud covered close to half the aquarium volume within 1 min. In the solitary aquaria the attractant was introduced at a point most distant from the prawn's head. All test chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri.

A response was considered positive only when a specific sequence of events relating to feeding behavior took place. These include increased antennular flicking as described by Schmitt and Ache (1979), followed by food searching motions carried out by the first pereiopods. The latter search the substratum in front of the animal in a sweeping movement with their chelae, frequently bringing them towards the mouthparts. This sequence usually ends with the animal's eventual arrival at the stimulant release point. For a detailed description, see Harpaz et al. (1987).

In a given assay, each prawn was scored only once unless stated otherwise. Each stimulant was tested at a minimum of five concentrations with at least 10 replicates per concentration. A total of 50 prawns took part in these experiments.

Where appropriate, the significance of differences between compounds was analyzed using ANOVA followed by the Student-Newman-Keuls test (Sokal and Rohlf, 1969), as well as by log probit analysis according to Daum (1970) for determination of ED_{50}.

Tests on Groups. Ten groups of six adult prawns each were kept in 50 liters of water contained in glass aquaria measuring 50 × 40 × 30 cm. A 5-cm layer of gravel, placed at the bottom of each aquarium, served as a biological filter, from which water was constantly circulated through the entire volume of the aquarium by an electrical air pump. Sections of plastic tubing (5 cm diameter, 10 cm long) were placed on the gravel layer as hiding sites to reduce cannibalism.

The group trials served as a first approximation in which the same concentration (0.1 M) was applied for every one of the 23 test chemicals. Attractiveness was expressed in terms of the time required for 50% of the group to respond positively. A ceiling limit of 6 min was set for a positive response, any later arrivals at the target were considered as accidental and nonsignificant under these experimental conditions.

When cannibalism occurred (rare and normally following molting), the whole group was disqualified.

Tests on Individuals. The individual tests were carried out only with prawns that were at responding stages of the molt cycle. Prawns at the nonfeeding stages (D_3 and A) were not tested. Designation of the different stages of the molt cycle was according to Peebles (1977). This was based on the recognized

phenomenon that many crustaceans, including *M. rosenbergii*, do not feed during the periods immediately before and after ecdysis. Moreover, during these particular nonfeeding stages of the molt cycle, the animals do not respond in the typical manner to chemical attractants (Harpaz et al., 1987).

The aquaria for individual prawns measured 30 × 15 × 20 cm and contained 7 liters of water each. A 300-ml cup filled with gravel and placed on the bottom of the aquarium was used as above for constant water filtration. Each of these aquaria was optically insulated from its neighbor to avoid visual-response interference arising from prawns learning and memorizing as reported by Singer et al. (1979). The molt cycle was recorded for each individual in this type of aquarium. The light–dark regime in these aquaria was 12L:12D to simulate the conditions of this prawn's tropical origin.

Based on preliminary tests, the time limit in this series for reaching the target in a positive response had to be raised to 10 min. In the control assays (pipets containing aquarium water only) included in each chemoattractant test, no more than 2% of the animals actually reached the pipet tip. However, practically all these "positive" responses should be regarded as chance arrivals since very rarely did any of these prawns go through the entire repertoire constituting a complete positive response. On the other hand, at very low concentrations (varying according to the attractants), the animal performed the behavioral repertoire, but finally failed to reach the target. Nevertheless, such cases were still recorded as positive response.

RESULTS

Test on Groups. Table 1 lists the chemicals eliciting a positive response in *M. rosenbergii* at an initial concentration of 0.1 M at the point of introduction.

TABLE 1. MEAN TIME REQUIRED FOR 50% OF GROUP-TESTED PRAWNS TO DETECT AND RESPOND TO 10^{-1} M CONCENTRATION OF VARIOUS CHEMOATTRACTANTS ($N = 10$ GROUPS FOR EACH ATTRACTANT)

Attractant	Time (sec, $X \pm$ SD)
Glycine	125.5 ± 36.3a[a]
L-Arginine	135.0 ± 41.9a
Trimethylamine (TMA)	142.2 ± 41.9a
Casein hydrolysate	143.5 ± 38.4a
Betaine	193.0 ± 75.2
L-Isoleucine	206.0 ± 79.0b
L-Proline	245.0 ± 84.2b

[a] Figures with the same letter do not differ significantly ($P > 0.05$).

Results are presented in terms of the time (in seconds) required for 50% of the prawns to detect and perform the food searching behavioral pattern while approaching the attractant source. The following chemicals either did not elicit any response, or the reaction was nonsignificantly positive at this relatively high concentration: adenosine, adenosine 5′-triphosphate (ATP), alanine, aspartic acid, cysteine, glutamic acid, glutathione, histidine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine. All amino acids were L forms.

Tests on Individuals. Except for casein hydrolysate and proline, the attractants eliciting a positive response in the group tests are indicated in Table 2, where the minimum molar concentrations required for these chemicals to attract at least 50% of the test prawns are presented. Taurine and nucleotides, which have been reported as powerful chemoattractants to other decapods (Case, 1964; Ache, 1972; Shepheard, 1974; Fuzessery et al., 1978; Heinen, 1980; Carr and Thompson, 1983; Derby et al., 1984), were tested as well. Among the amino acids, the most attractive were taurine, glycine, and isoleucine.

Table 3 presents the percentages of prawns positively responding to various nucleotides at ascending log concentrations. The most potent was AMP. However, at concentrations higher than 10^{-2} M, the response to this nucleotide decreased, indicating an inhibitory effect of AMP at higher concentrations. As shown in Table 3 (for 10^{-1} M), the potencies of GMP, ADP, and IMP were low and decreased in the indicated order. ATP and adenosine were inactive even at the highest concentration tested.

The results obtained with individual prawns actually confirmed the conclu-

TABLE 2. ED_{50}[a] VALUES OF VARIOUS CHEMOSTIMULANTS ELICITING POSITIVE BEHAVIORAL RESPONSES IN INDIVIDUALLY TESTED *Macrobrachium rosenbergii* PRAWNS

Test chemical	ED_{50}	Slope ± SE
TMA	3.03×10^{-8}	0.204 ± 0.032
taurine	4.55×10^{-8}	0.273 ± 0.040
glycine	2.78×10^{-7}	0.246 ± 0.031
L-isoleucine	2.34×10^{-6}	0.281 ± 0.033
betaine	4.98×10^{-6}	0.274 ± 0.042
L-arginine	1.14×10^{-5}	0.268 ± 0.041
AMP	3.29×10^{-4}	0.337 ± 0.057

[a] ED_{50} = mean molar concentration required for eliciting a positive response in 50% of the tested prawns, calculated from log probit regression according to Daum (1970). Statistical differences exist only between concentrations which are one log step apart. Saturation levels are similar except for AMP (see text).

TABLE 3. RESPONSE[a] OF INDIVIDUALLY TESTED *Macrobrachium rosenbergii* TO 10^{-1} M CONCENTRATIONS OF VARIOUS NUCLEOTIDES

Nucleotide	Rating
GMP	+++
ADP	++
IMP	+
ATP	no response
Adenosine	no response

[a] Since the response did not exceed 50%, rating was according to the following scale: +++ ≤ 50%; ++ ≤ 40%; + ≤ 30%.

sions drawn from the group trials in the case of glycine, arginine, TMA, betaine, and isoleucine. Also, the pattern of behavior towards the attractant's release point was observed to be identical in the two series of trials. At a high concentration of attractant, the entire sequence of events, as mentioned above, is performed with remarkable intensity and, upon reaching the target area, the prawns vigorously shake the pipet with their chelae. As the concentration decreases, the intensity of food searching movements diminishes, and the prawn at times gives up the search before reaching the target area, thus indicating that the mechanism involved is much more complex than a mere "all-or-none" response.

DISCUSSION

In this study, chemoattractants for *M. rosenbergii* were identified and their dose–response relations determined through the use of behavioral methods commonly employed in such studies: tests on prawn groups and individuals.

Many studies on feeding behavior of decapod crustaceans have been carried out on groups rather than individual animals (Carr, 1978; Deshimaru and Yone, 1978; Carr and Thompson, 1983; Carr et al., 1984). This is because, besides experimental convenience, results of group trials can at times be better related to field conditions, thereby making them more directly applicable to aquaculture. However, group experiments have some potential disadvantages; one of them is that all prawns are often not at the same stage of the molt cycle (see Methods and Materials). Another drawback is that at the beginning of each experiment animals are not equally distant from the pipet tip, so that the concentration gradient between the tip of the pipet and the site of the animal about to respond can influence the results. Furthermore, one cannot rule out the possibility of a responding animal stimulating other members of the group into

motion. Another source for possible error is the prawn's territorial behavior. This is displayed by its tendency, upon discovering a food site, to deter other prawns from approaching the site (Harpaz, 1986). Tests carried out on individuals offer a better chance of controlling experimental conditions, validating group test results, and avoiding most of their drawbacks.

Most of the chemicals tested in the present study have been reported as stimulants with various degrees of potency for other crustaceans (Case, 1964; Ache, 1972; Shepheard, 1974; Carr and Gurin, 1975; Carr, 1978; Fuzessery et al., 1978; Johnson and Ache, 1978; Heinen, 1980; Derby and Atema, 1982; Carr and Thompson, 1983; Santos-Filho, 1983; Derby et al., 1984). Some of these chemicals were also tested on *M. rosenbergii*, although only preliminarily (Holland, 1985). It is difficult to compare results obtained in different studies since the methods, experimental set-ups, and criteria used are not the same. Nevertheless, as regards *M. rosenbergii*, the ranking of the different chemoattractants is similar. The most potent were TMA, glycine, and taurine. In addition, we have found that betaine and, to a lesser extent, nucleotides are also active. The most potent nucleotide was AMP followed by GMP, ADP, and IMP in the listed order, while ATP and adenosine were not active. These results are similar to those reported by Carr and Thompson (1983) for *Palaemonetes pugio*, and by Derby et al. (1984) for the spiny lobster *Panulirus argus*. Yet, even the most potent nucleotide AMP was two to three orders of magnitude less effective than the amino acids for *M. rosenbergii*. For other arthropods, e.g., hematophagous insects, the trend of nucleotide potency ranking was found to be reversed (Galun et al., 1985).

In the present study, no attempt was made to discriminate between chemoreceptors associated with the senses of smell and taste. Following antennular removal, *M. rosenbergii*, like *Procambarus clarkii* (Ameyaw-Akumfi, 1977), regains (after a short recovery period) its ability to home in on several attractants. This points toward the possibility that other parts of the body (presumably the first pereiopods and maxillipeds) possess chemoreceptors which are able to substitute for those located on the antennules (Harpaz, unpublished). It seems that some of the amino acids and nucleotides, identified as chemoattractants for *M. rosenbergii*, have a dual function. All the amino acids eliciting a powerful response in *M. rosenbergii* stimulated (with varying degrees of activity) the chemoreceptors of the walking legs of the lobster *H. americanus* (Derby and Atema, 1982). Since, in Crustacea, leg and mouthparts contain the sensory structures involved in the sense of "taste" (gustation) (Atema, 1980), it seems that these amino acids may be powerful phagostimulants. Thus, incorporation of trimethylamine hydrochloride to a low-grade feed of adult *M. rosenbergii* brought about a 30–38% increase in food consumption (Costa-Pierce and Laws, 1985). In an attempt to improve the nutritional value of commercial pellets fed to adult *M. rosenbergii*, Farmanfarmaian and Lauterio (1979) supplemented

these pellets with various amino acids reported as essential for Crustacea. Among these amino acids, arginine appreciably stimulated growth. This beneficial effect may have been contributed also by the powerful attractancy of arginine to *M. rosenbergii* as shown in the present study.

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BEHAVIORAL EVIDENCE FOR MULTICOMPONENT TRAIL PHEROMONE IN THE TERMITE, *Reticulitermes flavipes* (KOLLAR) (ISOPTERA: RHINOTERMITIDAE)

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Abstract—Evidence is presented for a multicomponent trail pheromone in the eastern subterranean termite *Reticulitermes flavipes* (Kollar). Choice tests were used to compare strength and persistency of trails made by termites in glass tubes. Tubes connected to termite nests for 24 hr were marked by termites with an extremely long-lasting chemical that persisted for at least one year, and a highly volatile substance that decayed in 15 min. Individual termites varied the trail they deposited under different circumstances. When removed from the nest and placed in a clean tube, they deposited a highly volatile substance. They left stronger and more persistent trails when exploring clean tubes attached to the nest. When they discovered a new food source, they left trails that were more attractive and far more persistent than trails made by exploring or displaced termites. A food trail made by 15 workers was still effective after 24 hr, whereas a trail made by 50 displaced workers, walking one after another through a tube, lasted only 5 min.

Key Words—*Reticulitermes flavipes*, Isoptera, Rhinotermitidae, termite, trail pheromone, chemical communication.

INTRODUCTION

Chemical trails are important orientation cues for subterranean termite species, but also appear to function in recruitment communication. For example, in *Zootermopsis nevadensis*, workers lay trails to recruit nestmates for defense (Stuart, 1963, 1967). In *Nasutitermes costalis*, soldiers lay trails to recruit nestmates for exploration, defense, and food-harvesting (Traniello, 1981; Traniello and Busher, 1985). Trail pheromones are also used for recruitment to food by workers of *Trinervitermes bettonianus* (Oloo and Leuthold, 1979). These find-

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ings suggest that individual termites can regulate the amount and/or composition of trail pheromone deposited under different circumstances, but this has been clearly demonstrated in only one study. Oloo and Leuthold (1979) demonstrated that single workers returning to the nest from a new food-find laid trails that were far more attractive to nestmates, but shorter-lived, than trails laid by exploring workers before the discovery of food.

Although the principal component of trail pheromone has been identified for several termite species (reviewed in Prestwich, 1983), the high degree of species specificity in trails of closely related species has led several researchers to suggest the presence of secondary components in termite trail pheromones (Moore, 1974; Howard et al., 1976, 1982; Kaib et al., 1982; McDowell and Oloo 1984). These secondary components may be important for species or colony identification, but they could also function in the organization of colony activity. For example, Traniello (1982) and Traniello and Busher (1985) found that sternal gland extract of *N. costalis* contains both a highly volatile chemical that acts as an attractant, and a long-lasting component that functions as an orientation cue.

In this study, a behavioral bioassay has been used to compare the strength and volatility of trails deposited in different contexts by workers of *Reticulitermes flavipes* (Kollar).

METHODS AND MATERIALS

Reticulitermes flavipes is a subterranean termite species common throughout most of the eastern United States. It nests in the soil and feeds on wood. Termites for my experiments were collected in Chapel Hill, North Carolina in April 1981. Ten laboratory nests were set up in closed, quart-sized plastic canisters containing moist white play sand and blocks of partially decayed sweetgum wood. Each nest was started with 1500 workers (undifferentiated individuals beyond the third instar, see Esenther, 1969) and 30 soldiers collected from the same source colony. The nests were maintained at room temperature (19–23°C), in the laboratory, for two years. They remained healthy during this period and developed supplementary reproductives, young, and nymphal forms.

A glass tube (10 cm × 5 mm OD) was attached to a hole in the base of each nest canister where it was accessible to the termites in the nest at all times. The tubes were sealed at the far end with tight-fitting plastic caps and became, in effect, extensions of the nest galleries. A regular flow of traffic in and out of the tubes was present during most observation periods. These nest tubes facilitated observation of termites and made it possible to remove termites and to attach additional tubing or bait dishes with a minimum of disturbance to termites in the interior of the nest.

A bioassay chamber was used to examine trails laid by termites in glass

tubes. This chamber was a Lucite disk, 1 cm thick and 5 cm in diameter, which contained a Y-shaped tunnel (arms separated by 120°). Glass tubes containing termite trails were fitted into the two tunnels that formed the arms of the Y, and a termite was placed in the third tunnel which formed the stem of the Y. It would walk to the junction and was then faced with a choice between the two glass tubes. The arms of the Y-tunnel were approximately 5.1 mm in diameter, which was just large enough to accommodate 5-mm-OD glass tubes. The stem tunnel was 3 mm in diameter, which matched the inside diameter of the tubes used, so that a termite in the chamber could enter either glass tube without having to step up or down. The chamber was used to test for preferences between two trails laid in different contexts, or between an aged trail and an unused tube.

At the start of each testing session, several workers were removed from a nest tube and placed together in a small petri dish. For each trial, the glass tubes to be tested were first slipped into the two arms of the test chamber, and then a single termite was lifted out of the dish with a paint brush and placed in the chamber entrance. It would then walk to the junction and would enter one of the tubes presented. If the trails were similar, a termite would often poke its head and antennae into first one tube and then the other, but a choice was not recorded until it had fully entered (at least one body length) one of the two tubes. Usually it then continued down to the end of the tube and was removed along with the tubes being tested.

Because of the possibility of trail reinforcement by test termites, each trail-containing or control tube was used for only a single choice test. Tubes were then cleaned by soaking in dichromic acid for 24 hr and rinsing thoroughly with both tap and distilled water before being reused. A different set of tubes as well as a different test termite were used for every trial. Termites from several different nests were used to generate and test trails for each experiment, although termites used to test a particular trail were always from the same colony as those used to generate the trail. Because of the length of time required to generate some of the trails, experiments often required several days for completion.

Statistical analysis of trail-choice experiments was based on the binomial distribution (Hays, 1973). A probability of less than 0.05 was considered to be a significant result. If termites chose randomly between two glass tubes, they were presumed to contain trails of equal strength or attractiveness. For cases in which the control trail was a clean, unused glass tube, a random choice indicated that the trail offered had faded to the point where it was no longer perceptible to test termites. If the termites showed a statistically significant preference for one trail over another, then the preferred trail was considered to be stronger or to contain a more attractive component than the other trail.

Two groups of experiments were carried out. In the first group, trails along well-used passageways connected to the nest were examined for persistence and rate of decay. In the second group, trails laid by single termites (or small num-

bers of termites) in three different situations were examined for persistence and compared to each other in terms of termite preference.

Trail Markers in Well-Used Passageways Connected to Nest

Trail Persistence. Several short (5 cm) glass tubes were fitted tightly together by plastic "sleeves" to form longer tubes, and then these were attached to nest tubes. The ends of the long tubes were sealed with plastic caps. Termites were given 24 hr to explore and travel in the tubes. The tubes were then disconnected, and any termites that were in them were shaken out. Choice tests were made between these tubes and unused tubes. Some tubes were tested immediately while others were set aside (in an open glass jar) and tested at one of five elapsed times (10 min, 24 hr, 8 days, 3 months, 1 year).

Rate of Decay. Choice tests were performed between pairs of trails that differed in how recently they had been in use. Two short (5 cm) tubes were connected to a nest tube in series and were left in place for 24 hr. The outer tube was removed first and both ends quickly covered with plastic caps to minimize the loss of volatile chemicals. After waiting for different periods of time (from 10 min to two days), the second tube was removed and both tubes placed in the test chamber. Test termites chose between an older and a more recently used trail. Eight combinations, shown in Table 2, were tested.

Trails of Single Workers

Displaced from the Nest. To determine the presence of trail marker, a worker was removed from the nest tube, placed in one end of a clean 5-cm glass tube, allowed to walk to the other end, and then shaken out. This tube and an unused tube were immediately placed in the testing chamber and a different worker was allowed to choose between the two.

To determine the persistence of the single worker trail, single worker trails were tested 2, 4, 5, 6, and 10 min after they were laid in choice tests with unused tubes.

To determine the additive nature of trails, 10 workers were placed one after another in one end of a clean 5-cm glass tube and then shaken out as soon as they reached the other end. This procedure took less than 2 min since each termite takes only 5–6 sec to walk through the tube. Choice tests were run between 10 superimposed trails and a single worker trail.

To determine the persistence of superimposed trails, 10 workers were quickly placed one after the other in the end of a glass tube and then shaken out when they reached the other end. After 6 min (the expected duration of a

single trail) the trail was compared to an unused tube in the test chamber. This was repeated with 50 workers.

Starting from the Nest: Exploration Trail. A clean glass tube (terminus closed by a plastic cap) was attached to the end of a nest tube. After waiting for a single termite to enter this clean tube and walk to the far end, the termite was shaken out before it could turn around and retrace its steps. Although several termites invariably would have investigated the entrance to the clean tube, the far end of the tube contained only a single one-way trail. This was the end that was tested. This tube was compared in the testing chamber to a single displaced worker trail (as above).

To determine persistence, exploration trails were tested 10 min and 15 min after being laid in choice tests with unused tubes.

Starting from the Nest: Food-Find Trail. I was unable to obtain a single food-find trail for comparison with a single exploration trail because it is made by a termite on the return trip to the nest. Instead the following system was devised. A clean 2.5-cm tube was attached to a nest tube and a food dish containing damp sand and a wood block was connected to its terminus. A single termite was allowed to traverse the clean tube, antennate the wood block, and then return to the nest. This tube now contained an outbound exploration trail and a return trail from the food find. This tube was immediately removed and a second clean 2.5 cm tube was attached in its place. The second tube was sealed with a plastic cap at its terminus (i.e., no food present). Again, a single worker was allowed to traverse the full length of the tube and return to the nest. The second tube now contained a two-way (outbound and return) exploration trail. Test termites were given a choice in the chamber, between a "food-find" trail (with underlying exploration trail) and a two-way exploration trail (no food present).

To determine persistence, food-find trails were tested 60 min after being laid in choice tests with unused tubes.

As a control for tube contact with wood blocks, unused glass tubes were placed in direct contact with wood blocks for 30 min and then tested in choice tests with other clean tubes.

Multiple Food-Find Trails. For comparison with a well-used nest tube, a clean 5-cm tube was attached to a nest with a food dish at its far end and 15 workers were allowed to reach the food and return to the test. Choice tests were run between this tube and a nest tube (from a different part of the nest) that had been in use for a month and had presumably been traveled by hundreds of termites.

To determine persistence of superimposed food-find trails, tubes containing 15 superimposed food-find trails were tested 24 hr after being laid in choice tests with unused tubes.

RESULTS

Trail Markers in Nest Tubes

Tubes that had been connected to the nest for 24 hr were preferred to unused tubes at all ages tested (Table 1). A chemical marker was perceptible to workers even after one year, although not a single termite had entered the tubes during that time.

Fresh trails (used by termites within 30 sec of the test) were significantly preferred to trail tubes removed from the nest 15 min earlier (see Table 2). This indicates the presence of a volatile chemical that fades appreciably (to workers) in 15 min. Over the next 5 hr, the trail marker showed no significant further decay. Trails disconnected from the nest 15 min before being tested were not significantly different from trails that had been disconnected for 30 min, 2 hr, or even for 5 hr. They were, however, significantly preferred to trails that had been unused for 24 hr, indicating that the trail continues to fade over the 24-hr period, but does so at a much slower rate than during the first 15 min. Trails unused for 24 hr were equal in strength to trails unused for 48 hr, which indicates that the rate of decay becomes even slower after the first 24 hr.

Trails of Single Workers

Displaced from the Nest. Termites removed from the nest, and made to walk through clean glass tubes, left chemical markers clearly perceptible to other termites; tubes traversed by a single worker (in one direction only) were significantly preferred to unused tubes (21:9, $P < 0.02$).

The results in Table 3 show that the trail of a displaced worker lasts between 5 and 6 min; it was significantly preferred to an unused tube 5 min after it was deposited but was no longer preferred when tested after 6 min.

TABLE 1. SINGLE WORKER CHOICES IN TESTING CHAMBER BETWEEN TUBES CONNECTED TO NEST FOR 24 HOURS AND UNUSED TUBES

Elapsed time since tube disconnected	No. of tubes tested	Worker choices, used–unused	Binomial probability
1 h	16	14:2	0.002
1 day	16	15:1	0.0002
8 days	16	14:2	0.002
3 months	16	13:3	0.01
1 year	16	12:4	0.03

TABLE 2. SINGLE WORKER CHOICES IN TESTING CHAMBER BETWEEN OLDER AND MORE RECENTLY USED TRAILS

Elapsed time since trails last used		No. of trails tested	Worker choices, trail A–trail B	Binomial probability
Trail A	Trail B			
<30 sec	10 min	18	12:6	0.07
<30 sec	15 min	18	14:4	0.02
<30 sec	30 min	18	15:3	0.01
15 min	30 min	18	10:8	0.17
15 min	2 hr	18	9:9	0.19
15 min	5 hr	18	10:8	0.17
15 min	24 hr	18	15:3	0.01
24 hr	48 hr	18	9:9	0.19

Ten superimposed worker trails were significantly preferred to a single worker trail when tested immediately (14:4, $P < 0.02$). When these trails were tested 6 min after being made, in choice tests with clean tubes, no preference was found (10:8, $P < 0.17$). Similarly, 50 superimposed worker trails, when tested 6 min after being laid, were not significantly preferred by workers to unused tubes (10:8, $P < 0.17$).

Exploration Trail. The trail of a single worker exploring a clean tube attached to the nest was significantly preferred to the trail of a single displaced worker (15:3, $P < 0.01$). This "exploration" trail was preferred to an unused tube when tested 10 min after being laid (14:4, $P < 0.02$), but was no longer detectable by workers when tested after 15 min (12:6, $P < 0.08$), and so appears to last between 10 and 15 min.

Food-Find Trail. Food-find trails (which contained an underlying explo-

TABLE 3. SINGLE WORKER CHOICES IN TESTING CHAMBER BETWEEN TUBES TRAVERSED ONCE BY SINGLE DISPLACED WORKER AND UNUSED TUBES

Elapsed time before tubes were tested (min)	No. of tubes tested	Worker choices, used–unused	Binomial probability
2	18	13:5	0.04
4	18	13:5	0.04
5	18	14:4	0.02
6	18	7:11	0.12
10	18	9:9	0.19

ration trail) were significantly preferred to two-way exploration trails (14:4, $P < 0.02$) even though they were always older (4–25 min) than the exploration trails to which they were compared (since food trails were laid first).

Food-find trails were significantly preferred to unused tubes when tested 60 min after being laid (16:2, $P < 0.0006$). The two-way exploration trail decayed in 15 min after being laid (12:6, $P < 0.08$). This was consistent with the results for the one-way exploration trail (see above) which also lasted for only 10–15 min.

Workers chose randomly between clean tubes that had touched wood blocks for 30 min and other clean tubes (8:7, $P < 0.2$), indicating that tubes were not contaminated through contact with the food source.

Multiple Food-Find Trails. Test termites chose randomly between 15 superimposed food-find trails and nest tubes (15:9, $P < 0.08$). When tubes containing 15 superimposed food-find trails were tested 24 hr after being laid, they were significantly preferred to unused tubes (17:1, $P < 0.0001$), indicating the presence of a long-lasting component in the recruitment trail that persists for at least 24 hr.

DISCUSSION

This study was designed to examine the possibility that individual *R. flavipes* workers vary the amount or the composition of the trail marker deposited under different circumstances. The bioassay used, in which single termites were given choices between different trails (in glass tubes), in a Y-shaped intersection, enabled trails to be compared in terms of worker preference. In this bioassay, a statistical preference for one trail over another indicates that the preferred trail is either stronger or contains a more attractive component than the other trail. It should be noted that termite responses may be affected by the conditions of the bioassay (as test termites were removed from the nest). Nevertheless, the fact that termites prefer one trail over another clearly indicates that they can perceive a difference between the two trails. Choice tests between trail tubes and unused tubes allowed the longevity of different trails to be assessed. The lifetime of a given trail was considered to be the length of time until termites were no longer able to distinguish it from a clean tube.

Glass tubes used as passageways by *R. flavipes* colonies contain a chemical marker clearly perceptible to workers in choice tests with clean tubes. Although this chemical marker may be primarily a trail pheromone actively deposited from the sternal gland, it probably also contains salivary secretions, excrement, and substances passively rubbed off from the cuticle. Some component, or components, of this trail marker appears to be extremely persistent as it was still perceptible (to termites) after a year without reinforcement. It should be noted that the aging of trails under natural conditions may be very different from that

found for trails in glass tubes. It is possible that trail tubes may have picked up chemicals from the lab, as they were stored with ends uncapped, but this would be unlikely to account for the striking preference seen even in the 1-year-old tubes, especially as control tubes were also stored uncapped (although for a shorter time).

Traniello (1982) similarly found evidence for an extremely long-lasting component in passageways used by *Nasutitermes costalis*; an effective trail was produced from extracts of 4-year-old shelter-tube flooring. Tschinkel and Close (1973) found that extracts of trails laid on paper by *Trinervitermes trinervoides* produced trail-following for up to 7.5 hr. As suggested by Traniello (1982), persistent trails are probably found in termite species because these insects feed for extended periods on long-lasting food resources, and because, being blind, they rely heavily on chemical cues for orientation. In a temperate species such as *R. flavipes*, colonies appear to abandon chambers and foraging sites near the soil surface in winter and migrate to chambers below the frostline (Esenther, 1969). For these species, persistent trails may enable foragers to return to profitable foraging sites the following spring.

Comparison of older and more recently used trails revealed a rapid decline in trail attractiveness during the first 15 min of nonuse and a much more gradual decay after that time, a result which suggests that trails contain a volatile component. Some of the volatile material was probably dissipated in the time it took to disconnect and cover the tube ends, and plastic tube caps may have slowed, but would not prevent, their dispersion. These results must therefore be taken as an approximation of the natural decay curve.

A volatile trail pheromone has been reported in two other termite species. Oloo and Leuthold (1979) found that workers of *Trinervitermes bettonianus* laid highly volatile (15 min) recruitment trails following food discovery. Traniello (1982) found a volatile excitatory component in sternal gland extracts of *N. costalis* that produced a mass outpouring of workers from the nest. In these two species the volatile trail component is presumed to function as an excitatory pheromone that stimulates nestmates to forage and is therefore used primarily in recruitment communication. In the present study, a volatile pheromone was present in tubes that had never led to food sources (they were closed with tube caps) and that had been in use for 24 hr. It is therefore very unlikely that this component was related to recruitment effects. Instead, termites may reinforce all trails as they walk, and the volatile fraction may serve to distinguish trails currently in use from those that have been abandoned.

The presence of two or more components in the trail marker of *R. flavipes* is similar to the findings of Traniello (1982) for sternal gland extracts of *N. costalis*, which contain both a volatile recruitment component and a long-lasting orientation component although, in the present case, the functions of the two components have not been identified. The possibility of a multicomponent pheromone is consistent with sternal gland structure in *Reticulitermes*. Smythe and

Coppel (1966), in their histological study of the sternal gland of *R. flavipes*, first suggested that this species has some control over the amount and nature of trail substances emitted. Quennedey (1978), using electron microscopy, identified four different types of secretory cells in the sternal gland of *R. santonensis*, each containing a unique chemical secretion. The sternal cuticle forms a dome over the central region of the gland and encloses an extracellular space in which the secretion products of two cell types accumulate. Other secretion products are conducted through canals that pierce the dome and so reach the surface of the gland directly.

Comparison of trail markers left by single termites in three different situations revealed differences in both trail strength and volatility. Termites removed from the nest and placed in clean tubes left a marker that was clearly recognizable by other termites but that persisted for only 5 min. This highly volatile substance may function as a lifeline, to aid termites in maintaining contact with the colony, or it may be an alarm pheromone (pheromones which are characteristically short-lived; Wilson, 1971), as termites were undoubtedly agitated by their removal from the nest and by the lack of familiar odors in the clean tubes.

A worker exploring a clean tube attached to the nest left a trail that was intermediate in strength and persistence between that of a displaced worker and that of a food recruiter. It lasted for 10–15 min compared to 5 min for the trail of a displaced worker, and more than 1 hr for a food-find trail. However, when the tube remained attached to the nest for 24 hr, so that numerous termites traveled up and down its length, it came to contain a trail that persisted for a year. Therefore, although the initial worker laid a trail that lasted only 10–15 min, at some point during the 24-hr period, the extremely long-lasting component was deposited. Perhaps each exploring termite deposits only a small amount of the durable trail component because the stimulus is less exciting than, for example, a block of wood. Even so, with sufficient traffic, enough termite trails would accumulate so that the pathway would become permanently marked.

Although trails left by food-finders were stronger and much more long-lasting than exploration trails, this difference may be only quantitative. This is supported by the fact that termites chose randomly between 15 recruitment trails and a well-used nest tube. If the recruitment trail were qualitatively different, one would have expected even a very new recruitment trail to be chosen over a well-traveled pathway that did not lead to food. On the other hand, test termites were displaced from the nest and in this context might not be as attracted by a food recruitment trail as they might be otherwise. The bioassay used in this study did not distinguish between attraction and orientation, as trails were presented to single termites rather than to groups and because termite response was limited to movement within a tube. Therefore the results do not exclude the possibility that the food-find trail contains a volatile attractant or other components not present in other trails.

There does appear to be a qualitative difference between the trails of displaced workers and those of workers returning from a food find. Whereas a trail made by 15 food-finders lasted for 24 hr, the superimposed trails of 50 displaced workers lasted for only 5 min. The long-lasting component appears to be entirely absent in the trails of displaced workers.

These results clearly demonstrate the ability of individual workers to vary the amount or composition of trail marker deposited under different circumstances. Stuart (1964) first suggested, for *Zootermopsis nevadensis*, that termites may be able to regulate the amount of pheromone that is released from the extracellular reservoir by varying the amount of pressure exerted by the abdomen against the substrate. The cuticular dome of the sternal gland is studded with campaniform sensillae which provide proprioceptive information about the degree of compression of the dome. One may speculate that in *R. flavipes*, the secretions that accumulate in the extracellular space constitute the long-lasting component of the trail. Termites could vary the amount of persistent component that is deposited in relation to the nature of the stimulus, as in exploration and food-recruitment trails, by varying the pressure of the abdomen against the substrate. Volatile components may be conducted directly to the surface of the gland, and so be passively emitted at all times. This explanation would be consistent with the presence of a volatile component in well-used passageways, with the ability of workers to deposit a volatile component by itself and with their ability to vary the amount of long-lasting component that is deposited.

Directions for future work include identifying the separate components of the trail pheromone and assessing their functions in the organization of colony activity.

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ISOLATION, IDENTIFICATION, AND SYNTHESIS OF MALE-PRODUCED SEX PHEROMONE OF PAPAYA FRUIT FLY, *Toxotrypana curvicauda* GERSTAECKER (DIPTERA: TEPHRITIDAE)[1]

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Abstract—A male-produced sex pheromone of the papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker, was isolated from volatiles collected from air passed over calling males and was identified as 2-methyl-6-vinylpyrazine by comparative gas-liquid chromatographic and spectroscopic evidence. Synthetic 2-methyl-6-vinylpyrazine elicited typical pheromonal responses from unmated mature female flies such as walking, running, and flying in an arena bioassay; flying upwind with a zigzag flight pattern; and hovering in the pheromone plume in a wind-tunnel bioassay. These responses were similar quantitatively and qualitatively to responses to naturally occurring pheromone from calling male papaya fruit flies.

Key Words—*Toxotrypana curvicauda*, Diptera, Tephritidae, papaya fruit fly, male sex pheromone, pheromone, 2-methyl-6-vinylpyrazine.

INTRODUCTION

The papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker, is a major obligate pest of papaya fruit in south Florida, Central and South America, and the Caribbean. The behavior of this insect is of particular interest because, unlike other tropical frugivorous tephritids, it is very limited in its selection of hosts; the larvae usually infest only the fruit of papaya (Wolfenbarger and Walker, 1974).

[1]This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or the recommendation for its use by USDA.
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This tephritid, unlike many other fruit fly species, is not known to be attracted to or feed on yeast hydrolysate paste (Landolt, 1984) and is not attracted to aqueous solutions of brown or refined sucrose or to trimedlure, methyl eugenol, cue-lure, or vinegar (Sharp and Landolt, 1984). Thus there are no attractants or trapping materials available to monitor papaya fruit fly populations.

Landolt et al. (1985) reported the presence of a male-produced sex pheromone in *T. curvicauda* that elicited excitatory behavior in females such as walking, running, and flying in an arena bioassay and attraction in a wind-tunnel bioassay. The need for an attractant to monitor this pest insect and the potential of a female attractant for pest control led us to investigate the chemical nature of the sex pheromone produced by male papaya fruit flies.

This paper reports the isolation, identification, and synthesis of a male-produced sex pheromone of the papaya fruit fly that elicits both short- and long-range behavioral responses by unmated, mature females that are similar to responses from calling males in laboratory tests.

METHODS AND MATERIALS

Papaya fruit fly larvae were collected from field-infested papaya fruit from Dade County, Florida, and from fruit infested in the laboratory by field-collected flies. Larvae emerging from fruit were placed in wax-coated paper cartons filled with damp, heat-sterilized potting soil or vermiculite into which they burrowed and pupated. Emerging adults were sorted daily by sex and were held in aluminum screen cages supplied with sugar cubes and wet cotton dental wicks. All insects were held at 26 ± 1°C, 50 ± 5% relative humidity, and on a 12L : 12D photoperiod with lights on from 7:00 AM to 7:00 PM. Females used in bioassays were unmated and were reproductively mature (6–9 days old). They were kept in a room separated from males in 30 × 30-cm aluminum screen cages with sugar and water.

Arena and Wind-Tunnel Bioassays

Arena bioassays were conducted using single females in 500-ml clear plastic cups turned upside down, as described by Landolt et al. (1985). Doses of a candidate chemical or fraction on filter paper were introduced into the cups and the activities of the flies observed for a 2-min time period. The number of females responding by walking, running, and flying was recorded. The bioassays were conducted between 11:30 AM and 1:00 PM.

A preliminary test of gas–liquid chromatography fractions of volatiles collected from males (see later) was conducted with this arena bioassay. One male-hour-equivalent of each of three fractions was pipetted onto filter paper, and the behavior of the flies was observed and recorded. A dose–response test was con-

ducted using this bioassay at 0, 0.01, 0.1, 1, 10, and 100 μg of synthetic pheromone and its isomer with five flies tested per dose. This test was replicated three times.

Wind-tunnel bioassays were conducted in a tubular Plexiglas wind tunnel (0.44 m diam × 2.44 m long) with 22 cm/sec wind speed (measured in the center of the tunnel at the male release point) at 24–25°C under fluorescent lighting. Rubber septa (A.H. Thomas Co. 8753-D22, Philadelphia, Pennsylvania, 5 × 9 mm extracted with methylene chloride for 24 hr) were impregnated with the synthetic pheromone or its isomer in 100 μl of hexane. These septa were air dried in a fume hood for 24 hr after loading with the chemicals and then used only once. This pheromone source was suspended in the center of the upwind end of the tunnel by a clip hung on a steel stand. A female was placed at the downwind end of the tunnel in an upright 35-ml plastic vial and observed for 2 min. All behavior observed was recorded, particularly the time it took to leave the vial (activation time), the number of flights during 2 min, the upwind distance attained, plume tracking (upwind anemotaxis within the plume with casting or zigzagging), hovering (a stalled casting or circling flight pattern within 15 cm of the source), and landing at the pheromone source (septum or end of stand holding septum). Bioassays were conducted from 11:00 AM to 12:30 PM. Doses tested were 0, 1, 10, 100 μg, and 1 and 10 mg per septum. On each of nine days, five mature unmated females were tested per dose, beginning at the lowest dose of the series. Treatment means were separated with Duncan's new multiple-range test.

Comparison of Behavioral Responses to Synthetic and Natural Pheromones

A comparison was made of the responses of mature, unmated female papaya fruit flies (6–8 days old) to calling males and to synthetic sex pheromone in a flight-tunnel bioassay. Four males were held in a 3.8-liter glass jar through which air was pumped into the upwind end of the tunnel. Incoming air was passed through a charcoal filter and regulated with a flowmeter at 1 liter/min. Outgoing air was vented into the tunnel via 4-mm-diam steel tubing, with the vented air directed downwind from near the center of the upwind end of the tunnel. Synthetic pheromone was introduced into the tunnel using the same system and was dispensed from a 25-μl glass disposable micro-sampling pipet (Corning), sealed on one end, to provide a release rate of about 300 ng/hr. Release-rate determinations for pheromone from capillaries were made as described for rubber septa (see later). Batches of 10 female papaya fruit flies were tested alternately for responses to male-produced and synthetic pheromone. Each female was observed for 2 min following its introduction into the downwind end of the tunnel. Females were scored for plume tracking and for hovering in the plume near the pipe vent. A total of 20 females (two sets of 10) was tested per treatment.

Collection of Volatiles

Volatile sex pheromone produced by males was collected on Porapak N by the method of Landolt et al. (1985) for subsequent purification and spectroscopic analysis. Pheromone was collected by passing prefiltered (Porapak N) air over calling papaya fruit fly males held in glass jars and then through another Porapak N filter (pheromone trap). The collection system was operated 6–7.5 hr after the onset of the photophase, with 50, 3- to 8-day-old male papaya fruit flies placed in each jar. Each Porapak air filter and pheromone trap was extracted with 10 ml of hexane and then baked in an oven at 95°C for 2 hr before use. After volatile collection, pheromone traps were extracted with 10 ml of hexane and the extract was stored in a freezer. Similar volatile collections were made from mature, unmated female papaya fruit flies.

Male volatiles were also collected on charcoal filter traps with an apparatus similar to the one described by Tumlinson et al. (1982) for direct capillary GLC analysis. Compressed air, purified by passing it through two in-line charcoal filters, entered a glass chamber (4.2 cm OD × 4 cm long) where the insects were held. The upwind end of the collecting chamber was fitted with a glass frit to disperse the air and induce laminar flow. Air flow through the chamber was maintained at 1 liter/min. About 80% of the air entering the system and passing over the males was pulled by vacuum through a small charcoal (Aktivkohle für Gaschromat. nach Dr. Grob, 0.05–0.1 mm, Bender & Hobein AG, Zürich) filter trap similar to the one described by Grob and Zürcher (1976). Approximately 20% of the flow, measured by a flowmeter, was vented to maintain a slight positive pressure within the chamber.

Volatiles from a single mature (3- to 5-day-old) male papaya fruit fly were collected from 5 to 7 hr after the beginning of the photophase. Collection from a single male was replicated 20 times. Additionally, volatiles were collected from a group of five mature male flies in the same manner. Volatiles were extracted from the charcoal filter trap with 80 μl of dichloromethane (20 μl × 4). After adding *n*-nonane as an internal standard, the extract was concentrated slowly with a stream of N_2 to about 1 or 2 μl. Then 2 or 3 μl of hexane was added to the concentrate and the hexane solution was analyzed by capillary GLC.

Release rates of the synthetic pheromone from rubber septa were measured by collection of the volatiles released and analysis by GLC. Septa loaded with 100 μg and 1 mg of pheromone were placed in a 15 mm (ID) × 20 mm (long) stainless-steel tube, purified air was passed over them at 1 liter/min, and the volatiles were trapped on a charcoal filter. Each septum was loaded 24 hr prior to measuring the release rate and aired at room temperature until release rate measurements began.

Pheromone Purification

Crude pheromone extracted from Porapak N filters with hexane was concentrated to a small volume by a stream of nitrogen, and the concentrated material was subjected to micropreparative gas–liquid chromatography (GLC). Micropreparative GLC for purification of the pheromone was performed on a Varian model 1400 gas chromatograph equipped with a flame ionization detector. The glass column (2.3 mm ID × 2 m) was packed with 10% OV-101 on 60/80 mesh Chromosorb W. Temperatures of inlet, detector, and transfer line were 230, 250, and 80°C, respectively. Column temperature was maintained at 80°C. Carrier gas (N_2) flow rate through the column was 20 ml/min. The chromatograph was modified to accommodate a 95:5 effluent splitter and external outlet for collection (Brownlee and Silverstein, 1968). The effluent from the transfer line after the splitter was collected in 1.5-mm-ID × 305-mm-long U-shaped capillary glass tubes connected to the external outlet and cooled directly by an acetone–Dry-Ice bath to minimize the loss of the highly volatile pheromone.

Synthesized materials were purified by preparative high-performance liquid chromatography (HPLC) on a 1.25-cm-OD × 25-cm-long stainless-steel column packed with Lichrosorb 5 μm silica. A Lab Data Control Constametric II G pump delivered the hexane–ethyl acetate (75:25) mobile phase at 2.0 ml/min, and eluting components were detected with a Waters model 401 differential refractometer.

Pheromone Analysis and Identification

Naturally occurring pheromone and the synthesized chemicals were analyzed on 50-m × 0.25-mm-ID fused silica capillary columns as follows: an OV-101 column was maintained at 60°C for 1 min after injection, programmed at 5°C/min to 200°C, and then operated isothermally; a CPS-1 column was maintained at 60°C for 1 min after injection, programmed at 10°C/min to 180°C and then operated isothermally. A Carbowax-20M column was maintained at 60°C for 2 min after injection, programmed at 5°C/min to 210°C then operated isothermally. All columns were operated in the splitless–split mode (splitless delay 30 sec) with He carrier gas flow of 19 cm/sec in a Varian model 3700 gas chromatograph.

Mass spectral data were obtained with a Nermag R10-10C mass spectrometer equipped with a chemical ionization (CI)–electron impact (EI) source. A Varian Vista model 6000 gas chromatograph equipped with split-splitless injection system and 0.25-mm-ID fused silica capillary columns served to introduce samples to the ion source. For CI mass measurement, methane was em-

ployed as reagent gas. Helium was used as the GLC carrier gas in the EI and CI mass measurements. Data acquisition and reduction were accomplished with a Digital PDP 11/23 computer interfaced to the mass spectrometer.

Proton magnetic resonance spectra were obtained with a Nicolet 300-MHz Fourier transform NMR spectrometer interfaced to a Nicolet 1280 data system. All data were measured in benzene-d_6 solution using 16K data points and a 6-μsec pulse. The NMR spectrum of the natural pheromone was obtained from 10,000 scans on ca. 4 μg/20 μl benzene-d_6 solution in a 2-mm-OD NMR tube (Wilmad Glass Co., catalog No. 507 with WGS-5BL stem).

Synthesis of Pheromone

2-Methyl-6-dimethylaminoethylpyrazine. A mixture of 2,6-dimethylpyrazine (Pyrazine Specialties, Inc., Atlanta, Georgia, containing 2%, 2,5-isomer) (15.5 g) and dimethylamine hydrochloride (13.0 g) was heated until it began refluxing, and then formaldehyde (22.5 g as 38% aqueous solution) was added over a 30-min period. The resulting solution was refluxed for 2 hr. The solution was cooled to room temperature and diluted with water, made basic by the addition of 10% aqueous solution of sodium hydroxide, and extracted with chloroform. After removing the solvent, distillation of the residue gave 7.8 g of 2-methyl-6-dimethylaminoethylpyrazine with 5% of the 2,5-isomer (33% yield), 120–126°C/12 mm Hg: IR (cm^{-1}, CCl_4 solution); 3045 (m), 2980 (s), 2955 (s), 2870 (s), 2830 (s), 2770 (s), 1538 (s), 1465 (s), 1380 (m), 1270 (m), 1200 (s), 1180 (s), 1165 (s), 1080 (m), 1045 (m), 1020 (m), 880 (m), PMR (C_6D_6); 8.29 (1H, s), 8.14 (1H, s), 2.80 (1H, m), 2.65 (1H, m), 2.23 (3H, s), 2.12 (6H, s).

6-Methylpyrazylethyl-trimethylammonium iodide. Methyl iodide (6.4 g) was added to a stirring solution of 2-methyl-6-dimethylaminoethylpyrazine (4.6 g) in 20 ml of anhydrous ether under argon atmosphere. The mixture was allowed to stand overnight at room temperature. A yellow solid formed was filtered and washed with a small amount of ether to give 6.0 g of the quarternary iodide salt (70% yield). This hygroscopic compound was subjected to the next step without further purification.

2-Methyl-6-vinylpyrazine. The quarternary ammonium iodide (6.0 g) was dissolved in 40 ml of water. Then, 1.7 g of sodium hydroxide was added and the mixture was refluxed for 1 hr. The resulting solution was cooled to room temperature and extracted with chloroform. The chloroform solution was dried over Na_2SO_4 and evaporated carefully at atmospheric pressure. Microdistillation at 12 mm Hg gave 2.4 g of 2-methyl-6-vinylpyrazine (74% yield) with 5% of the 2,5-isomer. Pure 2-methyl-6-vinylpyrazine (100% purity) for bioassay was obtained by HPLC purification on the Lichrosorb column. Capacity factors (k') (Schram, 1980) of 2-methyl-6-vinylpyrazine and its 2,5-isomer were 2.0

and 3.2, respectively, under the conditions described in the experimental section). MS (m/z); 37 (43), 52 (72), 54 (26), 94 (26), 120 (M^+, 100).

IR (cm^{-1}, CCl_4 solution); 3105 (m), 3045 (m), 2940 (m), 1545 (s), 1456 (m), 1422 (s), 1395 (s), 1380 (m), 1275 (s), 1260 (s), 1228 (s), 1178 (s), 1160 (s), 1020 (s), 986 (s), 940 (s), 930 (s), 880 (s), PMR (C_6D_6); 8.16 (1H, s), 7.98 (1H, s), 6.50 (1H, dd, J = 17.4 Hz, 10.5 Hz), 6.33 (1H, dd, J = 17.4 Hz, 1.6 Hz), 5.22 (1H, dd, J = 10.5 Hz, 1.6 Hz), 2.08 (3H, S).

2-Methyl-5-vinylpyrazine. In the same manner, 2-methyl-5-vinylpyrazine was synthesized from 2,5-dimethylpyrazine (Pyrazine Specialties), containing 7% of the 2,6-isomer. Pure 2-methyl-5-vinylpyrazine (99.8% purity) for bioassay was obtained by HPLC purification as described in the experimental section. MS (m/z); 37 (52), 52 (79), 64 (29), 94 (26), 120 (M^+, 100).

IR (cm^{-1}, CCl_4 solution); 3080 (m), 3015 (m), 2930 (m), 1485 (s), 1410 (m), 1380 (m), 1320 (s), 1170 (s), 1032 (s), 985 (s), 940 (s), 925 (s), 905 (s), 880 (m), PMR C_6D_6); 8.20 (1H, s), 8.03 (1H, s), 6.54 (1H, dd, J = 17.4 Hz, 10.5 Hz), 6.31 (1H, dd, J = 17.4 Hz, 1.6 Hz) 5.21 (1H, dd, J = 10.5 Hz, 1.6 Hz), 2.09 (3H, s).

RESULTS AND DISCUSSION

Isolation and Identification

The capillary GLC analysis of the calling male papaya fruit fly volatiles collected on the charcoal filter indicated the existence of a single major peak (Figure 1), which did not appear in the analyses of volatiles from females. In an arena bioassay, only the GLC fraction that contained the major peak elicited the typical pheromone-stimulated responses by females (Landolt et al., 1985). The release rate of this compound, determined by GLC analysis of the volatiles collected from individual calling males, was 63.2 $\pm$ 33.2 ng/male-hr. The release rate from a group of five males was about 80 ng/male-hr.

Hexane extracts of the Porapak N filters were concentrated and subjected to micropreparative GLC purification as described in the experimental section to obtain sufficient material for PMR analysis. The purity of the isolated biologically active component of the volatiles was estimated to be 99.9% by capillary GLC analysis in which three different types of columns were used.

Structural Elucidation

The methane CI mass spectrum of the pure isolated major component established that the molecular weight was 120, with diagnostic peaks at m/z 121 (M+1), 149 (M+29), and 161 (M+41). In the EI mass spectrum (Figure 2), the presence of a strong parent peak at m/z 120 (M) and characteristic even

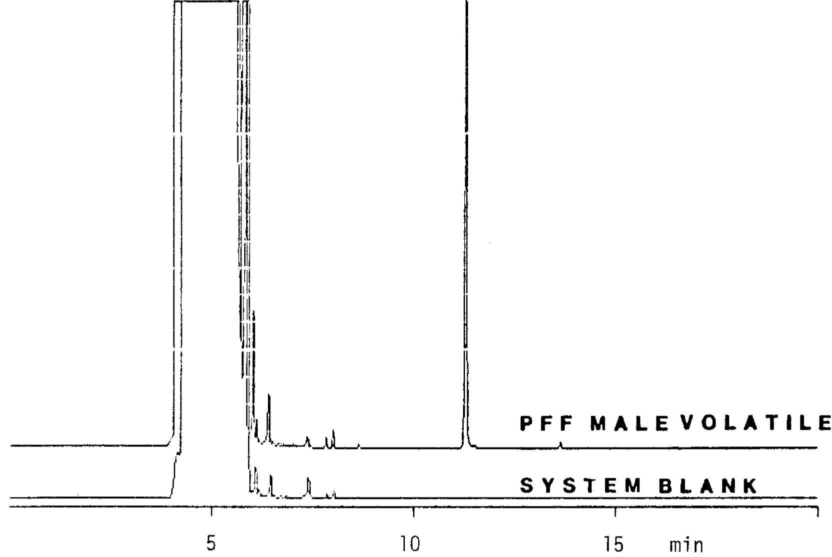

FIG. 1. Gas chromatogram of the volatiles from calling male PFF. Column: 50 m, capillary fused silica OV-101. Temp. program: 60°C for 1 min, 5°C/min to 200°C. Injection mode: splitless/split, split delay 30 sec.

fragment ions at *m/z* 52, 54, and 94 suggested that the pheromone has a heterocyclic structure involving two nitrogen atoms.

The 300-MHz [^{1}H]NMR spectrum of the pheromone (Figure 3) strongly supported the presence of a heterocyclic system in the structure. Two single-proton signals at 8.26 (s) and 8.08 (s) were attributable to the ring protons, the chemical shifts of which were consistent with those of a disubstituted pyrazine. A three-proton signal at 2.18 (s) and three one-proton signals at 6.59 (dd, J = 17.4 Hz, 10.5 Hz), 6.45 (dd, J = 17.4 Hz, 1.7 Hz), and 5.31 (dd, J = 10.5 Hz, 1.7 Hz), which appeared to be ABX systems, were attributable to a methyl and vinyl group, respectively, attached to the pyrazine ring. The presence of unsplit signals at 8.26 and 8.08 indicated that the substitution system should be 2,6 or 2,5. Due to slight downfield chemical shifts caused by the solvent dilution effect, exact assignments could not be made.

Both 2-methyl-6-vinylpyrazine (2,6-MVP) and 2-methyl-5-vinylpyrazine (2,5-MVP) were synthesized from 2,6-dimethylpyrazine and 2,5-dimethylpyrazine, respectively, employing a Hoffman exhaustive methylation procedure and modifications described by Kamal et al. (1962) (Figure 4).

The structure of the pheromone was deduced conclusively from the comparison of the 300 MHz [^{1}H]NMR spectra and the retention times on three different capillary GLC columns of the natural pheromone and synthetic 2,6-

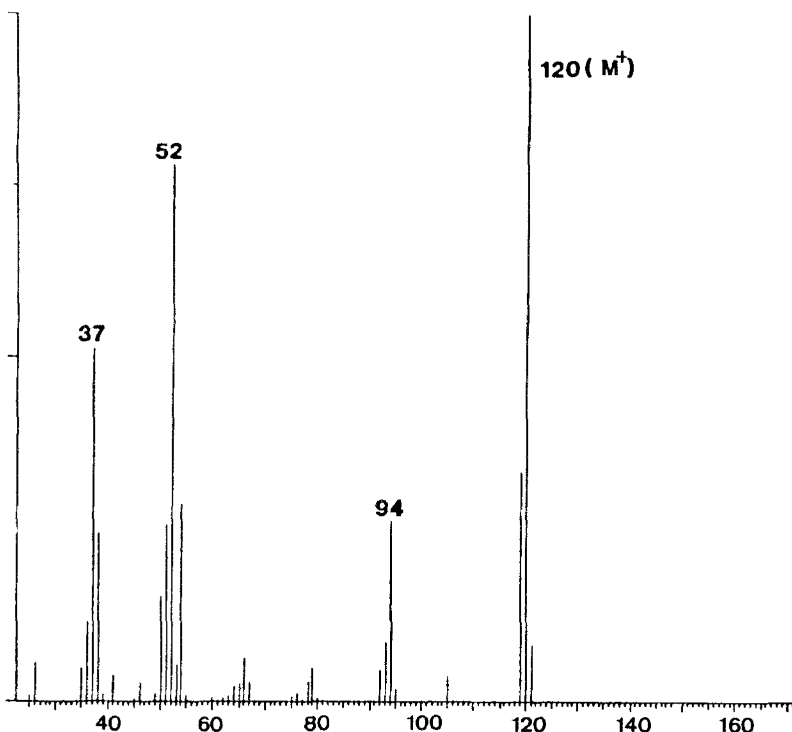

FIG. 2. Electron impact mass spectrum of the sex pheromone.

MVP and 2,5-MVP. The retention indices (Kovats, 1965) of 2,6-MVP and 2,5-MVP, respectively, on the three columns were: OV-101, 995 and 1000; Carbowax 20 M, 1455 and 1499; CPS-1, 1327 and 1355. The mass spectral data and GLC retention times of the natural pheromone were identical to those of 2,6-MVP, and thus the structure of the sex pheromone isolated from volatiles of the male papaya fruit fly was determined to be 2-methyl-6-vinylpyrazine.

Arena and Wind-Tunnel Bioassays

Synthetic 2,6-MVP was tested in the arena and wind-tunnel bioassays to compare its pheromonal activity to that of natural pheromone. Results of the arena bioassay, summarized in Table 1, indicated that synthetic 2,6-MVP elicited running and flying as did calling males and volatiles from calling males in a previous experiment (Landolt et al., 1985). Optimum response rates in the arena bioassay were obtained at 0.1- and 1-μg doses.

In the wind-tunnel dose–response experiment, summarized in Table 2, the

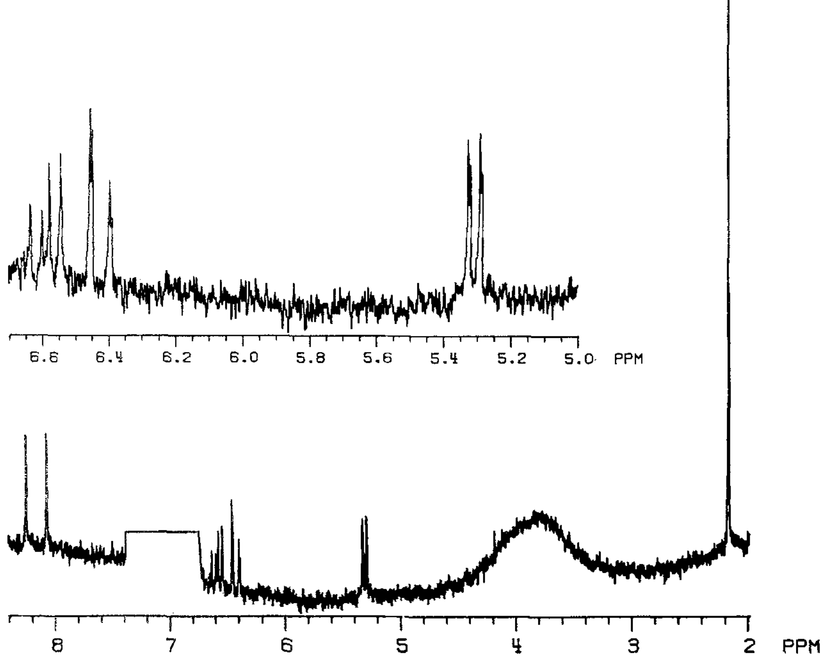

FIG. 3. 300 MHz-[^{1}H]NMR spectrum of the sex pheromone.

N
N
CH_2O
$(CH_3)_2\overset{H}{N}\cdot HCl$
1. reflux
2. 10% NaOH
N
N
N
CH_3I
I^-
N^+
N
N
aq. NaOH
reflux
N
N

FIG. 4. Synthesis of 2-methyl-6-vinylpyrazine.

TABLE 1. PERCENTAGE OF FEMALE PAPAYA FRUIT FLIES RESPONDING TO DOSES OF SYNTHETIC 2,6-MVP IN ARENA BIOASSAY ($N = 15$)

Dosage (μg)	Walking (%)	Running (%)	Flying (%)
0.0	80	0	20
0.01	90	50	30
0.1	100	100	90
1.0	100	90	100
10.0	100	70	70
100.0	100	100	80

first significant responses were attained at the 10 μg dose, with a sharp reduction in the activation time and an increase in the number of females tracking the plume and hovering in the plume near the septum. The peak response was attained with the 1-mg dose, which gave a 53% response rate for close-range hovering and 33% for landing. The 100-μg dose elicited 29% close hovering and 22% landing.

Release rate data from septa loaded with 100 μg and 1 mg of 2,6-MVP were obtained at 0.5-hr intervals over a 2-hr period that commenced 24 hr after loading. These data were subjected to linear regression analysis. A linear curve fit of the data for the 1-mg load resulted in the equation: release rate (ng/hr) = 602 − 2.5X (where X = time in minutes after commencing release rate measurement). Similarly, the equation for the 100-μg load was: release rate (ng/hr)

TABLE 2. MEAN TIME TO TAKE FLIGHT FROM RELEASE VIAL AND PERCENTAGES OF FEMALE PAPAYA FRUIT FLIES RESPONDING TO SYNTHETIC 2,6-MVP ON RUBBER SEPTA IN WIND-TUNNEL BIOASSAY ($N = 45$)[a]

Dosage (μg)	Time to flight (sec)	Plume tracking (%)	Close hovering (%)	Landing (%)
0	64.0c	0.0a	0.0a	0.0a
1	49.1c	0.0a	0.0a	0.0a
10	24.6b	26.7b	22.2b	11.1ab
100	12.9ab	40.0bc	28.9b	22.2bc
1000	13.4ab	60.0c	53.3c	33.3c
10000	5.1a	42.2bc	40.0bc	13.3ab

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

$= 93.6 - 4.5X$. While this release rate curve indicates a fairly rapid decrease in the rate of release over the 1- to 2-hr period during which the response of females to a septum was being tested, the release rate of 2,6-MVP from a 100-μg loaded septum is approximately the same as that from a calling male PFF.

To obtain a more accurate quantitative comparison of female responses to calling males with that to synthetic pheromone, neat 2,6-MVP was dispensed from glass capillary pipets to give a constant release rate of 300 ng/hr, approximately the same as the release rate, from four calling males (about 63 ng/hr per male). The responses of the females in the wind tunnel to both pheromone sources were similar. Sixty percent of the females entered zigzagging upwind flight and 40% hovered near the pipe vent in response to synthetic 2,6-MVP, vs. 55% and 20% for the same responses to pheromone from four calling males.

The response of male papaya fruit flies was also tested in the wind-tunnel bioassay using the 1-mg dosage. No response was evident in testing the males ($N = 10$), either as a general increase in activity, upwind movement, or orientation. Additionally, synthetic 2,5-MVP did not elicit pheromone responses from mature unmated females in a dose–response test conducted in the arena bioassay or the wind tunnel.

We conclude from the results of these bioassays that 2,6-MVP is a sex pheromone of the papaya fruit fly and is attractive to mature unmated females at a distance. Response rates were similar to those observed to natural material

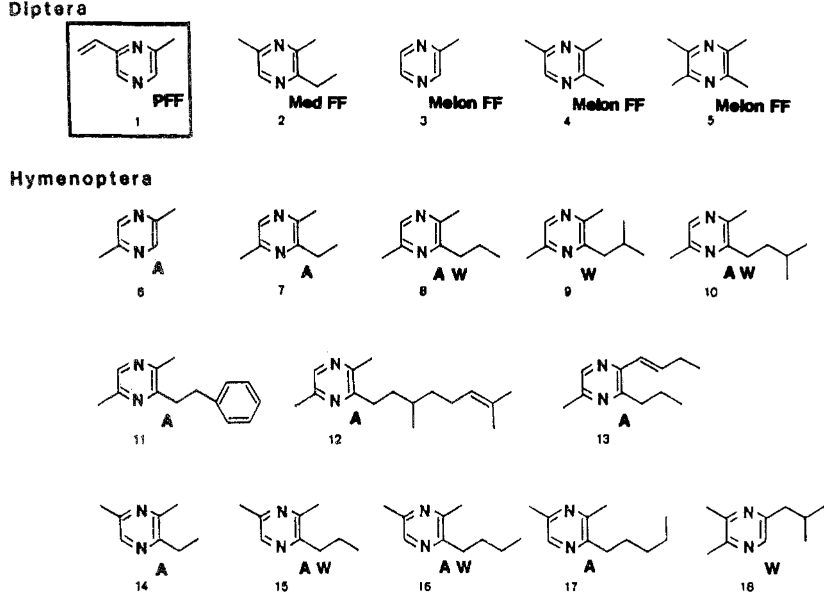

FIG. 5. Pyrazines from insects; FF = fruit flies, W = wasps, A = ants.

vented from calling males and thus the presence of other components is not indicated.

Previously, various alkylpyrazines (Figure 5) have been reported as mandibular and poison gland secretions from ants and wasps (Figure 5, 7–18) (Wheeler et al., 1982), and as trail pheromones of ants (6 and 7) (Attygalle and Morgan, 1984). Although pyrazines have been reported in the volatile components from male *Ceratitis capitata* (Wied.), the Mediterranean fruit fly (2) (Baker et al., 1985), and as rectal gland secretions of male *Dacus cucurbitae* Coquillett, the melon fruit fly (3–5) (Baker et al., 1982), the biological roles of these pyrazines were not determined. Also, 2-methyl-6-vinylpyrazine was reported as a flavor component of coffee (Bondarovich et al., 1967; Goldman et al., 1967) and roasted filberts (Kinlin et al., 1972). We present the first evidence that 2-methyl-6-vinylpyrazine is used in an insect communication system as a sex pheromone, which appears to function as a long-distance attractant.

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CUTICULAR HYDROCARBONS REGULATE MATE RECOGNITION, MALE AGGRESSION, AND FEMALE CHOICE OF THE ROVE BEETLE, *Aleochara curtula*

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Abstract—Immature, starved, or multiply mated males of the staphylinid beetle, *Aleochara curtula*, mimic their females chemically. The titer of the female sex pheromone components (Z)-7-heneicosene and (Z)-7-tricosene was quantified for various physiological types and both sexes by gas chromatography and correlated with the sexual response of males towards the cuticular hydrocarbon fractions. Modulation of intermale aggression by production of the female pheromone was shown by (1) reduction of the alkene titer of females kept at elevated temperatures, (2) treating live males with the synthetic female pheromone mixture, and (3) gradual amputation of male antennal segments. *A. curtula* males do not fight against members of other *Aleochara* species with a different hydrocarbon pattern. Contamination of *A. peschkei* males with the hydrocarbon fraction of *A. curtula* males, however, provoked the release of aggression. Choosy females reject mating attempts of males bearing the female sex pheromone.

Key Words—*Aleochara curtula*, Coleoptera, Staphylinidae, female sex pheromone, cuticular hydrocarbons, chemical mimicry, male aggression, female choice.

INTRODUCTION

In many insect species particular components of the cuticular hydrocarbons are used as female sex pheromones (for review see Howard and Blomquist, 1982; Jallon, 1984). Other communicative functions of surface hydrocarbons like caste recognition in social insects are mediated by the complex pattern of many compounds that vary in chain length, methyl branching, or unsaturation (Lok et al., 1975; Howard et al., 1978, 1982a; Clement and Lange, 1984). Deceptive in-

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formation is emitted by termitophilous or myrmecophilous beetles, mimicking the pattern of their hosts' cuticular hydrocarbons (Howard et al., 1980, 1982b; Vander Meer and Wojcik, 1982).

Chemical female mimicry by hydrocarbon pheromones has been demonstrated for males of the carrion-inhabiting rove beetle, *Aleochara curtula* (Goeze) (Peschke, 1985, 1987). (Z)-7-Heneicosene and (Z)-7-tricosene were identified as main components of the female sex pheromone (Peschke and Metzler, 1986). As measured only in a bioassay (Peschke, 1985, 1987), various physiological types of *A. curtula* males also elicit homosexual responses of other males by means of aphrodisiacs, and such behavior correlates with the avoidance of severe intermale aggression, which may lead to mechanical injuries, contamination with the toxic defensive secretion (Peschke and Metzler, 1982), and expulsion from the carcass. By female mimicry, however, the males get access to the carrion as a feeding and mating site (Peschke et al., 1987). The chemical identity of male releasers of homosexual responses with the female sex pheromone has so far only been demonstrated for immature beetles (Peschke, 1985). In the present paper, we report the chemical quantification of the alkene pheromone titer for other *A. curtula* individuals of various sex and physiological status, such as age, nutrition, previous copulations, and body size.

The evident positive correlation of the release of male homosexual responses by producing the female sex pheromone with the reduction of intermale aggression was confirmed by three sets of experiments: (1) The chemical information output of the females was affected by reducing their alkene pheromone content by keeping the beetles at elevated temperatures. (2) The chemical information output of the males was modified by contamination with the synthetic female pheromone mixture. (3) The sensory input of males was manipulated by gradual amputation of antennal segments.

Although the modulation of male contest by the female sex pheromone is fairly well understood, the essential releasers of aggression are unknown. Therefore, experiments on the role of the species-specific hydrocarbon composition in the recognition of male competitors have been conducted. We investigated the behavioral responses of *A. curtula* males towards other *Aleochara* species with different hydrocarbon patterns and the effect of their chemical manipulation.

While males releasing homosexual responses are protected from assaults of other males, females repulse mating attempts of these mimicking males. In this way they choose physiologically competent, nonmimicking males, which are capable of withstanding the aggressive interactions at the mating site and of transferring a large spermatophore (Peschke, 1985, 1987). In order to prove the hypothesis that female sex pheromone components also act as releasers of female repulsion behavior, we manipulated the chemical information output of males by contamination with the synthetic pheromone mixture.

METHODS AND MATERIALS

Field Collections and Laboratory Cultures

A. curtula (Goeze) and *Philonthus politus* (L.) were collected at rabbit carcasses in deciduous forests near Ochsenfurt, Bavaria (Peschke et al., 1987). *Aleochara (Euryodma) brevipennis* (Grav.) originates from the "Zeubelrieder Moor" (Ochsenfurt) and the Bodensee (fish bait). The species co-occurs with *A. curtula* on carcasses in middle Europe (Peschke and Fuldner, 1977). *A. peschkei* (Likovsky, 1983) was collected from fish carcasses from the banks of the river Comoe (Ivory Coast, West Africa).

These *Aleochara* species were continuously reared in the laboratory according to Fuldner (1968) and Peschke (1978, 1987) with puparia of *Calliphora erythrocephala* (Meig.) or *Lucilia sericata* (Meig.) serving as hosts for the parasitoid larvae (Peschke and Fuldner, 1977). Small beetles were obtained by using small puparia as hosts. Males were kept in groups of 10 in plastic boxes of 10 × 10 × 7 cm. Individual beetles and single pairs were kept in boxes of 5.5 × 3.5 × 1.5 cm. Three times a week, the beetles were transferred to freshly prepared boxes with moist filter paper and cut third-instar *Calliphora* maggots as food. Sexually isolated and well-fed beetles of both sexes at an age of 3 weeks were termed "standard laboratory beetles." The normal temperature and light regime were 22°C and 16:8 hr light–dark. Starved males were kept individually in order to avoid cannibalism.

Chemical Methods

Extracts were prepared according to Peschke and Metzler (1986). Thirty to 100 beetles were killed by freezing and extracted in 200 ml methylene chloride in a Soxhlet apparatus for 24 hr. The hydrocarbon fraction was purified on a silica gel column.

Gas chromatography was also conducted according to Peschke and Metzler (1986) on a Varian 3700 instrument with a 30-m DB-1701 capillary column (split 1:10, 1 ml He/min, 60°C to 300°C at 3°C/min, flame ionization detector). Quantitative measurements were carried out with a Kontron-Anacomp 220 computer system. The concentrations of the two pheromone alkenes were determined by using the corresponding *n*-alkanes as internal standards of constant titer and then related to the pheromone content of standard laboratory females [1 female equivalent (FE) (Z)-7-heneicosene = 2 μg, 1 FE (Z)-7-tricosene = 12 μg; Peschke and Metzler, 1986]. Both alkenes were synthesized according to Peschke and Metzler (1986). Some samples were also obtained from Dr. L.L. Jackson (Bozeman, Montana). For comparison of hydrocarbon patterns, we calculated the equivalent chain length for methyl-branched alkanes and plot-

ted the cumulative distribution functions of compounds versus increasing chain length (Hadley, 1977; Toolson and Hadley, 1977).

Behavioral Responses

At a distance of about 2.2 mm from the female, the male bends his mobile abdomen over his head and protrudes the genitalia with the tong-shaped parameres (grasping response; Peschke, 1978). Male aggression is characterized by pushing the opponent with head and mandibles and by drumming with the mobile abdomen on the other individual. Females that repulse the male grasping response do so by oscillating their mobile abdomen thereby avoiding the fixation of the male genitalia. In neutral encounters of two individuals, no conspicuous behavior could be observed (Peschke, 1987).

Bioassays

The sexual response of *A. curtula* males to the purified hydrocarbon fractions of beetles of various physiological status was tested in the model bioassay (Peschke, 1978). Soxhlet-extracted odorless beetles were glued to the tips of glass needles and treated with *n*-pentane solutions of 0.01 ml containing one beetle equivalent. The number of standard tester males responding to the model with the grasping response was specified in percentiles and statistically evaluated by the χ^2 test or the exact test of Fisher (Sachs, 1984). Confidence limits were calculated for the 95% level.

Beetles of both sexes and various physiological status were observed as single pairs in plastic boxes of 5.5 × 3.5 × 1.5 cm for a period of 30 min. The individuals were marked by dots of enamel paint on pronotum and both elytra. We recorded whether a certain behavioral response was seen at least once during the observation period, or we registered all encounters of beetles and specified the percentage in which a certain behavior was shown. Living beetles were treated with extracts or synthetic chemicals: 0.01 ml pentane solutions with one beetle equivalent concentration were applied to the pronotum of a chilled individual (6°C). As a control, beetles were treated with the pure solvent. One hour after contamination, the beetles were brought together and the pair was observed for 30 min as usual.

Antennal segments or palps of males 7 days old were cut off using pincers which were sharpened like scissors. Scapus and pedicellus were counted as segments 1 and 2, the flagellar segments as 3–11. After a further week, males of normal agility were observed individually together with single females (30 min).

For observation of interspecific reactions of beetles under natural conditions, we used an arena of 50 × 50 cm, in the center of which a small piece of carrion was placed (beef liver with *Calliphora* maggots, covered by a dish of

red glass; for details see Peschke, 1987). Individually marked *A. curtula* males and females (five each) were put together with five males and five females of another species (*A. brevipennis*, *A. peschkei*, and *Philonthus politus*). The beetles were observed at the carcass with a red-sensitive camera in a natural rhythm of white and dark red light (18:6 hr light–dark). The behavioral events at the carcass were recorded for 3 min every 60 min at normal speed over a period of 24 hr. For each encounter of two beetles, we recorded whether aggression, grasping, or neutral behavior was exhibited by *A. curtula* males.

RESULTS

Sex Pheromone Content in Beetles of Various Sex and Physiological Status

In former investigations (Peschke, 1985, 1987), all bioassays for testing pheromone contents had been conducted with entire beetles freshly killed by freezing. In the present experiments, sexual responses were also elicited by the purified total hydrocarbon fraction from extracts of females and males of various physiological status (model bioassay; Figure 1).

The cuticular hydrocarbon fraction from females collected in the field released the male sexual response at the same high rate as did extracts from standard laboratory females, from females just after copulation, and from those of small body size (Figure 1). Freshly emerged females yielded a somewhat lower grasping rate. The grasping response rate was drastically reduced by exposing the females to elevated temperatures (14 days at 27°C).

High behavioral release rates were also obtained with extracts from males captured in the field. The hydrocarbon fraction from young, starved, or multiply mated laboratory males also elicited homosexual grasping responses (Figure 1). On the other hand, hydrocarbons from sexually isolated, well-fed males reared in the laboratory did not trigger homosexual grasping responses, regardless of temperature (22°C or 27°C) and body size (large standard beetles: breadth of pronotum 2.0 $\pm$ 0.02 mm, small beetles: 1.4 $\pm$ 0.03 mm).

The quantitative determination of components of the hydrocarbon fractions used in the former bioassay gave a good correlation of grasping rates with the contents of (*Z*)-7-tricosene (Figure 1). Parallel to the determination of amounts (in micrograms), we preferred the scaling in terms of female equivalents in order to obtain a more lucid comparison of pheromone contents of mimicking individuals with that of standard laboratory females (defined as 1 FE). Females of various physiological status, apart from young beetles, had a constantly high tricosene content. In females kept at elevated temperatures, only trace amounts of this compound could be detected. Standard laboratory males had a concentration of 10^{-3} FE tricosene irrespective of their body size and the temperature at which they had been reared. Young, starved, and multiply mated males, however, yielded tricosene contents ranging from 0.08 to 1 FE.

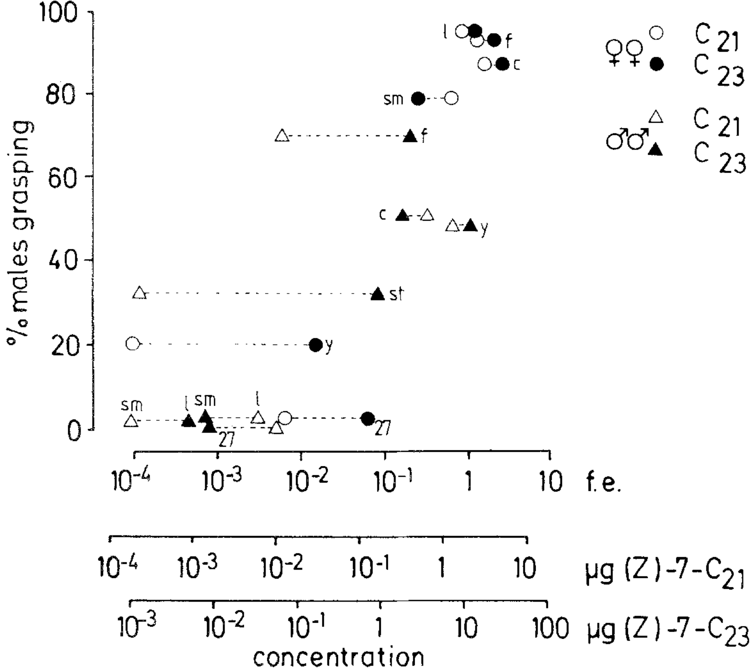

FIG. 1. Grasping response of *A. curtula* males towards models contaminated with one beetle equivalent of the total cuticular hydrocarbon fraction from extracts of males (triangles) and females (circles) of various physiological conditions: l: laboratory standard females (sexually isolated, well fed, 22°C); f: field-collected beetles; y: freshly emerged beetles; st: mature beetles starved for a period of 10 days; c: one copulation at the day of extraction; 27: beetles kept at elevated temperatures (27°C); sm: beetles reared on a small host. Thirty to 100 beetles were used per extraction; at least 100 males were tested for grasping in five replicates per sample. The grasping rate is plotted against the concentrations of the two pheromone compounds (Z)-7-heneicosene (open symbols) and (Z)-tricosene (solid symbols) in the hydrocarbon fraction as determined by quantitative gas chromatography. The concentration is scaled in terms of female equivalents as related to the content of laboratory standard females (= 1 FE). Because the combined action of compounds was tested in the bioassay, one value of grasping rate refers to two values of concentration of both pheromone compounds, which are linked by a dotted line.

The grasping rate is correlated with the concentration of (Z)-7-heneicosene in a similar way (Figure 1), except in the cases of field-collected or starved males and of young females, which release male sexual responses but have a low or even undetectable titer of the C_{21}-alkene. In these cases, the tricosene alone seems to be responsible for the release of the homosexual grasping response. Heneicosene could also not be detected in small males and males kept at 27°C.

Modulation of Male Aggression by Female Sex Pheromone

In the experiments described above, we demonstrated a correlation of homosexual responses with the production of female sex pheromone components by males. In the following experiments, the sex-specific chemical information was manipulated in order to investigate the correlation of female pheromone production with the release of male aggressive behavior.

Temperature Experiment. Males and females kept at 22°C or 27°C were observed as single pairs in various combinations (Table 1). As in the experiments with extracts, females kept at 27°C did not release male grasping at any encounter. On the other hand, the reduction of pheromone titer in females kept at elevated temperatures provoked a high aggression rate of males towards these females. The behavioral sequence was not different from that observed in intermale combats.

Contamination of Males with Synthetic Female Pheromone Mixture. A reduction of the release of aggression was demonstrated in observations (30 min) of pairs of males, to one of which the hydrocarbon fraction of a female surface washing had been applied; to the other, the pure solvent (Table 2). The male bearing the female pheromone attacked the other male in a high percentage of encounters, whereas the male without the pheromone only occasionally behaved aggressively towards the treated male. The treated male, however, released a high rate of homosexual graspings.

The same tendencies were observed when one male was contaminated with 1 FE of the synthetic female sex pheromone (Table 2). The rate of aggression is drastically reduced towards the treated male; however, more neutral encounters than homosexual grasping responses were observed in comparison to experiments with the total female hydrocarbon fraction.

Amputation of Male Antennal Segments. Males with their antennae amputated to various degrees were observed together with females during a 30-min period. The number of males exhibiting the grasping response towards

TABLE 1. AGGRESSION AND GRASPING RESPONSES DURING 30-MIN OBSERVATIONS OF SINGLE PAIRS OF *A. curtula* WITH MALES AND FEMALES KEPT AT DIFFERENT TEMPERATURES

Response of males kept at	Towards females kept at	Response (%)			$N_{encounters}$ (N_{pairs})
		Aggression	Neutral	Grasping	
22°C	22°C	11	31	58	160 (4)
22°C	27°C	52[a]	48	0[a]	84 (5)
27°C	22°C	0	28	72	93 (5)
27°C	27°C	33[a]	67	0[a]	30 (4)

[a] $P < 0.001$; χ^2 test: comparison to the preceding line.

TABLE 2. AGGRESSION AND HOMOSEXUAL GRASPING RESPONSES DURING 30-MIN OBSERVATIONS OF TWO *A. curtula* MALES, ONE CONTAMINATED WITH HYDROCARBON FRACTION OF FEMALE EXTRACT (1 FE) OR SYNTHETIC FEMALE SEX PHEROMONE (1 FE: 12 μg (Z)-7-TRICOSENE + 2 μg (Z)-7-HENEICOSENE), THE OTHER MALE (CONTROL) TREATED WITH SOLVENT (0.01 ml *n*-PENTANE)

Contamination with	Response of	Response (%)			$N_{encounters}$ (N_{pairs})
		Aggression	Neutral	Grasping	
Female hydrocarbon fraction	control males towards contaminated males	14	16	70	93 (4)
	contaminated males towards control males	88[a]	11	2[a]	85 (5)
Synthetic female sex pheromone mixture	control males towards contaminated males	10	73	17	60 (5)
	contaminated males towards control males	94[a]	6	0[a]	89 (5)

[a] $P < 0.001$; χ^2 test: comparison to the preceding line.

females was not reduced as long as four antennal segments were left (Figure 2). The next step of amputation (three segments left), however, gave a significant decrease of the grasping rate, which was continued with removal of further antennal segments or palps. Coincidentally with the stepwise decrease of sexual responses, the percentage of males behaving aggressively towards females increased, with a peak rate obtained when only the scapus was exempted from amputation. The rate of aggression was then decreased with further steps of removal of antennal segments and palps.

Releasers of Aggression

Sexual behavior as well as male aggression can be manipulated by the female sex pheromone titer. However, the simple absence of the female sex pheromone may be a signal for males to fight against another individual, or species-specific signals that may be other cuticular hydrocarbons may release male aggression. While in the former experiments the concentration of pheromone alkenes has been shown to vary considerably between the sexes, the saturated hydrocarbons have a very constant distribution (Peschke, 1985; Peschke and Metzler, 1986). The cumulative frequency function of the equivalent chain length of saturated hydrocarbon components also shows a very similar pattern for both sexes of *A. curtula* (Figure 3). In contrast, cuticular hydrocarbons of male *A. brevipennis* or *A. peschkei* have a distribution very different from that

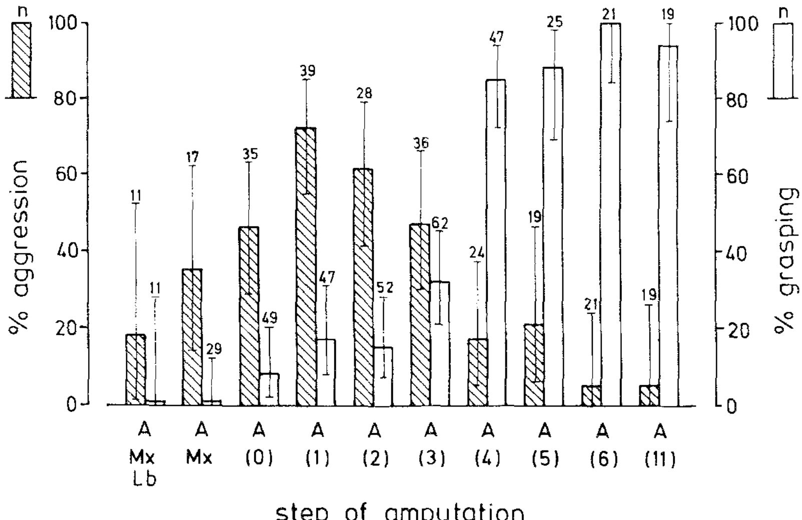

FIG. 2. Behavioral responses (grasping, aggression) of *A. curtula* males (various steps of symmetrical amputation of antennal segments and palps) to standard females during a 30-min observation (percent pairs showing the response at least once). Steps of amputation: A: antenna (in parentheses number of segments exempted from amputation, A11: control), Mx: maxillar palps; Lb: labial palps. Vertical bars: 95% confidence limits; n: number of pairs).

of *A. curtula*. Most noteworthy is the shift to higher carbon numbers and the total absence of C_{21} and C_{23} hydrocarbons. The identification of components of *A. brevipennis* and *A. peschkei* by mass spectrometry with the original gas chromatograms will be published elsewhere (Peschke and Metzler, in preparation).

A. curtula males and females were put together with individuals of other species in the video observation setup. *A. brevipennis* released a few grasping responses of *A. curtula* males (Table 3) which might be due to unsaturated C_{27} hydrocarbons. Aggression of *A. curtula* males against *A. brevipennis* individuals of both sexes was observed at an intermediate level. *A. peschkei* did not release interspecific grasping or aggressive responses of *A. curtula* males. Individuals of both sexes of *Philonthus politus*, a distantly related species from another subfamily of rove beetles (Staphylininae instead of Aleocharinae), also failed to provoke either aggression or sexual responses of *A. curtula* males (Table 3).

In conclusion, male aggression of *A. curtula* seems to be released by species-specific chemical cues, probably involving the saturated hydrocarbons of the cuticle. This hypothesis is supported by experiments with *A. peschkei* males contaminated with the hydrocarbon fraction of male *A. curtula* extracts (Table

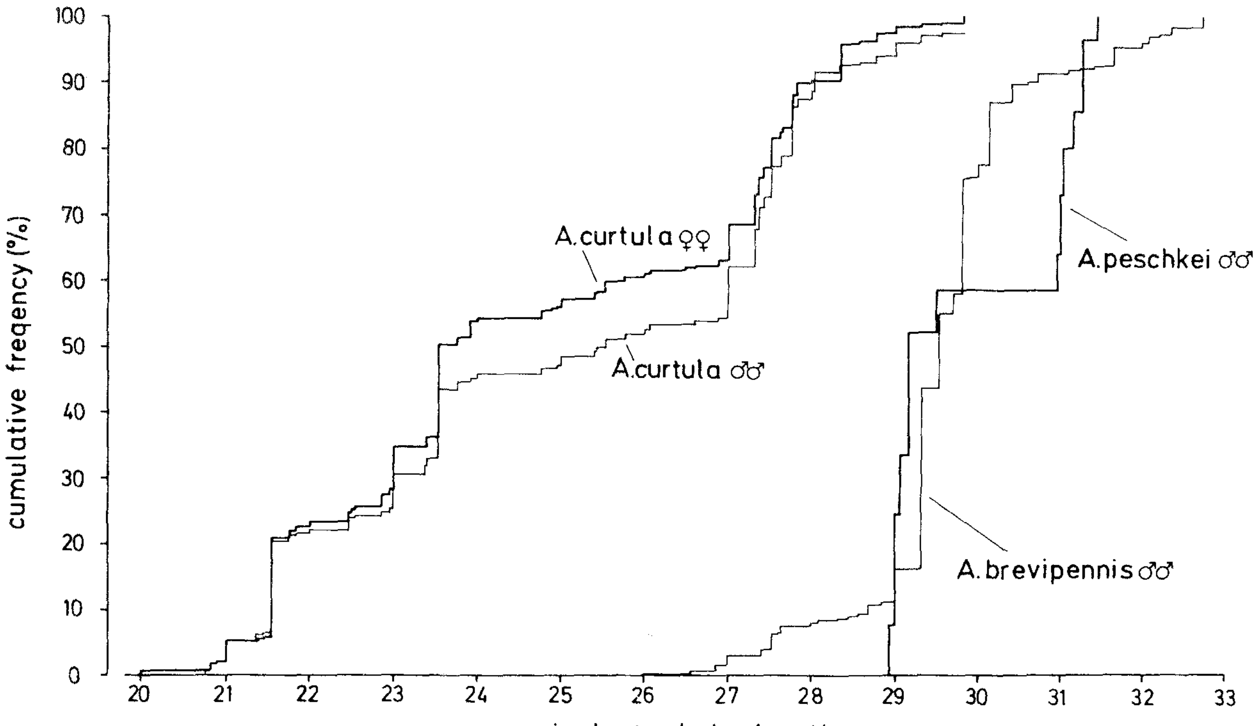

FIG. 3. Cumulative distribution functions of cuticular hydrocarbons of various equivalent chain lengths of carrion-inhabiting *Aleochara* species (*A. curtula* males and females; males of *A. brevipennis* and *A. peschkei*).

TABLE 3. BEHAVIORAL RESPONSES OF *A. curtula* MALES TO MALES AND FEMALES OF OTHER ROVE BEETLE SPECIES AT LABORATORY-SIMULATED CARCASS (VIDEO OBSERVATION).

	Response of males (%)[a]			
Response towards	Aggression	Neutral	Grasping	$N_{encounters}$ (N_{pairs})
A. curtula				
Males	95	5	0***	1206 (15)
Females	15***	66	19	437 (15)
A. brevipennis				
Males	38***	54	8 NS	13 (5)
Females	34***	54	12**	143 (5)
A. peschkei				
Males	6***	94	0***	145 (5)
Females	0***	100	0*	23 (5)
Philonthus politus	1***	99	0***	71 (5)

[a]$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; χ^2 test: comparison to *A. curtula* males (aggression) or to *A. curtula* females (grasping).

4). While *A. curtula* males did not behave aggressively towards *A. peschkei* individuals contaminated with the solvent in the 30-min observation bioassay, they exhibited a conspicuous fighting behavior towards *A. peschkei* specimens treated with male *A. curtula* hydrocarbons.

Female Repulsion Behavior

Males which release homosexual responses of other males may show the grasping response towards females. However, in comparison to nonmimicking

TABLE 4. AGGRESSION OF *A. curtula* MALES TOWARDS *A. peschkei* MALES CONTAMINATED WITH HYDROCARBON FRACTION OF EXTRACTS OF *A. curtula* MALES (1 ME, 0.01 ml) OR SOLVENT (*n*-PENTANE) DURING 30-MIN OBSERVATIONS OF SINGLE PAIRS

Response of *A. curtula* males towards	Response (%)			
	Aggression	Neutral	Grasping	$N_{encounters}$ (N_{pairs})
A. peschkei males				
Control	0	100	0	19 (4)
Contaminated	80[a]	20	0	45 (5)

[a]$P < 0.001$; χ^2 test, comparison to control.

TABLE 5. GRASPING OF *A. curtula* MALES CONTAMINATED WITH SYNTHETIC FEMALE SEX PHEROMONE (1 FE: 12 μg (Z)-7-TRICOSENE + 2 μg (Z)-7-HENEICOSENE) AND REPULSION OF GRASPING RESPONSES BY FEMALES DURING 30-MIN OBSERVATIONS OF SINGLE PAIRS

Males	Grasping of males (%)	Repulse by females (%)	$N_{encounters}$ (N_{pairs})
Contaminated with synthetic female pheromone	99 NS	80[a]	285 (6)
Control	94	32	83 (15)

[a] $P < 0.001$; χ^2 test: comparison to control.

males, they are more often repulsed by the female, who drums with her abdomen and thus avoids the fixation of male genitalia (Peschke, 1987). In order to investigate the releasers of female repulsion behavior, males were contaminated with 1 FE of the synthetic mixture of the female pheromone (Table 5). The treated males did not show an alteration in their own sexual response to females as measured by the grasping response rate during a 30-min observation period. On the other hand, the repulsion behavior of females towards such treated males is significantly increased.

DISCUSSION

Female mimicry in vertebrates using chemical (Mason and Crews, 1985) or behavioral and morphological cues (for review see Weldon and Burghardt, 1984) has been described for many species. Stealing sneaky copulations, avoidance of intermale aggression, and access to other males' territories are discussed as the main benefits of mimicking males. Behavioral transvestism in insects has only been described for scorpion flies where males behave like females in order to steal the nuptial gifts of other males (Thornhill, 1979).

Certain physiological types of males of the rove beetle, *Aleochara curtula*, mimic their females chemically in order to avoid intermale aggression. In this way they get access to a carcass and are able to feed on blow fly maggots in order to replenish their energy reserves for a forthcoming reproductive cycle (Peschke, 1987). On the other hand, females prefer males without the female pheromone. The adaptive significance of female coyness towards mimicking males seems to be the choice of an optimal mate: immature, starved, or multiply mating males, which all produce the female sex pheromone, need access to the

food resource; they transfer only small spermatophores and fertilize less eggs. On the other hand, physiologically competent males without the female sex pheromone have succeeded in numerous aggressive interactions with other males (Peschke, 1987).

(Z)-Heneicosene and (Z)-7-tricosene have previously been isolated and identified from mature *A. curtula* females as the main sex pheromone components (Peschke and Metzler, 1986). The pheromone molecules are components of the epicuticular waxes and are spread over the entire surface of the beetles (Peschke, 1978, 1986). Male antagonistic pheromones, which might be involved in the regulation of sex specificity of the pheromone information, could not be detected (Peschke, 1986). In the present work the pheromone alkenes were also found in the cuticular hydrocarbons of those males which release homosexual responses, e.g., young, starved, or multiply mated beetles. All these physiological types of males are mixed up in field collections (Peschke et al., 1987), where they also bear the female sex pheromones. Small males, which perform an alternative mating tactic as satellites in the surroundings of carrion (Peschke, in preparation), do not produce the female sex pheromone for protection.

The contents of the two pheromone components in various physiological types and both sexes of *A. curtula* range over three powers of 10 and are correlated with the release of male sexual responses. In most samples, both compounds contribute to the releasing effect of the total hydrocarbon fraction. In some cases, tricosene alone seems to be responsible for the release of sexual responses. In tests of individual synthetic substances, tricosene had its optimum at about 0.1 FE, whereas heneicosene released more sexual responses at the higher concentration of 1 FE (Peschke and Metzler, 1986). It is therefore difficult to quantify the contribution of the individual pheromone component to the releasing efficiency of the total hydrocarbon mixture of each physiological type. The combination of both compounds had no synergistic, only an additive, effect; the admixture of (Z)-9-heneicosene or (Z)-9-tricosene, which are produced together with the respective 7-isomers but have only little releasing efficiency (Peschke and Metzler, 1986), did not produce an antagonistic action (Peschke, in preparation).

Differences in the release of grasping rates towards synthetic compounds or towards the total hydrocarbon fraction containing the same amounts of pheromone components may be due to retardation of evaporation by the accompanying saturated hydrocarbons in the latter case (Peschke, 1986). This effect may also be responsible for different responses towards contaminated living males. Homosexual grasping is released at a lower rate after contamination with the synthetic pheromone mixture in comparison to males treated with the total hydrocarbon fraction of females. However, the aggression rate is reduced to the same extent in both cases. Individual (Z)-7-heneicosene and (Z)-7-tricosene

both contribute to the reduction of intermale aggression (Peschke, in preparation).

The modulatory effect of the female sex pheromone on the release of male aggression was also demonstrated by manipulation of the chemical information output of the female. Females which have been exposed to elevated temperatures show a significantly reduced content of cuticular alkenes in comparison to that of saturated compounds. This may be due to differential evaporation of saturated and unsaturated compounds. However, it cannot be excluded that the beetles actively alter the pattern of hydrocarbon synthesis at different temperatures (see Hadley, 1977; Toolson and Hadley, 1977). Males behave aggressively towards these females in the same way as towards other males.

A further indication of the modulatory effect of pheromone alkenes is the gradual increase of aggression accompanied by a decrease of sexual recognition with successive amputation of male antennal segments. In a preliminary investigation of the antennal morphology of *A. curtula* (Ungelenk and Altner, unpublished), no sex specific sensilla or striking quantitative difference in the distribution and number of various types could be found. The pattern of densely packed sensilla seems to be repeated in segments 5–11, whereas scapus, pedicellus, and the two proximal flagellar segments bear only a few sensilla. It seems likely that the sex pheromone information is perceived by numerous receptor organs sequentially distributed on the distal antennal segments. Removal of them gradually diminishes the input of sex pheromone information, whereas information on aggression-releasing chemicals, probably the saturated hydrocarbons, still can be perceived. Further steps of amputation reduce this input too. However, electrophysiological investigations of pheromone perception in *A. curtula* are urgently needed.

Females of *A. curtula* are apparently able to perceive the female sex pheromone too. However, the female behavioral response elicited by these chemicals, the release of aggressive repulsion of male mating attempts, is the reverse of the male reaction where aggression is reduced.

The releasers of intermale aggression of *A. curtula* may comprise components of the saturated hydrocarbon mixture that are present in both sexes. The shift to longer carbon chains in related species reduces aggression of *A. curtula* males; on the other hand, contamination of males of the other species with hydrocarbons of *A. curtula* males, which do not contain female pheromones, provoked intermale combats. At present, synthetic methyl-branched alkanes are not available, and therefore it is not possible to determine yet whether individual *n*-alkanes, methyl-branched alkanes, or the complex pattern of compounds of variable chain lengths serve as the releasers of male aggression. Saturated hydrocarbons modulate the information of alkenes acting as female sex pheromones in several muscid flies (e.g., Rogoff et al., 1980; Sonnet et al., 1975, 1977; Uebel et al., 1975a, b, 1976), or represent the major female pher-

omones, for example, in tsetse flies (e.g., Carlson et al., 1978; Huyton et al., 1980a, b) or butterflies (Grula et al., 1980).

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INTERACTIONS OF ALKALOIDS WITH GALEAL CHEMOSENSORY CELLS OF COLORADO POTATO BEETLE

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Abstract—The galeal chemosensory system of the adult Colorado potato beetle was used as a model to study the effects of alkaloids on insect gustation. Nine alkaloids, representing a wide range of structural types, were used. Their ability to stimulate chemosensory cells when presented in isolation and their ability to interfere with normal chemosensory processes were emphasized. None of the alkaloids stimulated chemosensory cells in a dose-dependent manner, although a few stimulated low-level activity from some cells. There was no evidence for a general "deterrent receptor" in these beetles. Some of the alkaloids had a marked inhibitory effect on normal chemosensory responses. Tomatine, solanine, papaverine, and sparteine significantly inhibited responses to amino acids (represented by GABA) while quinine and papaverine inhibited responses to sucrose. An attempt was made to correlate neurophysiological action of some alkaloids with their effects on feeding behavior. It was clear from this correlation that even a dramatic inhibition of sensory input by an alkaloid does not necessarily lead to measurable effects on behavior. The results are discussed in the context of current theories on the mode of action of alkaloids and other secondary plant compounds which may be involved in host recognition by phytophagous insects.

Key Words—*Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae, Colorado potato beetle, deterrent receptor, feeding deterrents, alkaloids, sugar receptor, amino acid receptor, chemoreception, steroidal alkaloids, antifeedants.

INTRODUCTION

Alkaloids represent one class of plant compounds that have come to be considered secondary because of the difficulty encountered in discovering a role for them in primary plant metabolism. The idea that secondary plant compounds

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might be important in protecting plants against natural enemies has generated a working hypothesis which suggests that insect herbivores will not feed on most plant species because of the presence of secondary compounds which inhibit feeding. One of the earliest and most comprehensive statements of this hypothesis came from Jermy (1961, 1966). More recently, several review papers have been published in which the relationships among secondary compounds, feeding deterrency, and chemoreception are discussed (Dethier, 1980, 1982; Jermy, 1983; Schoonhoven, 1982).

While it is clear from these papers that we now realize chemical messages signaling acceptance and nonacceptance are complex, the ideas of "specific deterrent receptors" (Schoonhoven, 1982); generalist receptors for deterrents, with the central nervous system responsible for decoding a complex message (Dethier, 1980); and an "inhibitory biochemical profile" for recognition of nonhost plants (Jermy, 1983) are given prominence. Implicit in each of the above hypotheses is the existence of receptors (in the pharmacological sense) evolved to interact with secondary plant compounds. These receptors may be found primarily on a cell which sends a negative message to the central nervous system (the so-called deterrent receptor) or they may be found spread variously over the membranes of a number of sensory cells requiring the central nervous system to decode the complex message. These ideas are largely based on data obtained from larvae of a number of lepidopteran species.

An additional hypothesis, which appears to better fit existing data on chrysomelid beetles, puts more emphasis on the general bioactive nature of many plant secondary compounds. Mitchell and Sutcliffe (1984) suggest that these compounds may not normally require a particular receptor type (specific or general) in order to have an effect. Instead, they may only need to be "capable of interfering with processes that are generally found in excitable membranes." These processes include receptors (in the pharmacological sense) for feeding stimulants as well as basic membrane properties associated with maintenance of a resting potential and production of a generator potential or an action potential.

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say) has been important in the development of ideas concerning the role of plant secondary compounds as feeding deterrents. Considerable effort has so far failed to reveal specific feeding stimulants which could account for the clear recognition of several solanaceous species as host plants by this insect (Ritter, 1967; Hsiao, 1968). On the other hand, the steroidal glycoalkaloids of the Solanaceae are well known, and the concept of host-plant selection by avoidance of deterrents has been associated with the Colorado potato beetle for some time. Because the suggested influence of alkaloids on plant avoidance by this beetle is generally accepted, its chemoreceptors provide an excellent system on which to test the various hypotheses discussed above.

In an earlier study (Mitchell and Harrison, 1985), we attempted to determine if the galeal chemosensory system of the adult Colorado potato beetle had cells sensitive to steroidal glycoalkaloids which could be interpreted as deterrent cells. Such cells were not found in this sensory field, instead the alkaloids elicited nonspecific responses from many cells, and caused at least short-term loss of sensitivity. The effect was similar to that caused by other surfactant chemicals such as saponins, and no differences were observed among effects of tomatine, solanine, and chaconine. In this paper, the possibility of alkaloid interactions with the stimulants sucrose and α-amino butyric acid (GABA) is addressed, together with an extension of the search for specific responses to alkaloids in this chemosensory system. In addition, an attempt is made to relate observed effects of some of these compounds on chemosensilla to their influence on feeding behavior in a complex sensory environment.

METHODS AND MATERIALS

Adult insects of both sexes were used for sensillar recording. To keep variability to a minimum, recordings were always made from adults which had emerged within 24 hr of the time of the experiment. Beetles were from a laboratory colony that is continuously maintained on fresh cut leaves of *Solanum tuberosum*. Wild *L. decemlineata* are collected each summer in the Edmonton area and added to this culture. Details of the rearing technique are provided in Mitchell and Harrison (1984) and in Harrison (1985).

Only the apical chemosensitive pegs on the galea of the maxilla were considered. This chemosensory field consists of 11–15 peg-like sensilla, usually with four chemosensory cells per peg (Sen and Mitchell, 1987). One of the sensilla in this field was termed the α-sensillum by Mitchell and Harrison (1984) because its sensitivity to amino acids was much greater than that of other galeal sensilla. Because of this sensitivity difference, the study of alkaloid interaction with GABA was conducted using the α-sensillum exclusively. All galeal chemosensilla have equal sensitivity to sucrose (Mitchell and Harrison, 1984). The tip-recording method originally described by Hodgson et al. (1955) was used throughout. The galeal sensilla studied have a very low sensitivity to NaCl and KCl, with concentrations as high as 150 mM stimulating very little activity. This feature allows a wide choice of saline concentrations for the carrier solution in the stimulating–recording electrode. In this study 50 mM NaCl was used. This concentration of salt rarely stimulates any activity from galeal chemosensilla in this insect. Alkaloids were dissolved in 50 mM NaCl made up in deionized water (pH approx. 6.7). When necessary (e.g., solanine and tomatine) solvent pH was lowered to 2 in order to dissolve the alkaloid, following which the pH was raised to 5 before testing. No effect related to pH alone in the range

5–7 has been observed (unpublished observations and Mitchell and Harrison, 1985).

Cell injury is always a possibility when working with alkaloids at the concentration used in this study (1 mM). Consequently, no more than three alkaloids were tested on any particular sensillum. In addition, regular applications of 10 mM GABA or sucrose were used to confirm that at least one of the cells in the sensillum was functioning normally during the entire experiment. Deterioration in response or signal-to-noise ratio terminated experiments on a preparation. In tests of alkaloid effect on responses to GABA or to sucrose, paired stimuli (e.g., GABA followed by GABA + alkaloid) were used to keep variability to a minimum. Responses were recorded on magnetic tape using a TEAC four-channel recorder and a Vetter FM recording adapter. Segments of each response were digitized using an Apple II computer fitted with an analog to digital card, and plotted on a Hewlett-Packard digital plotter for analysis and presentation. This system is described in Mitchell and McIntyre (1986).

Adult feeding on water-infused and alkaloid-infused potato leaves was measured using an assay described in Harrison (1985). Briefly, this method uses video analysis of prefeeding behavior and aspects of first meal consumption of individual, newly emerged adult beetles of both sexes. Care was taken to ensure that leaves maintained turgor pressure and other visible characteristics of good health during infiltration and during the feeding test. Analysis of the video data produced a number of measures related to plant acceptance and feeding (Harrison, 1985). For this study the following four were chosen: (1) time (seconds) spent in prefeed maceration activity, this is the first behavior that brings the plant sap into contact with mouthpart sensilla, probably the epipharyngeal sensilla in the upper part of the buccal cavity; (2) percent of beetles which rejected the leaf after some time spent in prefeed maceration behavior; (3) area of leaf consumed during first meal (mm^2, measured with a leaf-area meter), end of a meal was defined as more than 3 mm without feeding following some period of feeding; and (4) feeding rate (area/feeding time). Ten beetles were used in each experiment.

RESULTS

Stimulation with Alkaloids Alone. All the alkaloids shown in Figure 1 were tested at 1 mM for their effects on galeal sensilla. They were each applied to several sensilla on six preparations (four for atropine) using short (1–3 sec) application times. In no cases were there responses resembling the phasic–tonic responses normally obtained from insect chemoreceptors. In some cases a more or less regular, low-level response was elicited from a single cell. This was most consistent for strychnine (six of six animals) and occurred in three of six cases with arecoline, sparteine, and quinine. Atropine, papaverine, caffeine,

TOMATINE

SOLANINE

(steroidal glycoalkaloids)

PAPAVERINE
(isoquinoline)

SPARTEINE
(lupine)

ATROPINE
(tropane)

ARECOLINE
(pyridine)

CAFFEINE
(purine)

QUININE
(quinoline)

STRYCHNINE
(indole)

FIG. 1. Structures of the nine alkaloids used in this study.

tomatine, and solanine did not stimulate any cell in this manner. Irregular, bursting patterns of firing from one or more cells during short applications was seldom observed (four of 52 cases).

Because of the single-cell response in some preparations to 1 mM strychnine, arecoline, sparteine, and quinine, a limited dose–response study was con-

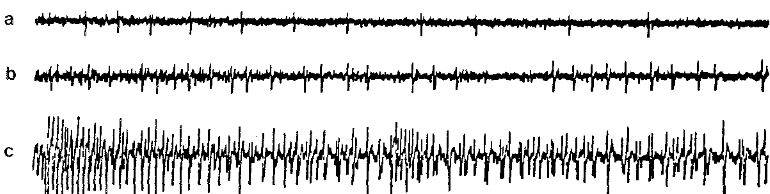

FIG. 2. (a, b) Response to sparteine sulfate (10 mM) from two sensilla on the same preparation. First second of response is shown. (c) burst of multicell activity after 4 sec of stimulation with 5 mM strychnine nitrate. Time bar = 100 msec.

ducted with these compounds. There was no increase in the low-level response over several concentrations between 1 and 10 mM for any of the compounds. Figure 2a and b shows a typical response to 10 mM sparteine sulfate from two preparations. The same four alkaloids were tested in long-term applications (approximately 30 sec). Strychnine and quinine, at 5 mM and 10 mM, elicited bursting activity after several seconds of application (Figure 2c). This activity is reminiscent of the delayed responses caused in this system by 1 mM concentrations of steroidal glycoalkaloids and saponins (Mitchell and Harrison, 1985). The high concentrations necessary to obtain this response with strychnine and quinine make it unlikely that the effect is behaviorally significant.

Inhibition of Responses to GABA and Sucrose. Table 1 documents the ac-

TABLE 1. INHIBITION OF RESPONSE TO GABA BY NINE ALKALOIDS[a]

Alkaloid	Response to GABA (10 mM)	Response to GABA (10 mM) plus alkaloid (1 mM)	Relative decrease in response	*N*
Tomatine	13.9 ± 3.9	3.8 ± 4.0	3.7[b]	6
Solanine	12.8 ± 3.2	7.3 ± 2.1	1.8[b]	4
Papaverine	15.8 ± 2.3	9.7 ± 5.1	1.6[b]	6
Sparteine	15.7 ± 3.4	11.2 ± 3.5	1.4[b]	6
Atropine	17.8 ± 5.4	12.4 ± 2.5	NS	5
Arecoline	16.3 ± 3.9	13.3 ± 1.9	NS	6
Caffeine	20.2 ± 3.1	17.8 ± 4.0	NS	6
Quinine	14.8 ± 3.9	13.5 ± 2.6	NS	6
Strychnine	15.3 ± 6.5	14.5 ± 4.5	NS	6

[a] Data represent impulses from α-sensillum occurring during 250–750 msec of 1-sec stimulations. Responses are expressed in impulses per 0.5 sec. Errors are ±SD.
[b] $P < 0.05$ Mann-Whitney U test. This survey was conducted using 18 adult *L. decemlineata*. Two to three alkaloids were tested per preparation. Multiple amino acid stimulations were distributed throughout the test period and each response to a mixture of GABA and alkaloid was compared to the preceding response to GABA alone.

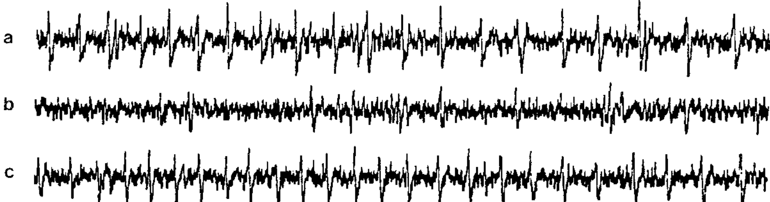

FIG. 3. Response of an α-sensillum to three sequential stimulus applications. (a, c) 10 mM GABA; (b) 10 mM GABA + 1 mM tomatine. Records show responses from 250 to 750 msec following stimulus application. Time bar = 100 msec.

tion of nine alkaloids when applied to the α-sensillum (Mitchell and Harrison, 1984) in the presence of 10 mM GABA. This sensillum contains a cell which is particularly sensitive to GABA and L-alanine, and Mitchell (1985) presents evidence that the same receptor is activated by both of these amino acids. Four of the alkaloids, when present at 1 mM, significantly reduced the response to 10 mM GABA. Tomatine was especially effective, reducing the response nearly fourfold (Table 1 and Figure 3).

The same cell in the α-sensillum responds to sucrose (Mitchell and Harrison, 1984), presumably involving a different receptor site (see Discussion). Most of the alkaloids tested at 1 mM had no significant effect on the response to sucrose, but quinine and papaverine were strong inhibitors (Table 2).

Stimulation of Additional Cells. The ability of these alkaloids to stimulate

TABLE 2. INHIBITION OF RESPONSE TO SUCROSE BY NINE ALKALOIDS[a]

Alkaloid	Response to sucrose (10 mM)	Response to sucrose (10 mM) plus alkaloid (1 mM)	Relative decrease in response	N
Quinine	11.8 ± 5.2	2.2 ± 3.5	5.4[b]	6
Papaverine	10.6 ± 4.2	3.2 ± 3.4	3.3[b]	5
Solanine	10.0 ± 4.8	5.6 ± 3.2	NS	5
Strychnine	10.4 ± 2.2	6.2 ± 7.6	NS	6
Sparteine	11.5 ± 3.2	8.3 ± 3.9	NS	4
Tomatine	11.1 ± 5.5	9.2 ± 4.4	NS	7
Caffeine	14.8 ± 7.4	12.3 ± 4.8	NS	4
Arecoline	10.2 ± 4.4	11.4 ± 6.1	NS	5
Atropine	10.0 ± 3.4	9.4 ± 3.3	NS	7

[a] Data represent impulses from α-sensillum occurring during 250–750 msec of 1-sec stimulations. Responses are expressed in impulses/0.5 sec. Errors are ±SD.
[b] $P < 0.05$ Mann-Whitney U test.

cells when presented alone may not necessarily predict their action on additional cells when one cell in the sensillum is already active. Therefore, the ability of alkaloids to stimulate additional cells when the amino acid-sensitive cell was already active was tested using mixtures of 10 mM GABA and 1 mM alkaloid. In some preparations, some alkaloids markedly stimulated additional cells when the amino acid-sensitive cell was active. This type of response was quite variable, but it occurred often enough and was of sufficient intensity when it occurred to warrant quantification. The data are presented in two ways: (1) average activity in non-amino acid-sensitive cell(s) during stimulation with GABA and GABA plus alkaloid are compared (Table 3); and (2) responses from preparations which showed a marked effect of alkaloid on non-amino acid-sensitive cell(s) under these conditions are presented alone (Table 4).

A marked effect was defined as a firing rate >20 impulses/sec from the non-amino acid-sensitive cell(s). During stimulation with GABA alone, spikes from cells other than the amino acid-sensitive cell were sometimes seen. These usually occurred at low frequencies ranging from one to five impulses in the 500-msec sample period (Table 3). Paired applications of 10 mM GABA and 10 mM GABA + 1 mM alkaloid on the same sensillum were used to keep variability to a minimum. Several alkaloids, notably atropine, did stimulate considerable activity in a second cell under these experimental conditions. Ref-

TABLE 3. AVERAGE RESPONSE FROM NON-AMINO ACID-SENSITIVE CELL(S) WHEN STIMULATED WITH 10 mM GABA (COLUMN 1) AND MIXTURE OF 10 mM GABA AND 1 mM ALKALOID (COLUMN 2)[a]

Response of additional cell(s) during stimulation with GABA	Response of additional cell(s) during stimulation with GABA + alkaloid[b]	Alkaloid used	Relative increase	N
0.9 ± 1.1	6.5 ± 6.6	Atropine	7.2	7
1.5 ± 1.7	6.8 ± 4.3	Arecoline	4.5	4
1.5 ± 0.8	6.2 ± 2.7	Quinine	4.1	6
2.7 ± 1.4	8.2 ± 3.7	Strychnine	3.0	6
2.2 ± 2.6	4.3 ± 4.1	Sparteine	2.0	9
2.8 ± 2.1	3.3 ± 1.0	Solanine	1.2	4
5.3 ± 3.5	3.5 ± 1.9	Papaverine	0	6
1.2 ± 1.0	1.2 ± 1.2	Tomatine	0	6
2.0 ± 0	1.5 ± 2.1	Caffeine	0	2

[a] Note increase in average response from these cells with some of the test mixtures. Errors are ±SD.

[b] Impulses counted between 250 and 750 msec of response and expressed as impulses per 0.5 sec.

TABLE 4. DATA FROM TABLE 3 TO EMPHASIZE RESPONSES FROM NON-AMINO ACID-SENSITIVE CELLS IN EIGHT PREPARATIONS PARTICULARLY IN COMBINATION WITH 10 mM GABA.[a]

Alkaloids	Number/total *N*	Response of additional cell(s)[b]
Atropine	3/7	13.3 ± 1.5
Strychnine	2/6	12.5 ± 2.1
Sparteine	1/9	14
Arecoline	1/4	11
Quinine	1/6	10

[a] A sensitive preparation is defined as one where the non-amino acid-sensitive cell(s) fired at a rate greater than 20 impulses/sec. during stimulation with the GABA–alkaloid mixture.
[b] Impulses counted between 250 and 750 msec of response and expressed as impulses per 0.5 sec.

erence to Table 4, however, shows that most of this additional activity, with atropine present, was produced in the α-sensilla of three of seven preparations. In these three animals, the response of the second cell averaged 26 impulses/sec. Figure 4 shows an example of this type of response. Two of the six preparations also had this kind of sensitivity to strychnine.

Behavioral Response to Alkaloid-Treated Food. Adult beetles, offered healthy leaves infiltrated with one of five different alkaloids, showed a variety of responses. The video bioassay used measured a number of parameters (Harrison and Mitchell, 1988), four of which are given in Table 5. Only atropine and papaverine had significant effects, with atropine being the most potent. Forty percent of adults refused the atropine-treated leaves after a significantly prolonged prefeed maceration time. The 60% which fed did not differ from controls in amount consumed or in feeding rate. Papaverine caused only a re-

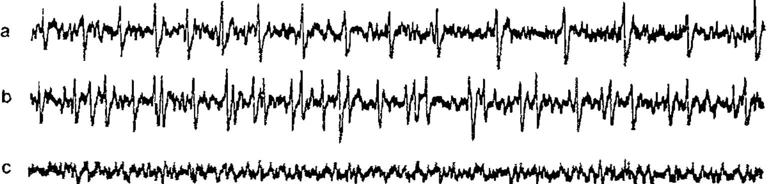

FIG. 4. Response of an α-sensillum to three sequential stimulus applications. (a) 10 mM GABA; (b) 10 mM GABA + 1 mM atropine sulfate; (c) 1 mM atropine sulfate. Note activity of a second cell during stimulation with GABA + atropine. Records and time as in Figure 3.

TABLE 5. FEEDING PARAMETERS MEASURED FOR ADULT BEETLES OFFERED ALKALOID-TREATED POTATO LEAVES, EXPRESSED RELATIVE TO BEETLES OFFERED WATER-TREATED CONTROL LEAVES[a]

Alkaloid (2 mM)	Relative prefeed maceration time	Rejection (%)	Relative amount consumed	Relative feeding rate
Water	1.00	0	1.00	1.00
Solanine	0.88	0	1.19	0.97
Tomatine	1.28	0	1.00	0.94
Atropine	2.62[b]	40[b]	1.00	0.63
Quinine	2.20	0	1.16	0.96
Papaverine	1.64	10	0.52[b]	0.81

[a] Data on solanine, tomatine, and atropine adapted from Harrison and Mitchell (1987) ($N = 10$).
[b] Significant at 5% level or less.

duction in amount consumed; other parameters remained indistinguishable from control beetles.

DISCUSSION

Deterrent Receptors. This study and a previous one on steroidal alkaloids and saponins (Mitchell and Harrison, 1984) provide no evidence for a receptor cell, which is sensitive to a wide range of potentially distasteful secondary plant compounds, in the galeal chemosensory field of the Colorado potato beetle. The idea of a deterrent-sensitive cell comes from work on several caterpillar species (Schoonhoven, 1982), and the best evidence for such a cell comes from *Bombyx mori* (Ishikawa, 1966) and *Pieris brassicae* (Ma, 1972; Blom, 1978). A variety of compounds stimulates these cells at concentrations in the micromolar range, and there is some evidence for a positive dose–response relationship. These two features strongly support the idea of high sensitivity to a number of structurally different secondary compounds in at least some lepidopterous species, but detailed studies are lacking on all but two species. Dethier (1980) questions the idea of a generalized deterrent receptor in lepidopterous larvae, stressing instead the fact that secondary compounds stimulate several cells in 59% of the cases he has studied.

The actions of repellents on mosquito antennal sensilla provide an interesting parallel to the situation in phytophagous insects. Davis (1985) reviewed the mosquito work and presented a list of possible actions of repellents which is remarkably similar to the one proposed by Schoonhoven (1982) for plant feeders. With the evidence to date in both systems, it is not possible to identify

any single mechanism that explains the effects of repellents or feeding deterrents. Given the vast array of chemical structures represented by these compounds, and the large number of potential molecular sites of action in a chemoreceptive system, each situation will require detailed study.

Comparative Aspects of Alkaloid Action. When the effects of a number of compounds on the same system are compared, some preliminary conclusions regarding mechanisms of action can be drawn. The evidence to date strongly suggests that sucrose, GABA, and L-alanine stimulate the same cell in the α-sensillum of the Colorado potato beetle (Mitchell and Harrison, 1984). Because of the difference in molecular structure of the amino acids and sucrose, and because of the high specificity of the amino acid response (Mitchell, 1985), there are probably at least two receptor sites on the same cell, one for the amino acids and one for sucrose.

The actions of the nine alkaloids given in Tables 1 and 2 can be interpreted against this background. Interestingly, except for papaverine, the compounds which significantly inhibit the response to GABA do not affect the response to sucrose. This suggests that tomatine, solanine, and sparteine have their effect on some site related to the amino acid receptor which is separate from the sucrose-receptor site. This observation also supports the hypothesis that the amino acid and sucrose receptors are separate entities. The fact that papavarine inhibits both responses does not exclude the possibility that it interacts with both receptor sites, but it is also possible that this alkaloid acts at sites other than the receptor site causing the entire cell or even all cells in the sensillum to be less sensitive. It is possible that the mode of action of papaverine, at the molecular level, is quite different from that of tomatine, solanine and sparteine. The data do not provide any information on the possible differences between the actions of the latter three compounds. It should be noted that the effect of tomatine and solanine described here is distinctly different from the long-term effects of these compounds which involve all cells in the sensillum (Mitchell and Harrison, 1985).

There are very few data on other phytophagous insect species with which to compare these results. Dethier (1982) emphasized the importance of peripheral integration in the context of host-plant recognition. He cited unpublished data from work on several lepidoptereous larvae which showed that tannic acid, quinine, piperidine, and caffeine inhibit electrophysiological responses to sugars. Apparently there is some variation in effects of these compounds across lepidopterous species. Frazier (personal communication) finds that caffeine excites a candidate ''deterrent'' cell in *Manduca sexta* confirming a similar result reported by Schoonhoven (1972). Caffeine was ineffective in all experiments reported here. Such differences in activity across orders may reflect differences in receptor mechanisms, and it should be possible to gain additional insight into the role of these compounds and the mechanisms underlying their action by

using a comparative approach. Interestingly, quinine has so far proven to be an effective inhibitor of sucrose stimulation in Diptera (Morita, 1959), Lepidoptera (Dethier, 1982), and Coleoptera (Mitchell and Sutcliffe, 1984, and present study). The fact that quinine inhibits the response to sucrose in the Colorado potato beetle, while leaving the response of the same cell to GABA unaffected, suggests that this alkaloid acts at a site specifically involved with sugar reception.

Correlation of Sensory Physiology and Behavior. The adult bioassay was limited to five alkaloids, because the results suggest that little is to be gained by looking at the remaining alkaloids which were studied electrophysiologically. It is assumed that the alkaloids introduced into the potato leaf remained intact for the duration of the test and that they were accessible to the beetle's chemosensory system. Only papaverine and atropine caused significant reduction of any of the feeding parameters measured (Table 5). Papaverine strongly inhibited responses to GABA and to sucrose, suggesting these effects may be causally related to its effect on feeding. It would be interesting to test papaverine more thoroughly. Solanine, tomatine, and quinine, although clearly effective at the cellular level, did not significantly disrupt consumption when presented in the whole-leaf context.

The lack of clear correlations between physiological and behavioral results in these, admittedly limited, data, is not altogether surprising. First, only the galeal sensilla are represented in the physiological study. Epipharyngeal sensilla are present in adult beetles (unpublished data) and probably in larvae as well. These sensilla may, in fact, contact leaf sap before the galeal sensilla, and their input is probably important in regulating feeding behavior as it is in lepidopterous larvae (de Boer et al., 1977). Unfortunately these sensilla are technically difficult to record from, making their inclusion in a general survey such as this a daunting task. Second, we still know very little about the nature of sensory codes in sensilla which mediate host-plant recognition. In a pioneering study, Dethier and Crnjar (1982) suggested a number of possible types of codes whereby lepidopterous larvae may recognize different hosts and nonhost plants. Their data did not allow firm conclusions, but they did indicate that detailed analyses of complex interactions at the chemosensory level will be required to make progress in this area. The results presented here lead to the same conclusion.

An interesting study by Derby et al. (1984) on *Homarus americanus* illustrates the generality and the complexity of the action of secondary compounds on sensory systems and their influence on behavior. They demonstrated that a number of chemosensilla on antennules and walking legs were sensitive to several of 14 secondary plant compounds tested. They also had difficulty in correlating the sensory effect with behavioral results. For example, ferulic acid stimulated a large response from leg and antennular sensilla but had no effect

on behavior, while tannic acid reduced food intake while only stimulating antennular sensilla.

It seems clear that future progress in this area will require in-depth study of a few well-chosen model systems. Prerequisites will be a good understanding of the basic responses of the chemosensory system in each preparation, an easily interpretable behavioral bioassay, and a thorough study of interactions of a few ecologically relevant secondary compounds with the sensory system in question. Success in correlating sensory and behavioral data will likely be enhanced if comparisons are made on an individual plant (defined substrate) and an individual insect basis.

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EFFECTS OF DOUBLE-BOND CONFIGURATION ON INTERACTION BETWEEN A MOTH SEX PHEROMONE COMPONENT AND ITS RECEPTOR:
A Receptor–Interaction Model Based on Molecular Mechanics

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Abstract—The dependence of the electrophysiological activity on the change of double-bond configuration of (Z)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*, and a dienic analog, (*E*)-2,(Z)-5-decadienyl acetate, have been investigated by single-cell measurements and molecular mechanics calculations (MM2). A previously reported model for the interaction between a moth sex pheromone component and its receptor has been refined. This new model gives an essentially quantitative correlation between the measured activities and the calculated conformational energies for a biologically active conformation defined by the model. Previously obtained structure–activity results for chain-elongated analogs of (Z)-5-decenyl acetate are significantly improved by the refined model. The effect of a change of the double-bond configuration on the substrate–receptor interaction is not additive but depends on the conformational properties of the entire molecule.

Key Words—Structure–activity, conformational energy, molecular mechanics, double-bond configuration, *Agrotis segetum*, Lepidoptera, Noctuidae, sex pheromone, single-cell recordings, receptor interaction.

INTRODUCTION

Straight chain monoolefinic acetates with a (Z) double bond constitute by far the largest class of known pheromone components of noctuid moths (Scheme 1) (Steck et al., 1982).

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$$CH_3-(CH_2)_n-CH=CH-(CH_2)_m-OAc$$

SCHEME 1.

Field trapping experiments and electrophysiological measurements on the response of male moth antennae (EAG) or single olfactory receptor cells have revealed that the (*E*) isomer of a pheromone component is significantly less active than the natural (*Z*) isomer. In an EAG screening of a large number of noctuid species, Priesner et al. (1975) reported that the (*E*) isomer is 1.8–5.6 times less active than the (*Z*) isomer. Single-cell measurements, which are better suited for this kind of comparison, show up to 10-fold less activity for the (*E*) isomer in noctuid species (Priesner, 1979). In tortricid species, this difference is usually about 100-fold (Priesner, 1979, 1980, 1983).

However, of all analogs of natural (*Z*) pheromone components so far tested in various studies, the corresponding (*E*) isomer is nevertheless among the most active. For instance, its activity corresponds to, or exceeds, the activity of analogs chain elongated or chain shortened by one methylene unit, which are geometrically more modest perturbations of the natural compound than the (*Z*) to (*E*) configurational change.

Previous structure–activity studies on moth pheromone components strongly indicate that the spatial relationships between the acetate group, the double bond, and the terminal methyl group are crucial for the biological activity (Kafka and Neuwirth, 1975; Kikuchi, 1975; Bestmann and Vostrowsky, 1982; Liljefors et al., 1985). Considering that the three-dimensional shapes of the thermodynamically preferred conformers of the (*Z*)- and (*E*) isomers are significantly different, the relatively high activity of the (*E*) isomer is surprising. However, straight-chain monoolefinic acetates are very flexible molecules. Rotations about single bonds of the (*E*) isomer may make it possible for this isomer to approach the three-dimensional shape of the corresponding (*Z*) isomer. This has previously been suggested by Kafka and Neuwirth (1975) in connection with the development of a receptor interaction model for pheromone components, but no attempts were made to fit (*E*) isomers to this model (Kafka and Neuwirth, 1975; Neuwirth, 1973).

Recently, we reported a new model for the interactions between a monoolefinic pheromone component and its receptor, employing structures and conformational energies calculated by the molecular mechanics method (Liljefors et al., 1985). This model was successfully used to rationalize the effects of chain elongation on observed electrophysiological single-cell activities of homologs of (*Z*)-5-decenyl acetate (**1**), a pheromone component of the turnip moth, *Agrotis segetum* (Bestmann et al., 1978; Arn et al., 1980; Löfstedt et al., 1982). In the present work, we report an extension of this model to include

the effects of a change of the double-bond configuration as well. This extension made necessary a refinement of the previously reported receptor interaction model (Liljefors et al., 1985). As will be described below, this refinement also significantly improves the quantitative aspects of the previously obtained results for chain-elongated analogs.

In connection with structure–activity studies on dienic analogs of (Z)-5-decenyl acetate (Bengtsson et al., 1987), we surprisingly observed that changing the configuration from (Z) to (*E*) at position 5 of (*E*)-2,(Z)-5-decadienyl acetate (compounds **4** and **5,** respectively) did not produce any significant change in the electrophysiological activity, in contrast to the effect of the corresponding configurational change in (Z)-5-decenyl acetate (see above). These observations provide a good test of the performance of the refined receptor interaction model.

We report electrophysiological single-cell measurements and conformational analysis of the compounds shown in Figure 1. Olfactory receptor cells specifically tuned to (Z)-5-decenyl acetate (**1**) are present in antennal sensilla type SW1 of the turnip moth, *Agrotis segetum*, and are readily accessible for single-cell recordings (Hallberg, 1981; Löfstedt et al., 1982; van der Pers and Löfstedt, 1983).

METHODS AND MATERIALS

Chemicals. The synthesis of (Z)-5-decenyl acetate (Figure 1, **1**), (*E*)-5-decenyl acetate (**2**), and the chain-elongated analogs (**6**)–(**12**) have previously been reported (Olsson et al., 1983; Liljefors et al., 1985). (*E*)-5-Dodecenyl acetate (**3**) was purchased from the Institute for Pesticide Research, Wageningen, The Netherlands. The synthesis of (*E*)-2,(Z)-5-decadienyl acetate (**4**) will be reported elsewhere (Bengtsson et al., 1987).

(E)-*2,*(E)-*5-Decadienyl Acetate (5).* This was prepared according to the method of Ando et al. (1982) from zirconocene monohydride (2.2 g, 9 mmol) (Schwartz's reagent), 3-(2-tetrahydropyranyloxy)-1-propyne (1.2 g, 9 mmol), 1-bromo-2-heptene (1.5 g, 9 mmol) and $Pd(PPh_3)_4$ (0.26 g) to yield 1.6 g (91%) of crude (**5**). δ_H(300 MHz; $CDCl_3$) 0.89 (3H, t, Me), 1.28–1.36 (4H, m, CH_2CH_2), 1.98–2.04 (2H, m, $CH_2C{=}$), 2.06 (3H, s, MeC=O), 2.72–2.78 (2H, m, $={CCH_2C}=$), 4.52 (2H, dd, OCH_2), 5.33–5.51 (2H, m, J_{AB} = 15.2 Hz, CH=CH), 5.51–5.62 (1H, m, J_{AB} = 15.2 Hz, C$\underline{H}$=CH), 5.72–5.83 (1H, m, J_{AB} = 15.2 Hz, CH=C$\underline{H}$), δ_C(15.03 MHz; $CDCl_3$) 14.0, 21.0, 22.2, 31.6, 32.2, 35.2, 65.1, 124.1, 126.9, 132.3, 134.9, 170.9, *m/e*: 136 (M^+-60; 5%), 107 (1), 93 (7), 79 (32), 67 (10), 55 (11), 43 (100).

The compounds were purified by argentation liquid chromatography (Houx et al., 1974) and by preparative GLC (column OV-351, 6 m). All compounds were at least 98.5% isomerically pure as determined by capillary GLC (column Supelcowax 10, 30 m). The double-bond configurations were confirmed by ^1H

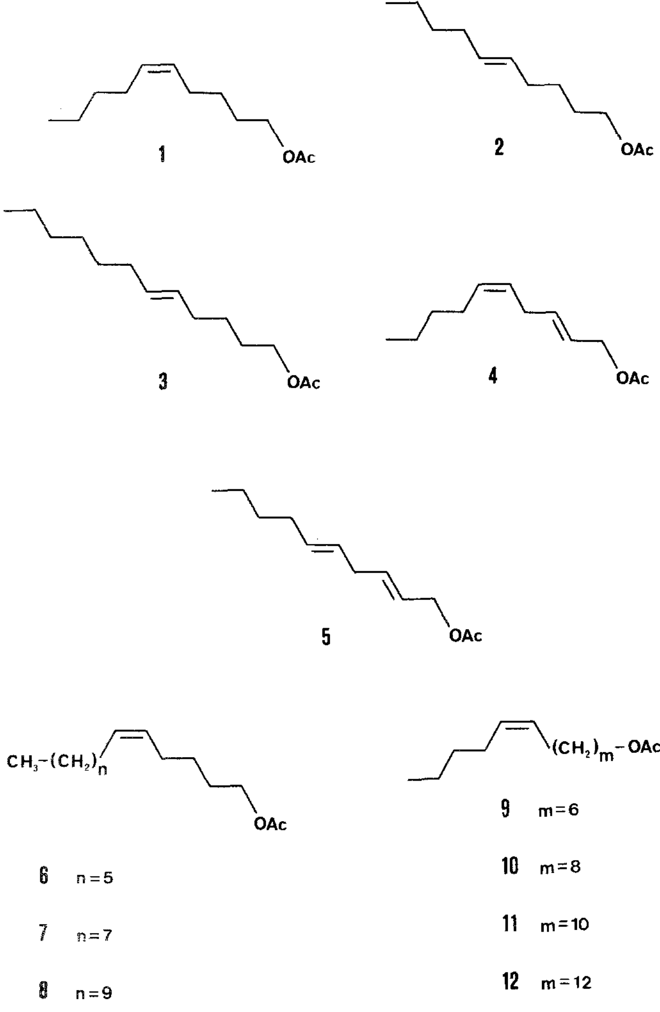


FIG. 1. Compounds studied.

and ^{13}C NMR spectroscopy using a Varian XL-300 or a Nicolet 360 WB spectrometer. Mass spectra were recorded on a Finnigan 4021 mass spectrometer.

Calculations. Energy-minimized geometries and conformational energies were calculated using the molecular mechanics program MM2 developed by Allinger and coworkers (Allinger and Yuh, 1980; Burkert and Allinger, 1982). Starting structures for the energy-minimization program were constructed by the molecular modeling system MIMIC (Liljefors, 1983; von der Lieth et al., 1984). This system was also used in the molecular superimposition studies and in the calculation and plotting of conformational energy maps.

Electrophysiology. The biological activities of the different compounds were established by measuring the electrical responses of receptors selective for (*Z*)-5-decenyl acetate (**1**) on the male *Agrotis segetum* antenna.

The measuring procedure was essentially the same as that described by van der Pers and den Otter (1978). A freshly excised antenna from a 2-day-old moth was placed with the base in a capillary electrode filled with Beadle-Ephrussi Ringer solution and connected to earth by an Ag–AgCl wire. The tip of an olfactory sensillum was cut off by a glass knife, and the recording electrode was placed in contact with the cut surface of the sensillum. The recording electrode was then connected to a high-impedance amplifier by an Ag–AgCl wire.

The stimulus was loaded onto a piece of filter paper and put into a plastic syringe. The amounts used ranged from 10^{-3} to 10^{2} μg. Two milliliters of the gaseous content of the syringe was injected into an airstream flushing the antenna continuously at a linear flow of 0.5 m/sec. Ten replicates were recorded with each different stimulus.

The response of the receptor cell was defined as the number of action potentials (nerve spikes) generated during 1 sec, starting from the onset of the stimulation. Dose–response curves using five different concentrations were made for all compounds tested.

The relative electrophysiological activities of the tested compounds were calculated from the dose-response curves, and expressed as the reciprocal of the relative quantities required to elicit the same response of the receptor cell.

Corrections for differences in vapor pressure were calculated as previously described (Liljefors et al., 1985), using data from Olsson et al. (1983).

Refined Substrate–Receptor Interaction Model

The new features of the refined model are: (1) the use of the entire pheromone component molecule **1** in the construction of the model instead of using only part of the molecule; (2) the use of the complete structure of the analogs in the calculations of conformational energies; and (3) addition of flexibility to the model with respect to the required location of the double bond (see Computational Procedure section below). As in our previously described model, the natural pheromone component **1** is used to define spatial relationships in the cavity of the receptor active site between positions of molecular parts crucial for full biological activity (Liljefors et al., 1985). The corresponding molecular parts in the studied analogs (**2–12**) may, through conformational rearrangements of their alkyl chains, be positioned in these space locations. The conformational energy required for such a rearrangement may then be related to the biological (electrophysiological) activity of the analog. A high conformational energy should correspond to a low biological activity and vice versa. As before, we assume receptor sites complementary to the acetate group, the double bond, and the terminal methyl group (Liljefors et al., 1985).

At the present stage of development our model is only applicable to com-

pounds analogous to the natural pheromone component and which have the ability to position the crucial molecular parts, as defined above, in the required positions. Thus, compounds such as chain-shortened analogs cannot be fitted directly to the model and its assumed interaction sites. It must also be borne in mind that the details of the transduction process are still largely unknown and that the process may be a complex multistep one (Kaissling, 1974, 1976, 1977). Our model is to be understood as a model for an "activated complex" related to the efficacy (intrinsic activity) rather than as model for the initial binding of the substrate.

In our previous model only those parts of the molecules which were changed in the test series were included in the calculations. This simplification facilitated the calculations for the chain-elongated analogs **6–12** in the structure–activity analysis previously reported (Liljefors et al., 1985) but is less satisfactory in the general case. In the present work the complete molecules are used in the calculations. This, however, introduces a complication since we now have to take the conformational properties of the natural pheromone component **1** into account.

Conformational Analysis of Compound 1. Compound **1** is a very flexible molecule with a large number of conformers within 1 kcal/mol of the energy of the thermodynamically most stable one. However, a study of conformationally restricted dienic analogs of compound **1** strongly indicates that the alkyl chains of **1** should have an all-*anti* conformation in the biologically active state (Bengtsson et al., 1987). This reduces the problem to the conformation with respect to the C=C—C—C fragment. It is then implicitly assumed that one of the stable conformers in this respect corresponds to the biologically relevant structure of compound **1**.

A calculated conformational energy map for the rotation about the vinylic bonds in (Z)-4-octene, used as a model for the olefinic part of a monoolefinic pheromone component, is shown in Figure 2. It shows a degenerate double-minimum for the *cisoid* conformation (Figure 3) and a single minimum for the *transoid* one with an energy difference of 0.11 kcal/mol, favoring the *transoid* conformation. The populations of the two types of conformations are thus very similar, in agreement with experimental data on other (Z)-olefins. An electron diffraction study on (Z)-3-hexene indicates the presence of both types of conformations with comparable populations (van Hemelrijk et al., 1981). This has also been concluded from infrared data on liquid (Z)-3-hexene (Shimanouchi et al., 1971). Raman spectra of crystals of (Z)-monoolefinic fatty acids show the molecules to have either the *cisoid* or the *transoid* conformation depending on the crystalline modification (Koyama and Ikeda, 1980).

The energy barrier between the two *cisoid* conformers in (Z)-4-octene is calculated to be very low, 0.12 kcal/mol. The interconversion between the two *cisoid* forms may thus be described as a large amplitude torsional motion. The energy barrier for the *cisoid* to *transoid* interconversion is calculated to be 0.84

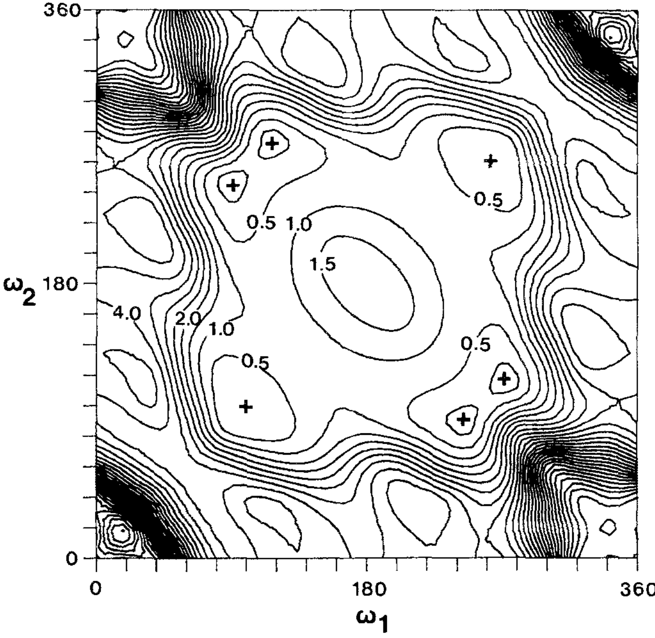


FIG. 2. Conformational energy map for the rotation about the vinylic bonds of (*Z*)-4-octene. ω_1 and ω_2 are C=C—C—C dihedral angles in degrees. Local minima are denoted by +. Isoenergy contours are shown with an energy difference of 0.5 kcal/mol. At the degenerate minima at ω_1, ω_2 = 90,240 and 120,270 (and the symmetry related pair) the 0.2 kcal/mol level also is shown.

kcal/mol, in good agreement with the experimental value 0.60 ± 0.06 kcal/mol for the analogous barrier in (Z)-2-pentene (van Eijk, 1981). The above analysis of (Z)-4-octene implies that there are three conformers of the natural pheromone component **1** which are candidates for the biologically active structure within the context of our model.

The energy-minimized geometries of these conformers are shown in Figure 4. The *cisoid 1* and *cisoid 2* conformers differ mainly in the torsional angles about the (C=C)-(C—C) bonds. In the *cisoid 1* structure the C=C—C—C dihedral angles are calculated to be 116.1 and −87.2 degrees for the *n* and *m* chains, respectively. The corresponding values for the *cisoid 2* conformer are 88.4 and −114.9 degrees. The difference in calculated conformational energy

cisoid transoid

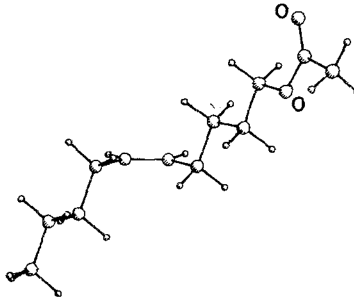
FIG. 3. Energy-minimized *cisoid* and *transoid* conformers of (*Z*)-4-octene.

O
O

cisoid 1

O
O

cisoid 2



transoid

FIG. 4. Energy-minimized geometries of the *cisoid* and *transoid* conformers of (*Z*)-5-decenyl acetate (**1**) with all-*anti* alkyl chains.

between the three conformers is less than 0.06 kcal/mol. As there are presently no data from which it is a priori possible to choose which of these conformers to use as a model for the biologically active conformation of **1**, all three were used in turn and all calculations were done for each of the three models. These models will, in the following, be denoted *cisoid 1, cisoid 2,* and *transoid* according to Figure 4. The use of all three models in the calculations gives a good check of the sensitivity of the calculated results to the precise geometry of the substrate–receptor interaction model.

Computational Procedure. As in our previous model, the pheromone component analogs studied were assumed to interact with the receptor with the terminal methyl group and the acetate group in the relative positions in space defined by the natural pheromone component. Computationally this is accomplished by restricting the encircled atoms in Figure 5 to these fixed positions during the energy-minimization procedure. In our previous model the double bond and the vinylic carbons were also restricted to fixed positions. However, the results obtained indicated that these constraints are too severe in the general case (Liljefors et al., 1985). To add more flexibility to the model, the double bond and the vinylic carbon atoms were allowed to move during the energy minimization process in the plane defined by the C—C=C—C fragment in the reference molecule **1**. The restriction to a common plane ensures that the pi orbitals have the same direction in all molecules studied, and thus may interact with the corresponding binding site in a similar way. Before energy-minimization, the double bond in the studied analog was placed in a position as close to the double bond in the reference molecule **1** as possible.

With these restrictions, the molecules were energy-minimized with respect to all remaining degrees of freedom. A large number of starting structures was employed, and the lowest energy one after energy-minimization was used in the further analysis. The construction of trial structures for the chain-elongated analogs, employing a diamond lattice, has previously been described (Liljefors et al., 1985). Next, the unconstrained global energy minimum for each molecule was calculated. The conformational energies required by the molecules to acquire their "biologically active conformations" could then be evaluated by

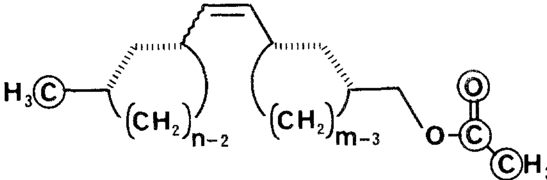


FIG. 5. Encircled atoms are held in fixed positions in the calculations of structures and energies for biologically active conformations. The dashed lines indicate the alkyl chains in the natural pheromone component **1**. For constraints on the double bond, see text.

taking the difference in calculated energy between the lowest energy conformationally rearranged structure that fits the requirements of the receptor–interaction model and the unconstrained global energy minimum. These energies are then compared to the observed electrophysiological activities relative to the reference compound **1**.

Note that in the context of our model the interaction energies between the terminal methyl group, the double bond, and the acetate group and their receptor counterparts are the same or at least very similar for all molecules included in the present study. This is due to the requirement that the three receptor-interacting parts of the substrate molecule are at fixed positions in space or, in the case of the double bond, only allowed a very limited freedom of motion. However, the conformational energy required to attain the biologically active structure is different and depends on the structure of the molecule. Thus, the differences in total receptor–interaction energies along the series of molecules are, according to our model, determined by the different conformational energies. The calculated conformational energies correspond to enthalpies. The entropy terms have not been explicitly considered in the present work, but differences in the conformational entropy contributions for the molecules in the series investigated should largely be compensated by differences in hydrophobic binding (Liljefors et al., 1985).

RESULTS AND DISCUSSION

Chain-Elongated Analogs. The new model described above was first used to recalculate the conformational energies required for the previously studied chain-elongated analogs **6–12** to acquire their "biologically active conformations". For details of the results obtained previously, see Liljefors et al. (1985). The purpose of this recalculation was to study the performance of the refined model in relation to the old one and to investigate if the calculated results are dependent on the choice of biologically relevant conformer for the natural compound **1** (*cisoid 1, cisoid 2* or *transoid*, see Figure 4).

The results are shown in Figure 6. The calculated energies are clearly not very dependent on the conformer assumed to be the biologically active one for the natural pheromone component **1**. The observed minima in activity for compounds **7** and **10** correspond to calculated conformational energy maxima in all three cases, and the results are qualitatively similar to those previously obtained (Liljefors et al., 1985). However, very satisfactorily, the refined model now makes it possible to put the results for chain elongation on either side of the double bond on the same energy scale. Our previous model clearly exaggerated the conformational energies required for the analogs chain-elongated between the double bond and the acetate group, by as much as 8–10 kcal/mol (Liljefors et al., 1985). This was due to the overly severe constraints imposed on the molecule in the previous model.

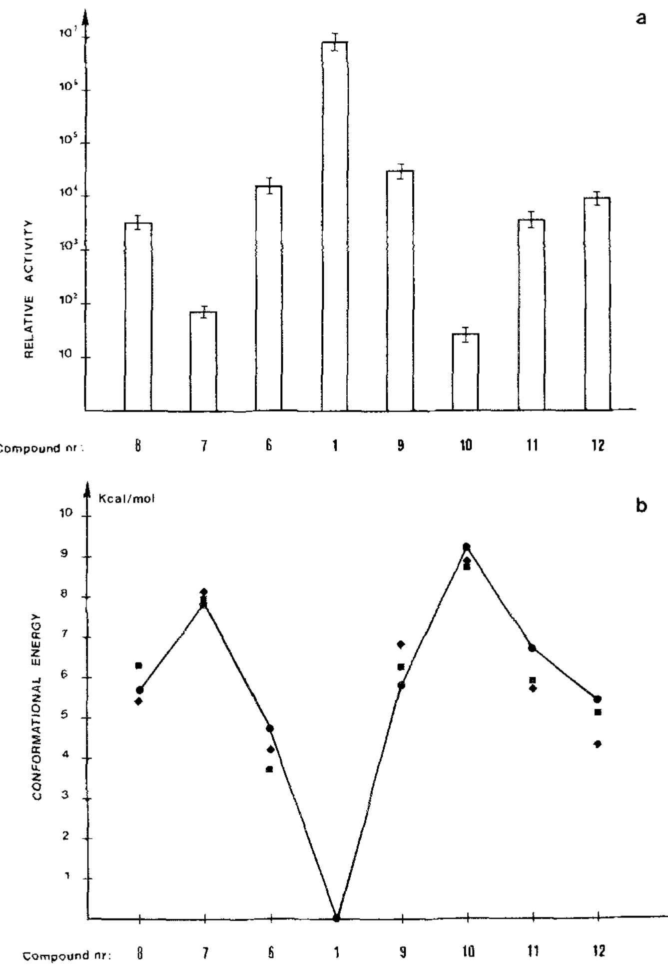


FIG. 6. (a) Experimental single-cell activities (from Liljefors et al., 1985 and (b) calculated conformational energies for the biologically active conformations of chain-elongated analogs of **1**. ♦ *cisoid 1;* ● *cisoid 2;* ■ *transoid* model. The calculated energies for the *cisoid* 2 model are connected by a solid line.

From the results in Figure 6 it is possible to calculate the conformational energy corresponding to a decrease of the biological activity by a factor of 10. On average this becomes 1.7, 1.7, and 1.6 kcal/mol for the *cisoid 1, cisoid 2,* and *transoid models*, respectively. For all three models and for all seven analogs the maximum calculated deviation from these numbers is 0.5 kcal/mol. The refined model thus significantly improves the results for the chain-elongated analogs **6–12,** and gives an essentially quantitative relationship between calculated conformational energy and observed biological activity. The similar re-

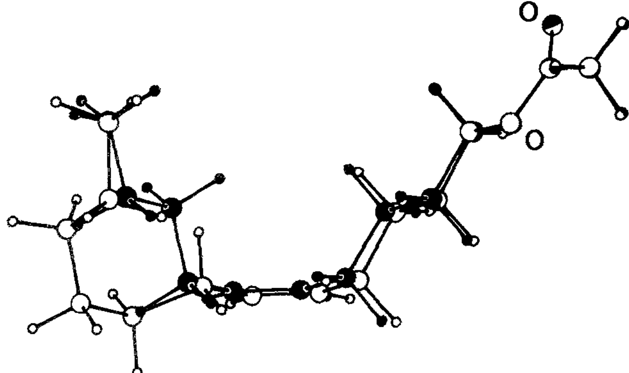


FIG. 7. Superimposition of the *cisoid* 2 conformer of **1** (filled atoms) and the calculated biologically active conformation of compound **6**.

sults obtained with this series of compounds for the three different models precludes the possibility of selecting one of them as the most probable biologically active structure of the natural pheromone component **1**.

As an example of the structure of a calculated biologically active conformation, a superimposition of the calculated "active structure" of compound **6** and the natural pheromone component **1** in its *cisoid* 2 conformation is shown in Figure 7. To fit the geometrical requirements of the receptor–interaction model, **6** is forced to adopt *gauche* conformations about two adjacent bonds. The difference in double bond positions of **1** and **6** is quite small. The distance between the midpoints of the double bonds in **1** and **6** in the superimposition shown is 0.6 Å. The calculated conformational energy of **6** in this "biologically active" conformation relative to the lowest energy one is 4.7 kcal/mol, which may be compared to the corresponding value using our previous model, 5.8 kcal/mol. For compounds **9–12**, the difference between the calculated energies using the old and new models is even larger, as much as 5–10 kcal/mol. This implies that a substantial reduction of the conformational energy may be achieved with a modest adjustment of the double-bond position in the cavity of the receptor "active site." It should be noted that the calculated biologically active conformations of **6–12** do not correspond to local energy minima of the unconstrained "free" molecules.

Double-Bond Configurational Isomers. The measured electrophysiological single-cell activities for compounds **2–5** relative to the natural pheromone component **1** are shown in Figure 8a. The (*E*) isomer **2** is less active by a factor of 100 than the corresponding natural (*Z*) isomer **1**. Chain elongation by two methylene units (compound **3**) further lowers the activity by about a factor of 100 (including corrections for differences in vapor pressure). This may be compared

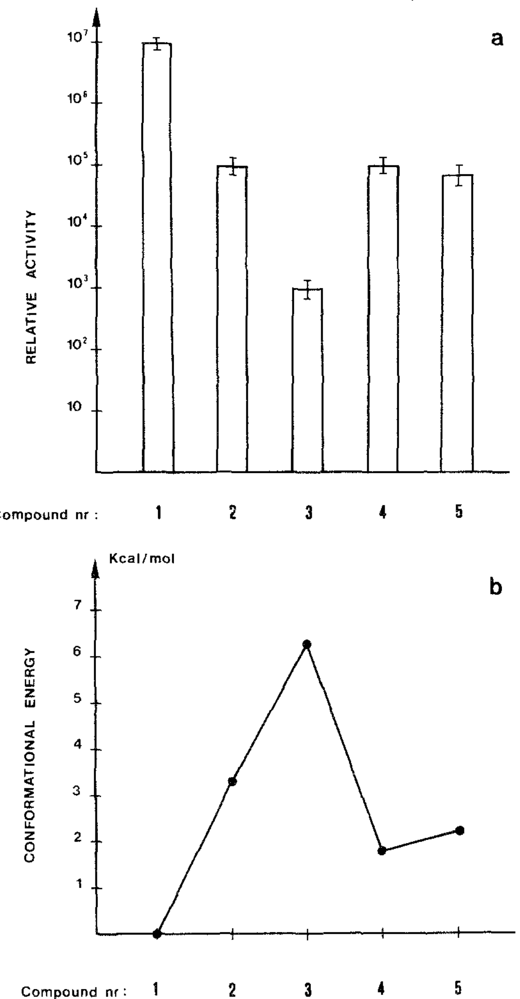


FIG 8. (a) Experimental single-cell activities for compounds **1–5**. (b) Calculated conformational energies for the biologically active conformations (*cisoid* 2 model) of compounds **1–5**.

to the relative activity for **6** with respect to **1**, which is ca. 1000 (Figure 6). The loss of activity due to chain-elongation is thus somewhat less in the (*E*) series than in the corresponding (*Z*) series.

Introduction of an (*E*) double bond in the 2 position of the natural pheromone component (compound **4**) has approximately the same effect as the *Z*/*E* configurational change (Figure 8a); the observed activity is reduced by a factor of ca. 100. Surprisingly, no further significant change in the activity was ob-

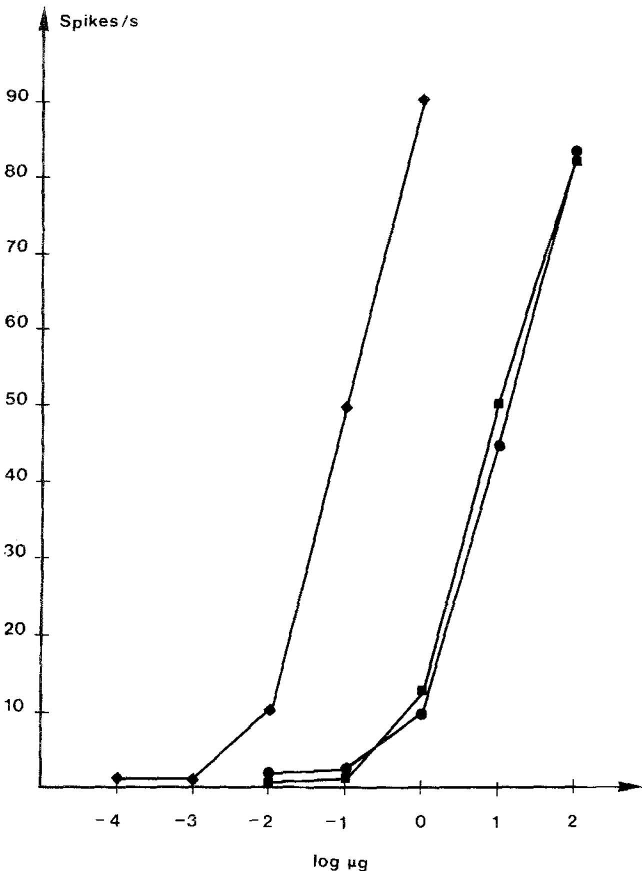


FIG. 9. Dose–response curves for compounds **1**, ♦; **4**, ■; and **5**, ●; obtained by electrophysiologic single-cell recordings.

served for the (*E*)-5 isomer **5** of this diene. The close similarity of the electrophysiological response of the two compounds is demonstrated in Figure 9, which shows the dose–response curves for the natural pheromone component **1** and the two diene analogs **4** and **5**. The effect of configurational change is thus not additive, but depends on the properties of the entire molecule.

The conformational energies required for compounds **2–5** to acquire their biologically active conformations, according to the receptor–interaction model described above was calculated, and the results are shown in Figure 8b. (The results for the *cisoid 2* model according to Figure 4 are shown. The results for the other two models in this series are also very similar).

The calculated conformational energies clearly have a very close correla-

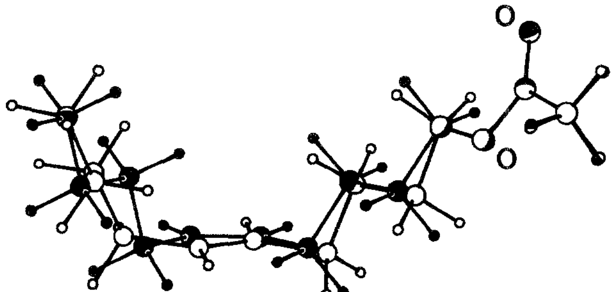
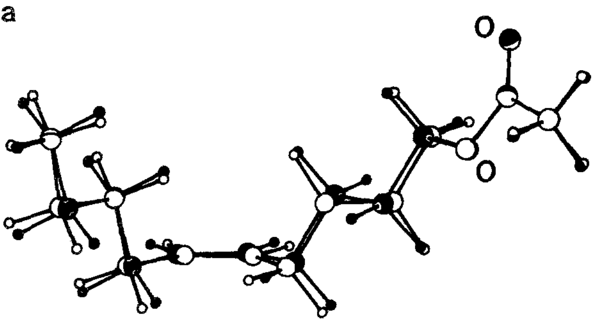


FIG. 10. Superimposition of the *cisoid 2* conformer of **1** (filled atoms) and the calculated biologically active conformation of compound **2.**

tion to the measured electrophysiological activities. The calculated conformational energy for compound **2** is 3.3 kcal/mol, which corresponds to 1.65 kcal/mol for each power of 10 of decrease in activity compared to **1.** This is identical to the number obtained for the chain-elongated analogs. A superimposition of compound **2** in its calculated "biologically active conformation" and compound **1** is shown in Figure 10. The (*E*) isomer is forced to adopt a *gauche-anti* conformation of the alkyl chain connecting the double bond and the methyl group. The position of the (*E*) double bond in **2** is sufficiently close to that of the (Z) double bond in **1** to assure similar interactions with the binding site.

The chain-elongated (*E*) isomer **3** is calculated to have a conformational energy requirement of 6.3 kcal/mol to reach its "biologically active conformation." Using the value 1.65 kcal/mol for a 10-fold decrease in activity as obtained above, this should give a reduction of the activity by a factor of ca. 100 compared to that of **2,** which is also observed (Figure 8a). Furthermore, comparing compounds **2** and **3,** the conformational energy for reaching the biologically active conformation is increased by 3.0 kcal/mol due to chain-elongation by two methylene groups in the (*E*) series. This may be compared to the corresponding value of 4.7 kcal/mol in the (Z) series (Figure 6, compound **6**). The calculations thus also reproduce the observation that chain elongation in the (*E*) series leads to a smaller decrease of the activity than in the (Z) series.

Gratifyingly, the unexpected similarity of the activities of compounds **4** and **5** is also well calculated. After conformational rearrangements which increase the energy by 1.8 kcal/mol (Figure 8) the (*E*)-2, (Z)-5 diene **4** becomes an extremely good "mimic" of the natural pheromone component **1,** as is demonstrated by the superimposition of the two molecular structures in Figure 11a. The (*E*) double bond in **4** closely mimics the *anti*-conformation of the corresponding saturated fragment in **1.** The (*E*)-2, (*E*)-5 diene **5** requires a conformational energy of 2.2 kcal/mol, only slightly higher than that for **4,** to mimic the molecular shape of **1,** as can be seen in Figure 11b.



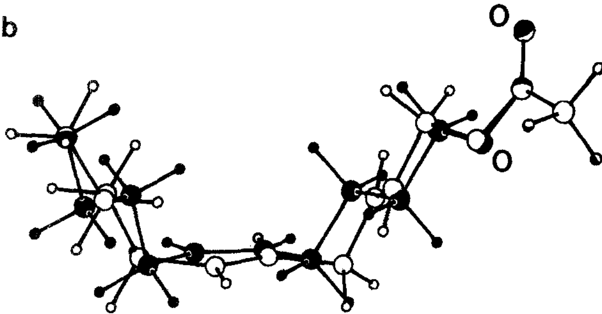


FIG. 11. Superimpositions of the *cisoid 2* conformer of **1** (filled atoms) and the calculated biologically active conformations of (a) compound **4** and (b) compound **5.**

CONCLUSIONS

The model for the interaction between a pheromone component and its receptor presented in this work gives an essentially quantitative correlation between the measured electrophysiological single-cell activities and conformational energies calculated by molecular mechanics. The calculated conformational energies correspond to the energies required for the molecules to acquire a biologically active conformation as defined by the model. It should be noted that the resulting conformations for the analogs of the natural pheromone component are not local energy minima for the "free" molecules.

The effect of a change of double-bond configuration on the biological (electrophysiological) activity is not additive, but depends on the conformational properties of the entire molecule.

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COUMARINS IN *Prunus mahaleb* AND ITS HERBIVORE, THE SMALL ERMINE MOTH *Yponomeuta mahalebellus*

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Abstract—Larvae of the small ermine moth *Yponomeuta mahalebellus* were reared on foliage of *Prunus mahaleb*, a plant known to contain coumarins. Thin-layer chromatography and gas chromatography showed that coumarin, umbelliferone, and herniarin were present in leaves of *P. mahaleb* and in pupae and adults of *Y. mahalebellus*. Overall concentrations of simple coumarins in the plant and insect were, respectively, 0.54% and 0.003–0.004% (dry weight). The possible role of coumarins in the chemical defense of both the plant and insect is discussed.

Key Words—*Prunus mahaleb*, Rosaceae, *Yponomeuta mahalebellus*, Lepidoptera, Yponomeutideae, coumarin, umbelliferone, herniarin, plant–insect interaction, chemical defense.

INTRODUCTION

In preliminary feeding trials, small ermine moths appeared to be unpalatable to birds (unpublished observations). This has prompted us to search for distateful chemicals in these insects (Fung et al., in preparation). In our screening, we found that extracts of pupae of *Yponomeuta mahalebellus* (Lepidoptera: Yponomeutideae) contained fluorescent compounds when inspected in UV light. Larvae of this small ermine moth feed on leaves of *Prunus mahaleb* (Rosaceae), a plant known to accumulate simple benzo-α-pyrones like coumarin and the hydroxycoumarins umbelliferone and herniarin (Figure 1) (Hegnauer, 1964–1973; Murray et al., 1982). As some coumarins are strongly fluorescing compounds, we decided to examine *Y. mahalebellus* and its host plant for the presence of simple coumarins.

Several classes of chemicals are known to occur in arthropods, i.e., alkaloids, cardiac glycosides, phenols, and cyanogenic glycosides (Rothschild,

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R	
H	coumarin
OH	umbelliferone (7- hydroxycoumarin)
OCH_3	herniarin (7- methoxycoumarin)

FIG. 1. Simple coumarins.

1973; Harborne, 1982). These secondary metabolites are often sequestered from the food plant, and they may offer the insect protection against predators on account of their toxicity and/or bitterness. To our knowledge, there has been no earlier report on the presence of coumarins in insects. The occurrence of coumarins in *P. mahaleb* and *Y. mahalebellus* will be discussed in view of their possible ecological function.

METHODS AND MATERIALS

Insect and Plant. P. mahaleb and *Y. mahalebellus* are not indigenous to The Netherlands, but occasionally *P. mahaleb*, presumably originated from cultivated plants in parks, is present among the natural vegetation. *Y. mahalebellus*, offspring of a strain originally collected from Reims, France, was reared on its host plant *P. mahaleb* found in the dunes of Meijendel, The Netherlands. In May 1986, fourth-instar larvae were collected from this shrub and reared further in the laboratory on the same foliage at 20°C. Adult moths were killed 24 hr after emergence. Leaves, pupae, and adults were freeze-dried and stored in a desiccator until chemical analyses were performed.

Extraction and Purification. Leaf powder (0.5 g) and insect material (0.7–0.8 g) were extracted with 15 ml 50% methanol (40–50°C; 1 hr). After filtration, the residue was rinsed with 3× 5 ml 50% methanol. The combined aqueous methanol solutions were extracted with 3× 25 ml chloroform. The organic layers were combined and concentrated under reduced pressure. Plant extracts were then ready for analyses, whereas insect extracts had to be further purified in order to remove interfering fatty substances. Insect extracts were cleaned by preparative thin-layer chromatography on silica gel plates (60 F_{254}, 1 mm layer thickness; Merck, Darmstadt, Germany). After development of the plates in toluene–ether (1 : 1) saturated with 10% acetic acid solution, bands with coumarin (R_f = 0.68), umbelliferone (R_f = 0.42), and herniarin (R_f = 0.60), detected in UV light at 254 nm and 365 nm, were scraped off and powdered. The

combined powder was washed with 200 ml ethanol, and the filtered alcoholic solution was concentrated under reduced pressure.

Thin-Layer Chromatography (TLC). Analyses of plant extracts, dissolved in 400 μl methanol, and purified insect extracts, redissolved in 200 μl methanol, were carried out on silica gel plates (60 F_{254}, 0.25 mm layer thickness; Merck). The solutions (4–12 μl) were applied and the plates were developed in toluene–ether (1 : 1) saturated with 10% acetic acid solution (Wagner et al., 1983). Coumarins were detected in UV light at 254 nm and 365 nm, before and after spraying with 5% potassium hydroxide in ethanol (Wagner et al., 1983).

Gas Chromatography (GC). After TLC analyses, extracts were dried and silylated with 300 μl *N,O*-bis(trimethyl)-acetamide and 100 μl dimethylformamide. Sensitivity of umbelliferone in GC analyses was enhanced after silylation. After heating at 50°C for 15 min, the solutions were ready for injection. The presence of coumarin, umbelliferone, and herniarin in the extracts was confirmed by coinjection with commercially available reference compounds. Coumarins were also assessed in a semiquantitative way by comparing peak areas of the extracts with peak areas of known concentrations of reference compounds.

Analyses were carried out on a Perkin-Elmer 3920 B gas chromatograph equipped with a FID detector and a capillary WCOT SE 52 fused silica column (0.32 mm ID × 25 m; Chrompack, Middelburg, The Netherlands). On-column injection was performed. Nitrogen was used as carrier gas at a rate of 1 ml/min. A temperature program was applied: 4 min isotherm at 130°C, consequently 16°C/min until 230°C, isotherm at 230°C. Temperature of the injection block and FID interface was kept at 250°C.

RESULTS AND DISCUSSION

Coumarins are secondary metabolites that are widespread in the plant kingdom. There are several types of coumarins, differing in the substitution pattern of the benzo-α-pyrone nucleus. Simple coumarins which lack additional fused ring systems are the most common representatives of this group (Hegnauer, 1964–1973).

It has been suggested that under the selective pressure of herbivory, the production of novel types of coumarins in plants was stimulated. New paths in biosynthesis led to the formation of more complicated coumarins, i.e., linear furanocoumarins and angular furanocoumarins, which are more toxic to insects than the simple coumarins (Berenbaum, 1983). For example, larvae of the southern armyworm *Spodoptera eridania* grew normally on a diet containing umbelliferone, while larval development was inhibited when the linear furanocoumarin xanthotoxin was incorporated in the diet (Berenbaum, 1978). Still, simple coumarins are not harmless to phytophagous insects. Coumarin in high

dose generally inhibits feeding of a number of insects. At a concentration of 0.2%, coumarin was an antifeedant to caterpillars of the gypsy moth *Porthetria dispar* (Meisner and Skatulla, 1977). At a level of 1.5% in an artificial diet, coumarin inhibited feeding of larvae of the cotton leafworm *Spodoptera littoralis*; at lower concentrations food intake was decreased and pupal weight was reduced as the efficiency of assimilated food conversion was inhibited (Mansour, 1981). Coumarin inhibited completely larval development of *S. littoralis* at higher concentrations and affected greatly growth and adult fertility at lower dosages (Mansour, 1982). When broad-bean plants were treated with 0.01% and 0.02% coumarin and offered as food to *Aphis craccivora*, the natality and natality rate of this aphid decreased significantly (Mansour et al., 1982). As for small ermine moths, coumarin was toxic to larvae of *Y. padellus* and *Y. rorellus* (van Drongelen and van Loon, unpublished results).

Our analyses showed that high concentrations of coumarin and herniarin, and a trace of umbelliferone, were present in leaves of *P. mahaleb* (Figure 2). The amount of coumarin was 0.27% dry weight. Since coumarin at this level is known to be a feeding deterrent to a number of insects, the presence of simple coumarins in *P. mahaleb* must confer some chemical protection against herbivory. It is interesting to note that *P. mahaleb* is poisonous to *Y. malinellus* and *Y. padellus*: larvae of these species died after being fed with this plant (Gerrits-Heybroek et al., 1978; Gerrits-Heybroek and Ulenberg, unpublished results).

Obviously *Y. mahalebellus* has overcome this toxic barrier as their larvae are specialist feeders on *P. mahaleb*. The tested pupae of this small ermine moth contained umbelliferone along with some coumarin and herniarin; these three coumarins were also present in the adults (Figure 2). Overall concentration of simple coumarins in *Y. mahalebellus* was low: 0.003–0.004%. Variations in coumarin contents in the plant and insect remain unknown, since lack of insect material prevented us from carrying out replicate analyses. Coumarins in *Y. mahalebellus* are most likely derived from the plant as they are absent in larvae of *Y. evonymellus*, *Y. malinellus*, and *Y. rorellus*, which feed on plants lacking coumarins.

At this stage it is not clear whether such small amounts of simple coumarins can protect *Y. mahalebellus* against predation. In the literature injurious effects of coumarins were reported at higher dosages. Coumarin and hydroxycoumarin exert toxic actions to several organisms (Murray et al., 1982). Coumarin produced liver damage in rats and dogs; for rats it was toxic at a 0.25% dietary level (Hazleton et al., 1956). Several workers suggest that some secondary substances, i.e., aristolochic acids and cardiac glycosides, are sequestered in insects because they are protective agents against microbes and parasites (Urzúa et al., 1983; Smith, 1978). Tests have shown that umbelliferone and its derivatives inhibit fungal and bacterial growth at 0.05% level and that herniarin is a more potent antimicrobial agent than umbelliferone (Jurd et al., 1971). Antimicrobial activity declines substantially with decreasing concentra-

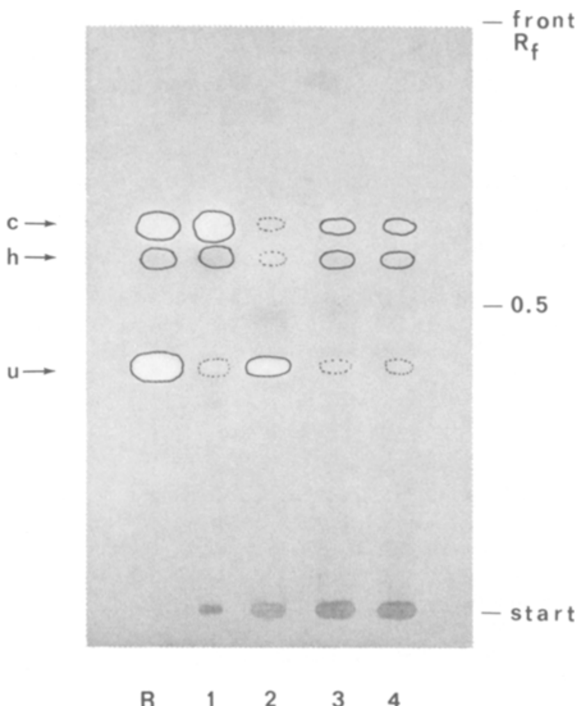

FIG. 2. TLC of simple coumarins in *P. mahaleb* and *Y. mahalebellus*. For TLC conditions see Methods and Materials. The plate was photographed in UV light, 365 nm. R: solution of reference compounds coumarin (c), herniarin (h), and umbelliferone (u); 1: leaves of *P. mahaleb*; 2: pupae of *Y. mahalebellus*; 3: male moths of *Y. mahalebellus*; 4: female moths of *Y. mahalebellus*.

tions of hydroxycoumarins, so it is uncertain whether they are still active at 0.003–0.004%, the levels found in *Y. mahalebellus*.

Recently high concentrations of carotenoids were recorded for *Y. mahalebellus* (Rothschild et al., 1986). It was suggested that carotenoids, which have antioxidant properties as they are effective quenchers of singlet oxygen, might function as photoprotective agents for this small ermine moth against the harmful effects of coumarin in its body (Rothschild et al., 1986). Coumarins in *Y. mahalebellus* and its host are simple coumarins, and simple coumarins are much less photoreactive towards nucleic acids than furanocoumarins (Marciani et al., 1971). Since only a small amount of simple coumarins is detected in pupae and adults of *Y. mahalebellus*, it seems unlikely that in these stages a protective function against coumarins can be attributed to the high levels of carotenoids found in this moth.

In summary, we conclude that some coumarins are present in *Y. mahalebellus* but that it is doubtful whether they play a significant role in the chemical defense of the insect. It seems that when the larvae feed on leaves of *P. mahaleb*, some coumarins pass through the gut and are not totally eliminated from the insect's body. For cocaine, it was demonstrated that most of the ingested alkaloid was excreted by larvae of the lymantriid moth *Eloria noyesi*, while some unchanged base was retained in the body during metamorphic development to adults (Blum et al., 1981). The significance of low levels of cocaine in these moths is also unknown.

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Book Review

Social Odours in Mammals, Volumes 1 and 2. Richard E. Brown and David W. Macdonald (eds.). Oxford and New York: Clarendon Press, Oxford University Press, 1985. $60 (£55) and $45 (£35), 882 pp. illustrated.

Richard Brown and David Macdonald have brought together a uniquely valuable and most timely zoological compendium, illustrated with line drawings and nearly 70 photographic plates, which demands a place on the shelf of anyone with an interest in the interdisciplinary study of chemical signalling in mammals.

No one scanning this encyclopedic two-volume work of 20 independent chapters, each with its own detailed bibliography, can doubt the pervasive importance of chemical cues in the lives of mammals. Brown and Macdonald have rendered future students a very great service indeed by gathering together a veritable mine of information which will be worked and reworked by many in this emerging field of study.

Their work is a catalog par excellence, with all the strengths and weaknesses of such a text. It is an encyclopedic work of reference, encompassing a vast amount of information, but without uniting this information very satisfactorily. Although aspiring to be interdisciplinary, it is also very much a zoologist's book.

The field has been divided by taxon, the 13 authors confining themselves to giving detailed accounts of what is known of the role of scent in the lives of specific groups of mammals, and while this subdivision has its merits, it has not encouraged a unified discussion of broader issues. Only in the introduction and in the competent first chapter by Peter Flood on the anatomical sources of significant mammal scents is the presentation free to range widely. Integration is helped a little by general indices of author, species, and odor source (principally the names of various glands) repeated at the end of each volume.

The text is particularly strong in cataloging the occurrence and structure of scent glands and other scent sources across the whole of the Mammalia, including orders which have not figured widely in such discussion previously. The social functions of odors in the lives of mammals, including scent-marking patterns and behavioral responses, are also documented, and in some chapters the anatomic organization of the olfactory organs is detailed.

Other facets of the subject are dealt with less well. Thus, a consideration

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of the molecular aspects of the subject is largely absent or is dealt with peripherally and inadequately, even for those relatively few species which have been studied in some depth chemically. The molecular dimension is one in which the authors seem not to be confident, one mentioning the possibility of volatile peptides for example. Even so, this work will provide an invaluable source of background zoological information for molecular scientists and others entering this interdisciplinary field. A notable exception to the absence of chemistry is provided by the chapter by Gisela Epple and Amos Smith in which they bring together their findings on the chemistry and biology of communication in the saddle-backed tamarin, *Saguinus fuscicollis*.

In addition to the chapters already mentioned, there are useful chapters on the monotremes and marsupials by Eleanor Russell, on the insectivores (including the tree shrews and the elephant shrews) with a separate chapter as a specific case study on *Tupaia belangeri* by Dietrich Holst, and on the bats by Uwe Schmidt. Richard Brown has provided a chapter on primer effects under the heading of rodents, as well as further chapters on myomorph rodents; on the primitive ungulates (elephants, hyraxes, and the aardvark); on armadillos, sloths, anteaters, and pangolins; and on marine mammals. David Macdonald has written on the hystricomorph rodents and on the carnivores. Zuleyma Halpin has provided a chapter on the sciuromorph rodents, Diana Bell on rabbits and hares, Patricia Moehlman on the Perrisodactyla, Morris Gosling on the Artiodactyla, Gisela Epple on the primates, and the work is concluded by a chapter by Richard Doty on man.

Valuable as the work is, I did feel that the presentation was noticeably uneven even for a multiauthor work, and that, perhaps with more ruthless editorial direction, it could have been condensed into a more balanced and more manageable single volume. Some authors have given concise summaries of whole orders, while others have dealt with single species, and others still have presented their own primary research findings as they might in a journal, and quite often a sharper, crisper presentation could have been achieved. I also noticed one or two places where I felt the text to be in error, but slips cannot be avoided in such a work.

But the merit of this text easily survives such criticisms. As the editors write:

> The literature on social odours lies widely scattered in the vaults of several disciplines. A profusion of loose ends juts out from the literature to overwhelm the reader; by considering all the mammalian families within these two volumes, we hope that some of these loose ends will be knotted together, while the remainder will be considerably untangled.''

While the text may not have fully lived up to this utopian aspiration of

interdisciplinary synthesis, it has none the less provided a most important zoological contribution to that end.

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Book Review

The Science of Allelopathy. A.R. Putnam and C.-S. Tang (eds.). New York: Wiley Intersciences Publication, John Wiley & Sons, 1986. $52.50, xi + 317 pp., illustrated.

This book consists of 17 chapters comprising the contributions of 21 of the participants in a symposium on allelopathy convened by the two editors at the International Congress of Pacific Basin Societies, held in Hawaii in December 1984. The initial chapter, by A.R. Putnam and C.-S. Tang, is devoted to an overview of the science. This is a stimulating introduction to the subject based upon early and recent history, the present state of knowledge and techniques, and the editors' views on the needs and the probable future of the science. The very strong emphasis on agriculture, industrial interests, and interdisciplinary requirements of the subject are predictable from the editors' interests, but they are probably well earned by the events in the discipline. It did seem to me, however, that if such a major part of basic allelopathic research as its implication in ecology was to be omitted from this chapter on the basis of its having been reviewed in E.L. Rice's second edition of *Allelopathy*, several other entire chapters might have been omitted from the book on the grounds that their entire subject matter was recently published in the *Journal of Chemical Ecology*. I am, of course, opposed to either or both omissions. On balance, this initial chapter is a valuable introduction to the subject of allelopathy for the advanced student or researcher.

The remaining 16 chapters are divided into groups constituting three parts of the volume. Part 1, comprising Chapters 2 through 6, is entitled "Field Observations of Allelopathy and Autotoxicity" and actually contains much more.

In Chapter 2, E.L. Rice gives a thorough review of the literature on "Allelopathetic Growth Stimulation" to which he appends a brief but convincing research report establishing a grotesque stimulation of growth by unidentified allelochemical compounds. Mature leaves of ground-ivy decaying in soil stimulated the growth of radish shoots by as much as 1064% and downy brome shoots by as much as 770%.

In Chapter 3, A.R. Putnam and L.A. Weston review "Adverse Impacts of Allelopathy in Agricultural Systems" in limited detail. With most of the weeds listed in tabular form, the subject is still massive.

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"The Role of Allelopathy in Subtropical Agroecosystems in Taiwan," by C.-H. Chou, constitutes Chapter 4. The great preponderance of allelopathic studies issued from Chou's laboratory, but some have been the work of two other groups, especially that of T.S.C. Wang. At the end of his review Chou offers a brief proposal of a theory of the origins of allelopathy and autotoxicity, their evolution, natural selection, and value to the species in which they occur. His proposal fell far short of its mark.

In Chapter 5, J.V. Lovett discusses "Allelopathy: The Australian Experience" in a well-balanced and comprehensive presentation. Australia has, at least in its drier districts, some landscapes richly dappled with "halo" patterns associated with shrubs and trees long suspected of allelopathic complicity. Yet, the studies of species interactions have concentrated upon weed impacts on pastures and various field crops and, among native species, crop residues, forest tree inhibition, etc., a strong bias toward economically important questions. Although some neglect of basic science necessarily resulted, it is remarkable how readily the investigators recognized fundamental questions and contributed to their solutions. The rhizosphere, the intricate dynamics of weed–pasture interactions, and the allelopathic peculiarities of insect frass incidental to *Eucalyptus* defoliation are better known than the widely visible "halo" patterns in coastal health vegetation of southern Australia.

C.-C. Young presents "Autointoxication of *Asparagus officinalis* L." in Chapter 6. The literature is reviewed and the first steps in an analysis of an allelopathic hypothesis are presented, while identification and modes of action of phytotoxins are projected.

Part 2, entitled "Techniques for Studies of Allelochemicals and Their Modes of Action" comprises Chapters 7 through 11.

C.S. Tang describes in detail "Continuous Trapping Techniques for the Study of Allelopathy in Higher Plants" in Chapter 7. The trapping action is based on the passage of excess or cycling aqueous medium through columns of Amberlite XAD-4 resin and the eventual elution of adsorbed chemical compounds from the resin by suitable organic solvents. This method is precise when rigorously applied, but it lacks the flexibility required in most allelopathic studies. Aside from root exudation, it is difficult to envision any natural process in plant interaction to which it would be applicable without relaxation of the rigorous conditions of isolation for the sake of purity.

G.R. Leather and F.A. Einhellig offer "Bioassays in the Study of Allelopathy" in Chapter 8. They introduce the subject with a strong argument for standardization of bioassays. While extreme care ensuring uniformity within a bioassay is doubtlessly mandatory, requiring that standardized bioassays be employed whenever, wherever, and whoever investigates biochemical interactions of whatever plant species can only stifle experimental innovation. I hope that was not the intent. However, under "Seed Germination" the authors complain that "Even reports originating from the same laboratory differ in the conduct

of the assay." This is an excellent way to put an end to experimental originality. Although the authors recognize the fact that some allelopathic studies employed both seed germination and seedling growth bioassays, they apparently did not recognize that several of the studies they listed under "seed germination" in their table 8.1 employed only seedling growth and offered no seed germination data at all. Their descriptions of assays utilizing aquatic, cryptogamic, and miscellaneous other receiver plants include evidence that they, too, have practiced some innovative experimental design. The chapter is very useful.

H.G. Cutler writes on "Isolating, Characterizing, and Screening Mycotoxins for Herbicidal Activity" in Chapter 9. He offers a stimulating and highly instructive introduction to the subject which is intriguing to the broadly interested biologist and should be invaluable to the novice in this specialty.

F.A. Einhellig reviews "Mechanisms and Modes of Action of Allelochemicals" in Chapter 10. This is an excellent review of a complex and very incompletely known facet of allelopathy. The great variety of allelochemicals and the diversity inherent in their modes of release, movement, and accumulation, playing upon differential susceptibilities of multiple processes constituting growth in divers receiver plants almost defy definition. However, what little is known is shown to relate very likely to membrane perturbation, hormone and enzyme disruptions, and a long list of absorptive, retentive, and metabolic processes issuing from these disruptions. Just enough is known about these effects of certain phenolic and terpenoid allelochemicals to suggest a multitude of future research projects for plant physiologists for much longer than their interest will likely persist.

In Chapter 11, "Allelochemical Mechanisms in the Inhibition of Herbs by Chaparral Shrubs" is treated by the late W.H. Muller.[1] The physiologic mechanisms described are based almost entirely upon his own work and that of collaborators under his leadership. The field-directed research involved collaboration with others and established the environmental elements here treated. In the effects of *Salvia* monoterpenoids (especially cineole) on growth of a series of seedling species, a mechanism involving membrane perturbation and disruption of respiration and absorptive control is as nearly fully established as any allelopathic mechanism yet proposed.

Part 3 treats "Chemistry and Potential Uses of Allelopathy" in Chapters 12 through 17.

"The Function of Mono- and Sesquiterpenes as Plant Germination and Growth Regulators" by N.H. Fischer occupies Chapter 12. It is a rather detailed review of the literature with particular attention given to relations between structural qualities of regulatory molecules and their specific biologic activities. The treatment should remain of general value long after its coverage has been outdated by the passage of time.

[1] Professor Walter H. Muller died on May 28, 1986.

''Polyacetylenes as Allelochemicals,'' by K.L. Stevens, constitutes Chapter 13. This is an instance of the chemistry of a group of compounds being far ahead of the corresponding ecology, partly because of the ephemeral nature of the compounds. Their biotic activity has been repeatedly demonstrated in vitro and plants that produce them appear to be allelopathic, but these extremely toxic compounds are too dynamic for the application of anything approaching Koch's postulates. The plants may be telling us things never dreamed of by Koch.

''Qualitative and Quantitative Determination of the Allelochemical Sphere of Germinating Mung Bean'' is treated in Chapter 14 by C.S. Tang and B. Zhang. The authors used the continuous trapping method with Amberlite XAD-4 to quantify the exudation of allelochemicals by germinating mung beans. On the basis of this quantity, the size of a mung bean, and the degree of toxicity revealed by bioassays of exudates, they calculated the thickness of allelochemical spheres of germinating mung beans. Considering the several highly variable factors in the environment of the germination process, this might be highly approximate. The authors conclude, among other things, ''Based on the data obtained, we introduced and established the concept of allelochemical spheres.'' I once thought that concept originated in my work, but then I encountered an older writer as follows: ''For they say that the vine scents the cabbage and is infected by it. Wherefore the vine-shoot, whenever it comes near this plant, turns back and looks away as though the smell were hostile to it'' (Theophrastus, ''Enquiry into Plants'', IV, xvi, 6.). Modern terminology, identification, allelochemicals, and experimental methodology we can claim for our own generation (or that of our grandfathers), but we have no monopoly on ideas.

In Chapter 15, ''Caffeine Autotoxicity in *Coffea arabica* L.'' is treated by G.R. Waller, D. Kumari, J. Friedman, N. Friedman, and C.-H. Chou. This is a summary treatment of a program embracing studies of allelochemicals of *Coffea arabica* (of which caffeine is the principal one), biologic activity, metabolism, source, incidence in the environment, absorption and translocation in *Coffea* seedlings, isolation of susceptible tissues from toxins, and autotoxic deterioration of old plantations from work in Waller's laboratory and from the recent literature.

In Chapter 16, ''Allelopathic Activity of Rye (*Secale cereale* L.)'' is presented by J.P. Barnes, A.R. Putnam, and B.A. Burke. This also is a summary statement based upon extensive literature, a significant part of which issued from Putnam's laboratory. The most powerful toxicity was exhibited by the benzoxazines which, although they accounted for a small proportion of rye toxicity, occurred in small quantities and therefore must have been extremely potent.

In Chapter 17, ''Microbially Produced Phytotoxins as Herbicides—A Perspective'' is offered by S.O. Duke. This is an impressive subject, comprising much evidence of the implication of microbial species in what appears at first

examination to be a simple case of higher plant allelopathy. The prospect of significant herbicides from this source seems very promising.

The usual weaknesses of symposium volumes are a bit more serious in this book than they are in most of the genre. The style, quality, significance, and inclusiveness of the several chapters are far from uniform. One gathers from the Preface that publication was an afterthought or at least that it was not assured before the gorup was assembled, thus limiting the opportunity of the editors to integrate the volume. These complaints are minor, however, compared to the positive qualities of the book. Its avowed purpose of reviewing the science of allelopathy, assessing its state and its future, and supporting the concept of interdisciplinary research is well met by the several writers. There remains, however, ample room for alternative opinion. I am particularly pleased to see in some of the powerful ephemeral phytotoxins evidence supporting the theory of A.G. Winter,[2] that some cases of allelopathy may involve fleetingly present toxins that one can never hope to find accumulated in the environment. Winter theorized that plant roots had equal chance with decomposition microorganisms to absorb ephemeral inhibitors. First proposed in 1942 and repeatedly supported by its author for almost 20 years, Winter's very logical proposal finds strong support in some of the chemical advances issuing from the new ambience of team research so strongly emphasized in this volume. If the book did nothing else than lead to an accommodation of modern allelopathic thinking to the subtle logic of one of the best theorists of the preceding generation, it would be proven a success.

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[2] Winter, A. G. 1961. *Symposia of the Society for Experimental Biology* 15:229–244.

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INHIBITION OF FEEDING BY A GENERALIST INSECT DUE TO INCREASED VOLATILE LEAF TERPENES UNDER NITRATE-LIMITING CONDITIONS

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Abstract—Nitrogen-limited plants of *Heterotheca subaxillaris* accumulate greater quantities of leaf volatile terpenes than do nitrogen-rich plants. A series of feeding trials were performed to determine if such nitrate-limited plants are better defended against generalist-feeding insect herbivores. Soybean looper (*Pseudoplusia includens*) larvae were fed leaves from *H. subaxillaris* rosettes grown under high and low nitrate supply regimes. Larval consumption, growth, and survival declined as the leaf volatile terpene content increased. Larval consumption and growth were enhanced by higher plant nitrate supply and with increasing leaf age. The results suggest that the higher quantity of volatile terpenes in the leaves of nitrate-limited plants may better defend these leaves against generalist-feeding insects.

Key Words—*Heterotheca subaxillaris*, Asteraceae, *Pseudoplusia includens*, Lepidoptera, Noctuidae, volatile terpenes, nitrogen, herbivory.

INTRODUCTION

Plants growing under nitrogen-limiting conditions generally have a slower growth rate than those growing under nitrogen-rich conditions (Chapin, 1980). Comparable loss of leaf nitrogen to herbivores by nitrate-limited and nitrate-rich plants presumably has a greater impact on the growth of nitrogen-limited plants. Carbon supply does not limit plant growth under low nitrate conditions, and consequently, increased quantities of carbon-based defenses should be selected for as nitrate availability decreases (Janzen, 1974; McKey et al., 1978; Bryant et al., 1983; Coley et al., 1985; Mihaliak and Lincoln, 1985).

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Leaf mono- and sesquiterpenes are known to function as carbon-based chemical defenses against herbivores (Eisner, 1964; Rice et al., 1978; Mabry and Gill, 1979; Langenheim and Hall, 1983). Increased quantities of leaf mono- and sesquiterpenes occur as plant nitrate availability decreases (Mihaliak and Lincoln, 1985) and may provide greater defense against insect herbivores. The quantity of leaf volatiles is also known to decline with leaf age (Crankshaw and Langenheim, 1981; Mihaliak and Lincoln, 1985). This decrease coincides with the lower productivity and nitrogen content of older leaves (Mooney, 1972; Mihaliak and Lincoln, 1985). The high volatile terpene content of young leaves under nitrate-limiting conditions (Mihaliak and Lincoln, 1985) suggests a pattern of increased carbon allocation to the defense of those leaves which represent the largest relative proportion of the potential photosynthetic capacity of a plant. Plant allocation to defense should reflect leaf value (Rhoades, 1979), assuming that leaf value is measured by its relative contribution to future plant productivity and the cost of replacement (Mooney and Gulmon, 1982).

The objective of this study was to determine if the increased quantity of leaf mono- and sesquiterpenes under nitrate-limiting conditions better defends plants against a generalist insect herbivore. A further goal was to investigate whether patterns of insect consumption and growth are consistent with the prediction that a leaf which represents a high relative proportion of the photosynthetic capacity of a plant is better defended than a leaf which will contribute less to future plant productivity. Despite an often observed increase in herbivore consumption on leaves with a relatively low concentration of nitrogen (Mattson, 1980; Scriber and Slansky, 1981; Lincoln et al., 1982, 1986), we predict that insect consumption rates should decline as leaf terpene levels increase under nitrate-limiting conditions. Further, since the potential contribution of a leaf to plant productivity is greatest when a leaf is young, and decreases with leaf age, (Mooney and Gulmon, 1982), we expect that consumption and growth of herbivores will be less on these young leaves than on older, less defended leaves.

To test these predictions, we have performed feeding trials using the generalist lepidopteran *Pseudoplusia includens* (soybean looper) and young and mature leaves from *Heterotheca subaxillaris* rosettes grown under high and low nitrate supply regimes. *Pseudoplusia includens* is recognized as a polyphagous species (Kogan and Cope, 1974) and has been observed feeding on *H. subaxillaris* (personal observation).

METHODS AND MATERIALS

The plant species used in this experiment, *Heterotheca subaxillaris* (Lam. Britton and Rusby) (Asteraceae) (camphorweed), is a herbaceous annual/biennial (Awang and Monaco, 1978) which occurs in old fields, disturbed sites, and open sandy areas of the southeastern United States. Forty-one mono- and ses-

quiterpenes have been identified from the leaves of *H. subaxillaris* (Lincoln and Lawrence, 1984). *Pseudoplusia includens* (Walker) larvae used in the feeding trials were obtained from a laboratory colony which was maintained on a pinto bean–soy protein–wheat germ diet (Greene et al., 1976) in an environmental incubator under a thermophotoperiod of 14 hr light–10 hr dark (26°C–20°C).

The plants were grown in a greenhouse using seed germinated on filter paper in Petri dishes. Seedlings were transferred into a potting medium of perlite, vermiculite, and sand (1 : 1 : 1) within one week of germination and maintained under a photoperiod of 14 hr day–10 hr night using 1000-W coated metal halide and high-pressure sodium lamps as supplemental light (690 μmol/m^2/sec). The plants were watered on alternate days with either tap water or a nutrient solution. The nutrient solution contained either 0.5 mM or 5.0 mM nitrate and provided the nitrate-limited and nitrate-rich treatments, respectively. These solutions consisted of equimolar concentrations of KNO_3 and $Ca(NO_3)_2$. $CaCl_2$ and KCl were added to the nitrate-limited solution to maintain equal Ca^{2+} and K^+ concentrations. Both nutrient solutions contained 1.65 mM K_2HPO_4, 1.35 mM KH_2PO_4, 2.0 mM $MgSO_4$, micronutrients, and chelated iron.

Feeding trials were performed by removing leaves from individual 12-week-old *H. subaxillaris* rosettes, enclosing the petiole in a folded moistened tissue, and then placing the larvae and leaf into a plastic Petri dish. All leaves and insects were kept in the environmental incubator throughout the feeding trial under the thermophotoperiod described above. A second matched leaf was removed from each plant and used to determine the leaf volatile terpene content, the leaf nitrogen content, and the leaf water content. No perceivable changes in the leaf volatile terpene or nitrogen content occur when leaves are detached from the plant.

Larvae were fed either an immature (1-week-old) or fully expanded (4-week-old) leaf from a *H. subaxillaris* plant grown under either the 0.5 mM or the 5.0 mM nitrate treatment. For comparison, feeding trials were also performed using 1-week-old leaves from soybean plants (*Glycine max* L. Merr. cv. Ransom) grown under the 5.0 mM nitrate treatment. Leaf age was determined by placing small rings over the petiole of newly emerging leaves. All of the feeding trials began with larvae which had recently molted to the fourth instar and had not begun to feed. The larvae were kept with the leaf until they either molted to the fifth instar or died. Seventy feeding trials were performed on *H. subaxillaris* leaves, and ten were performed using soybean leaves.

The initial fresh weight of each larva and leaf used in the feeding trials was recorded. At the end of the feeding trial, the leaf was dried and weighed, and larva was frozen and then dried to determine the final dry weight. The initial dry weight of the larvae was computed using a fresh weight–dry weight conversion calculated from matched larvae not used in the experiment. The initial dry weight of the leaves was calculated from a regression analysis of the fresh and dry weights of the matched leaves which were also used to determine leaf

terpene, nitrogen, and water content. The relative growth rate (RGR), relative consumption rate (RCR), and efficiency of conversion of ingested food (ECI) (Waldbauer, 1968) were computed on a dry weight basis over the entire fourth-instar feeding period.

Leaf volatile terpene content was determined for the matched leaves using gas chromatography by adding a known amount of an internal standard (*n*-tetradecane) to a pentane extract of each leaf. After extraction, the sample was centrifuged to remove all leaf material and then concentrated to a volume of approximately 100 μl under a stream of nitrogen. Analyses were performed using a gas chromatograph equipped with a SP-1000 fused silica capillary column (0.25 mm $\times$ 30 m), a flame-ionization detector, and a digital integrator (temperature program from 50 to 175°C at a rate of 3°C/min with a 5-min initial and 15-min final isothermal period). Leaf volatile terpene contents were calculated without response factors for individual compounds.

The nitrogen content of the residual leaf material collected after pentane extraction was measured using a Hewlett-Packard Carbon-Hydrogen-Nitrogen Analyzer and a digital integrator. The nitrogen content of each sample was computed using a response curve generated with cystine (U.S. Department of Commerce, Bureau of Standards). Measured leaf nitrogen contents were not corrected for the materials extracted with pentane.

All statistical analyses were performed using the Statistical Analysis System, SAS Institute Inc.

RESULTS

The leaf volatile terpene content was higher under nitrogen-limiting conditions ($\bar{X} = 4.73$ mg/g, SD = 1.39) than under nitrogen-rich conditions ($\bar{X} =$ 3.73 mg/g, SD = 2.38; $F = 7.87$, $P < 0.01$), and the leaf nitrogen content was reduced when plant nitrate availability was limited (Table 1; $\bar{X} = 24.4$ mg/g at high nitrate, $\bar{X} = 18.5$ mg/g at low nitrate; $F = 19.6$, $P < 0.001$). As leaves aged, reduced quantities of leaf volatile terpenes ($F = 21.7$, $P < 0.001$) and leaf nitrogen ($F = 4.22$, $P < 0.05$) occurred (Table 1). The volatile leaf terpene content changed less with leaf age under nitrate-rich conditions than under nitrate-limiting conditions ($F = 5.61$, $P < 0.05$ for nitrogen treatment by leaf age interaction). Under nitrate-limiting conditions, leaf nitrogen content was near the level suggested as the minimum necessary for larval growth (Fox and McCauley, 1977).

Larval consumption (RCR), growth (RGR), and survival were lower on the leaves of nitrate-limited plants (Table 1; nitrate treatment effect: RCR, $F =$ 31.5, $P < 0.001$; RGR, $F = 55.97$, $P < 0.001$; survival, $\chi^2 = 16.6$, $P <$ 0.01). These leaves of nitrate-limited plants contain more volatile terpenes and

TABLE 1. CHARACTERISTICS OF LEAVES OF *Heterotheca subaxillaris* AND SOYBEAN, AND PERFORMANCE OF *Pseudoplusia includens* LARVAE OVER ENTIRE FOURTH INSTAR[a]

	0.5 mM Nitrate		5.0 mM Nitrate		
	Immature	Mature	Immature	Mature	Soybean
Leaf nitrogen content (mg N/g leaf)	20.5[ab]	16.6[a]	24.8[c]	23.5[bc]	34.8[d]
Leaf volatile content (mg volatiles/g leaf)	6.25[a]	3.22[b]	4.02[b]	3.03[b]	
Leaf weight consumed (mg dry wt.)	6.1[a]	13.3[a]	27.0[b]	48.9[c]	10.8[a]
Leaf tissue consumed* (% of total leaf)	25[a]	44[ab]	50[bc]	67[c]	
Relative consumption rate (mg consumed/mg larva)	8.9[a]	16.9[ab]	24.6[bc]	31.6[c]	7.6[a]
Relative growth rate (mg growth/mg larva)	−0.28[a]	−0.02[a]	0.59[b]	0.96[b]	1.01[b]
Conversion efficiency (mg growth/g eaten)	−47[a]	−27[a]	22[a]	28[a]	234[b]
Insect survival (%)	14	38	78	89	100

[a] Larvae were offered either immature (1-week-old) or mature (4-week-old) leaves from plants grown using nutrient solutions which contained either 0.5 mM or 5.0 mM nitrate. *Pseudoplusia includens* performance on 1-week-old leaves of soybean plants grown using 5.0 mM nitrate is given. Means in each row followed by the same letter are not significantly different (Tukey's studentized range test, $P < 0.05$, * arcsin square root transform).

less nitrogen than comparable leaves of nitrate-rich plants. The negative mean growth rate for larvae which fed on leaves of nitrate-limited plants is due to weight loss of some larvae prior to their death during the feeding trial. The RGR of the surviving larvae which were offered leaves from the 0.5 mM plants averaged 0.50 mg/mg larva over the fourth instar. Consumption and growth were reduced on immature leaves and increased on older leaves (leaf age effect: RCR, $F = 7.61$, $P < 0.01$; RGR, $F = 6.72$, $P < 0.01$).

Leaf volatile terpene content has a negative effect on the growth and consumption rate of *P. includens*, while larval growth was positively related to leaf nitrogen content (Table 2). The absence of a relationship between consumption and leaf nitrogen contrasts with the often observed compensatory feeding response of herbivores which encounter nitrogen-poor leaves (Mattson, 1980; Scriber and Slansky, 1981; Lincoln et al., 1982, 1986).

Leaf water content, measured as percent of leaf fresh weight, increased significantly under nitrate-rich conditions (for 0.5 mM plants, $\bar{X} = 75.3\%$, SD $= 3.2\%$; for 5.0 mM plants, $\bar{X} = 78.1\%$, SD $= 1.8\%$; $F = 11.6$, $P < 0.005$).

TABLE 2. EFFECT OF LEAF VOLATILE TERPENE CONTENT AND LEAF NITROGEN CONTENT ON RELATIVE GROWTH RATE AND RELATIVE CONSUMPTION RATE OF *P. includens* LARVAE OVER ENTIRE FOURTH INSTAR[a]

	Leaf nitrogen content		Leaf volatile content	
	Slope	P	Slope	P
Relative growth rate	0.03	<0.05	−0.09	<0.01
Relative consumption rate	0.33	<0.19	−2.00	<0.006

[a]Multiple regression analysis, $df = 2, 67$.

However, when added to a multiple-regression analysis with leaf volatile terpene and leaf nitrogen content, leaf water content did not significantly influence either RCR ($P < 0.44$) or RGR ($P < 0.56$) of the larvae.

The total amount of leaf tissue consumed by larvae was lowest on leaves from nitrate-limited plants (Table 1; $F = 31.5$, $P < 0.001$). Increased larval consumption on leaves from nitrate-rich plants resulted in both a greater absolute amount of leaf tissue being eaten and a larger proportion of each nitrate-rich leaf being consumed by the herbivores. Further, more leaf tissue was consumed by larvae feeding on mature leaves than on immature leaves.

The conversion efficiency (ECI) of larvae was significantly lower when offered leaves of nitrogen-limited plants than when offered nitrogen-rich leaves ($F = 5.03$, $P < 0.05$). Further, ECI was lower when larvae fed on *H. subaxillaris* leaves than on soybean leaves (Table 1). The growth rate of *P. includens* larvae on soybean leaves was not different from their growth rate on *H. subaxillaris* leaves grown under nitrate-rich conditions. However, a significant increase in consumption occurred when feeding on the *H. subaxillaris* leaves.

DISCUSSION

The higher leaf volatile terpene content combined with the reduced leaf nitrogen content of nitrate-limited plants resulted in decreased performance by a generalist herbivore. These results suggest that leaves of nitrate-limited plants may be better defended from generalist-feeding insect herbivores than leaves of nitrate-rich plants and that young leaves are better protected than older leaves.

The concentration of leaf nitrogen is usually considered to be a major determinant of insect herbivore feeding and nutrition (Mattson, 1980; Scriber and Slansky, 1981; Lincoln et al., 1982; Scriber, 1984). However, the results of this study and of previous investigations of the interactions between leaf nitrogen and carbon-based chemical defenses suggest that both nitrogen and allelochemicals are important factors in determining the nutritional quality of

plant tissue for herbivores (Lincoln et al., 1982; Redak and Cates, 1984; Lincoln, 1985; see, however, Morrow and Fox, 1980).

In the current study, leaf water content was lower under nitrate-limited conditions but failed to explain the variance in either RCR or RGR. Low leaf water content (ca. 60%) can suppress larval growth and nitrogen utilization efficiency (Scriber, 1977; Scriber and Slansky, 1981). However, slower larval growth due to variation in water content is not likely to occur on leaves from herbaceous plants (Scriber, 1977; Slansky and Feeny, 1977).

An abundance of leaf nonstructural carbohydrates appears to increase production and/or decrease turnover of volatile terpenes (Croteau et al., 1972; Croteau, 1984). Higher concentrations of nonstructural carbohydrates accumulate in leaves as plant nitrate availability declines (Chapin, 1980; Fritsch and Jung, 1984). Thus, the ecological hypothesis regarding increased carbon allocation to defense under nitrate-limiting conditions (Rundel, 1982; Coley et al., 1985; Mihaliak and Lincoln, 1985) and the current results are consistent with the hypothesized physiologic controls on plant terpene biosynthesis (i.e., increased availability of carbon for the production of volatile terpenes under nitrate-limiting conditions).

The results agree with the predictions of the leaf value hypothesis (Rhoades, 1979). Nitrogen-limited plants commonly have a slower growth rate than nitrogen-rich plants. Loss of leaf material by a nitrogen-limited plant should have a greater impact on growth than a comparable loss of leaf tissue by a nitrogen-rich plant. In the current study, less consumption occurred on the leaves of nitrate-limited plants than on leaves of nitrate-rich plants, and the quantity of leaf volatiles was highest under nitrate-limiting conditions. Thus, leaves which potentially contribute a high relative proportion to total plant productivity appear to be better defended. Similarly, the potential contribution to productivity is greatest in immature leaves and declines with leaf age. Young leaves contained higher levels of volatile terpenes, and consumption was reduced on these leaves.

The high leaf terpene content in leaves with a relatively low nitrogen content (i.e., those of nitrate-limited plants) increases the quantity of terpenes per unit nitrogen an insect must consume when feeding on these leaves. If leaf volatile terpenes are toxic, herbivores may be able to consume only small quantities of the immature leaves of nitrate-limited plants or, if the terpenes were repellent, the herbivore would move in search of another food source. In either case, the increased allocation to carbon-based defense by the slower growing, resource-limited plant (Coley et al., 1985; Mihaliak and Lincoln, 1985) would be successful in retaining leaf nitrogen.

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SIGNIFICANCE OF SAGUARO CACTUS ALKALOIDS IN ECOLOGY OF *Drosophila mettleri*, A SOIL-BREEDING, CACTOPHILIC DROSOPHILID

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Abstract—*Drosophila mettleri* is a soil-breeding, cactophilic drosophilid which lives in the Sonoran Desert. Several chemical constituents of cacti in this region have been identified as having major roles in insect-host plant relationships involving *Drosophila*. For example, isoquinoline alkaloids, which are present in senita cactus, have been shown to be toxic to seven of the nine species tested. The two tolerant species are *D. pachea*, the normal resident, and *D. mettleri*. Necroses of senita cacti are often used as feeding substrates by *D. mettleri* adults, but this species has never been reared from senita rots. Soil, which have been soaked by juice from saguaro and cardón rots, are the typical breeding substrates of this species. The tissues of both of these cacti also contain alkaloids, chemically related to those in senita, but at much lower concentrations. Alkaloid concentration in saguaro-soaked soil was found to be 1.4–27 times the average concentration in fresh tissue. Alkaloids were extracted from saguaro tissue and used in tests of larva-to-adult viability, developmental rate, and adult longevity. Elevated concentrations of saguaro alkaloids had no significant effect on the longevity of *D. mettleri*, but significantly reduced the longevity of *D. nigrospiracula* and *D. mojavensis*, two nonsoil breeding cactophilic species. Viability and developmental rates of all three species were affected, but the effect on *D. nigrospiracula* was comparatively greater. It is argued that the adaptations that allow *D. mettleri* to utilize the saguaro soil niche also convey tolerance to alkaloids present in senita tissue. The ability to utilize senita necroses as feeding substrates represents an ecological advantage to *D. mettleri*, in that the density of potential feeding sites is increased as compared to species which are more specific in their host-plant relationships.

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Key Words—*Drosophila*, *D. mojavensis*, *D. nigrospiracula*, *D. mettleri*, Diptera, Drosophilidae, cactus, alkaloids, viability, development, longevity, host-plant relationships.

INTRODUCTION

The study of the chemical ecology of the four cactophilic *Drosophila* species that live in the Sonoran Desert of North America has led to a reasonably complete picture of the chemistry involved in host-plant utilization (Fogleman and Heed, 1987, and references therein). These drosophilids feed and breed in the necroses of columnar cacti and are highly host-plant specific. Briefly, the primary *Drosophila*-cactus relationships are: *D. mojavensis*-*Stenocereus gummosus* (agria) or *S. thurberi* (organ pipe); *D. pachea*-*Lophocereus schottii* (senita); *D. nigrospiracula*-*Carnegiea gigantea* (saguaro) or *Pachycereus pringlei* (cardón); and *D. mettleri*-soils which have been soaked by saguaro or cardón (Heed, 1978).

The latter species, *D. mettleri*, exhibits a novel behavioral characteristic, i.e, ovipositing in soils which have been soaked by cactus rot exudate. The only other reported example of soil-breeding *Drosophila* involves a Hawaiian species, *D. heedii*, which oviposits in soil that has been inundated by tree drippings (Kaneshiro et al., 1973).

Drosophila mettleri is also the only one of the four species that is not limited in its substrate utilization by host-plant chemistry (Fogleman and Heed, 1987). The chemical constituents of cacti that have been shown to have toxic effects on adults and larvae of nonresident *Drosophila* species include medium-chain fatty acids (C_8 to C_{12}) and dihydroxy sterols in agria and organ pipe cacti and high concentrations (3–15% dry weight) of alkaloids in senita cactus (Kircher, 1969, 1982). These fatty acids and sterol diols are toxic to *D. nigrospiracula* (Fogleman et al., 1986; Fogleman and Kircher, 1986), and senita alkaloids are toxic to both *D. nigrospiracula* and *D. mojavensis* (Kircher et al., 1967). Although larval viability is significantly reduced in some cases, laboratory experiments have demonstrated that *D. mettleri* can successfully complete larval development in necrotic tissues of all five cactus species mentioned above (Fogleman, 1984).

Despite its chemical tolerance, *D. mettleri* appears to be behaviorally restricted to soil-breeding, but not necessarily to soils soaked by a particular species of cactus. Adults have been reared from naturally occurring organ pipe-soaked soil (Fogleman et al., 1981) and from artificially produced senita-soaked soil in the field (Fogleman et al., 1982). It probably also uses agria-soaked soil, but no rearing records are available for this substrate. Cardón- and saguaro-soaked soils, however, are more commonly used because, being much larger cacti than the others, rots in these species are more likely to produce an adequate substrate. In essence, *D. mettleri* is an opportunistic generalist.

The ability of *D. mettleri* to use all of the cactus species as feeding substrates represents an obvious ecological advantage over the other, more restricted cactophilic *Drosophila*. Since several of the columnar cactus species are present in any given area of the Sonoran Desert, the density of feeding substrates for *D. mettleri* is greater. Its penchant for opportunistically using any soaked soil is also of ecological value in the stringent environment of the desert. The question of current interest is how *D. mettleri* became tolerant to the alkaloids present in necrotic tissue of senita cactus in light of the fact that other cactophilic *Drosophila* (i.e., *D. nigrospiracula* and *D. mojavensis*) have remained intolerant and that the evolutionary history of *D. mettleri* does not support the idea that it evolved its tolerance in the same manner as the resident species, *D. pachea*.

The cacti which produce soaked soils more commonly used by *D. mettleri*, saguaro and cardón, also contain alkaloids (Gibson, 1982). Brown et al. (1972) reported that 95% of the alkaloids in saguaro are the isoquinolines, carnegine (1,2-dimethyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline) and gigantine (1,2-dimethyl-5-hydroxy-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline). The remaining 5% are trace β-phenylethylamine and isoquinolines (Ordaz et al., 1983). The chemical structures of the major saguaro alkaloids are depicted in Figure 1. The senita alkaloids, lophocereine (1-isobutyl-2-methyl-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline), pilocereine, and piloceredine (two lophocereine trimers), were first isolated and identified by Djerassi et al. (1958, 1962). The majority of the alkaloid fraction of senita exists as the trimers which are more stable than the monomer, lophocereine (Kircher et al., 1967). The structures of lophocereine and pilocereine are also shown in Figure 1. The fitness measurements made by Kircher et al. (1967) demonstrate that the toxicity of senita alkaloids is mainly due to pilocereine since lophocereine (at 1% dry weight of the medium) has essentially no effect on adult longevity and a comparatively minor effect on larval viability. A subsequent study of the effect of senita alkaloids on *D. mettleri* showed that this species was less affected with respect to adult longevity and larval viability than *D. pachea* at concentrations which are fatal to other desert *Drosophila* (Fogleman et al., 1982). This same study reported that, in collections of adults feeding on rotting senita in nature, *D. mettleri* can be present in significant proportions, sometimes outnumbering *D. pachea*.

In contrast to senita, the alkaloids in saguaro are only present at 1–1.7% of the dry weight of the tissue (Brown et al., 1972) and, at these concentrations, are apparently not toxic to the larvae of any of the desert *Drosophila* (Fellows and Heed, 1972). However, the actual breeding site of *D. mettleri* represents a situation where the alkaloids may be much more concentrated. As rot juice continually drips on the soil, water evaporates—producing a substrate in which all solutes become more concentrated than in the tissue itself. Therefore, the alkaloids in saguaro- and cardón-soaked soils could be in sufficiently high con-

FIG. 1. Chemical structures of saguaro and senita alkaloids: (A) carnegine, (B) gigantine, (C) lophocereine, (D) pilocereine.

centration that the adaptations which accompanied the evolutionary invasion of *D. mettleri* into the soaked-soil niche convey tolerance to similar compounds in other substrates, e.g., necrotic senita tissue. This was suggested by Fogleman et al. (1982) as a possible explanation of *D. mettleri*'s ability to tolerate senita alkaloids. The similarity in chemical structure of saguaro alkaloids and lophocereine can be clearly seen in Figure 1.

This paper reports the results of experiments designed to test the following questions: (1) is the concentration of alkaloids in samples of saguaro-soaked soil greater than that of the tissue? and (2) do elevated concentrations of saguaro alkaloids have any effect on fitness parameters of cactophilic *Drosophila*? Fitness was estimated through the standard parameters of adult longevity and larval viability and developmental rate. *Drosophila nigrospiracula* and *D. mojavensis*

were also tested as controls since their response to senita alkaloids is known and neither species is capable of utilizing soaked soils as larval substrates.

METHODS AND MATERIALS

Strain Derivation. Strains of all three *Drosophila* species were multifemale lines initiated by rearing adults from natural substrates. Cultures had been maintained in the lab on Carolina Instant *Drosophila* Medium (Formula 4-24) for approximately two years. All experiments were carried out under ambient laboratory temperature (ca. 25°C) and humidity. Although these parameters varied during the experimental period, all replicates were subject to identical conditions.

Alkaloid Extraction. Fresh saguaro tissue (ca. 30 kg) was extracted first with methanol and then with a 2 : 1 chloroform–methanol solution in a continuous Soxhlet extractor. The evaporated extracts were added to a 5% HCl solution and extracted several times with ether. The aqueous phase was made alkaline with NH_4OH and extracted with ether. The ether was evaporated and the crude alkaloids were purified through another ether–HCl–NH_4OH–ether sequence followed by evaporation. A total of 38.51 g (0.9% of the dry weight of the plant) of purified alkaloids was obtained in this manner. Since soaked soil and cactus tissue differ greatly with respect to percent dry weight, alkaloid concentration is expressed as milligrams of alkaloids per milliliter of water (total weight minus dry weight) so that direct comparisons can be made between substrate types. The concentration of alkaloids in the fresh tissue was determined to be 1.50 mg/ml.

Alkaloid solutions were made up by dissolving the purified alkaloids in slightly acidic water (pH 6.0 via phosphoric acid). These solutions had alkaloid concentrations of 5, 10, and 15 times the concentration of fresh tissue based on the following parameters: dry weight = 14.27%; alkaloid concentration = 1.0% dry weight (1.67 mg/ml). The 5×, 10× and 15× concentrations represent 8.35, 16.7, and 25.04 mg/ml respectively. A control solution was composed of the acidic water without alkaloids.

Three soaked-soil samples weighing approximately 1337, 344, and 593 g respectively, were collected from beneath rotting saguaros in the vicinity of Tucson, Arizona. Only the top 2.5 cm of soil was taken, and all three samples contained *D. mettleri* larvae. Alkaloids were extracted and their concentrations in the soil samples were determined in the manner described above.

Adult Longevity Experiments. Three replicate sets of four vials (10 × 6 cm) were set up for both sexes of *D. mettleri*, *D. nigrospiracula*, and *D. mojavensis*. The vials contained a total volume of 10 ml of *Drosophila* medium consisting of 5 cc of dry *Drosophila* medium (Carolina Formula 4-24) and 5 ml of one of the solutions (0, 5×, 10×, or 15×). Approximately 10 grains of

bakers' dry yeast were added to each vial. Thirty-three adult flies (5–10 days old, sexes separate) were placed in each vial, and the number of dead flies in each vial was recorderd daily. Twice a week, the flies were transferred to vials containing fresh food. The experiments were terminated after 30 days or when half the flies had died (LT_{50}), whichever period was longer.

Larval Viability and Developmental Rate Experiments. First-instar larvae were obtained by allowing adults of the three species to oviposit in water–agar Petri dishes which contained a 2.5 cm patch of a paste made from dry saguaro powder and water. The flies were transferred to new plates daily. Plates containing eggs were stored for 24 h until the first-instar larvae hatched. Larvae were transferred to experimental media with a small probe.

Three replicate sets of four Petri dishes (60 × 15 mm) were set up for *D. mettleri*, *D. nigrospiracula*, and *D. mojavensis* larvae. Each dish contained 10 cc of dry *Drosophila* medium, 10 ml of one of the alkaloid solutions (0, 5×, 10×, or 15×), several grains of bakers' dry yeast, and 50 first-instar larvae. The number of eclosing adults was recorded daily until eclosion ceased. These data represent the viabilities of the larvae in media containing different alkaloid concentrations. Arcsine transformation was performed on the data before statistical analysis. Developmental rate was calculated as the average number of days required for eclosion for all flies eclosing from each concentration.

RESULTS AND DISCUSSION

Table 1 contains the data on alkaloid concentration in senita and saguaro tissue that have been reported in the literature and our determination of the average concentration present in field samples of saguaro-soaked soil. The concentration in soaked soils ranged from about 1.4 to 27 times the typical alkaloid concentration in fresh saguaro tissue (1% dry weight or 1.67 mg/ml). One of the three soil samples (concentration = 45.4 mg/ml) is probably close to an upper limit in alkaloid concentration since this sample was very tarry and contained only one *D. mettleri* larva. These results support the hypothesis that the

TABLE 1. ALKALOID CONCENTRATIONS (IN mg/ml OF AVAILABLE WATER) IN *Drosophila* SUBSTRATES

Substrate type	*N*	Average	Range	Reference
Senita tissue (mature stems)	12	10.7	6.5–20.5	Kircher (1969)
Senita tissue (young stems)	10	12.7	1.8–36.8	Kircher (1969)
Saguaro tissue (mature stems)	not reported		1.4–2.4	Kircher (1982)
Saguaro-soaked soils	3	17.8	2.4–45.4	

plant solutes are more concentrated in the soaked-soil substrate than in the plant itself. Furthermore, the range of alkaloid concentrations measured in saguaro soil is greater than and includes the entire range of values reported for senita.

The results of adult longevity experiments are given in Table 2. The parameter, LT_{50}, was obtained by averaging the three replicate vials for each alkaloid concentration. Although average longevity of both sexes of all three species decreased with increased alkaloid concentration in the medium, this trend was much more pronounced (and statistically significant) for *D. nigrospiracula* and *D. mojavensis*. The percent reduction in longevity (averaged over sex) for *D. nigrospiracula* and *D. mojavensis* with the 5× treatment was 56.7 and 48.9, respectively, as compared to only 91.4 for *D. mettleri*. *Drosophila nigrospiracula* was also the only species in which there was a difference in longevity between sexes. Males consistently exhibited greater longevity than females. In two-way analyses of variance for all three species, no significant interactions were detected for either sex by alkaloid concentration or species by alkaloid concentration. The ability of adults to tolerate saguaro alkaloids may be summarized by: *D. mettleri* > *D. mojavensis* > *D. nigrospiracula*.

The results presented above may be compared with those obtained by Kircher et al., (1967) and Fogleman et al. (1982) in their investigations of the effect of senita alkaloids on adult mortality. Kircher demonstrated that *D. mojavensis* and *D. nigrospiracula* were intolerant of senita alkaloids (or purified pilocereine) at 1% of the dry weight of the medium, while Fogleman and his co-workers revealed that *D. mettleri* was less affected than the resident species, *D. pachea*, by alkaloid concentrations up to 10% dry weight. Although these studies and the present investigation may be considered parallel and both saguaro and senita cacti contain isoquinoline alkaloids, it should be emphasized that senita's alkaloid composition is much more complex. The majority of the alkaloid fraction of senita is composed of the trimers, pilocereine and piloceredine, which are more toxic than the monomer (Kircher et al., 1967). The toxicity of lophocereine at levels above 1% dry weight is not known, but it is reasonable to expect that it would be directly comparable to that of the simple saguaro alkaloids.

The results of the investigation of the effect of saguaro alkaloids on larval viability are shown in Table 3. The statistical analyses indicate that, unlike adult longevity, larval viability of all three species is significantly affected by increasing concentrations of alkaloids in the medium. *Drosophila nigrospiracula* was, by far, the most affected since no adults eclosed from media containing alkaloid concentrations of 10× and 15×.

For *D. mettleri* and *D. mojavensis*, it is of interest to determine the alkaloid concentration at which they first suffered a significant reduction in viability. A Student-Newman-Keuls procedure, which is a multiple comparison test for differences between means (Sokal and Rohlf, 1969), showed that *D. mettleri* ex-

TABLE 2. EFFECT OF SAGUARO ALKALOID CONCENTRATION ON ADULT LONGEVITY[a]

Species	Replicates	Alkaloid Concentration[b]				Sex	Alk.
		0	5×	10×	15×	F_s	F_s
D. mettleri							
Males	3	19.7 ± 6.1	19.3 ± 3.7	15.7 ± 0.7	13.7 ± 4.6	0.158	0.843
Females	3	19.7 ± 1.5	16.7 ± 2.3	14.0 ± 2.5	13.0 ± 8.5		
D. nigrospiracula							
Males	3	35.3 ± 3.8	22.7 ± 3.8	18.0 ± 2.5	16.7 ± 3.8	35.730[c]	14.882[c]
Females	3	21.0 ± 1.0	10.3 ± 1.2	8.0 ± 1.7	7.3 ± 3.1		
D. mojavensis							
Males	3	17.3 ± 5.2	7.7 ± 1.7	8.7 ± 2.2	8.0 ± 3.0	0.143	4.296[d]
Females	3	16.3 ± 3.3	8.7 ± 3.7	6.3 ± 0.3	7.0 ± 3.0		

[a]The figures given are the average number of days until half the flies had died (LT_{50}) ± the standard error. Results of two-way ANOVAs (F_s for differences between males and females; F_s for differences between alkaloid concentrations) are also given.
[b]Times the concentration in fresh tissue (1.67 mg/ml).
[c]$P < 0.001$.
[d]$P < 0.05$.

TABLE 3. EFFECT OF SAGUARO ALKALOID CONCENTRATION ON AVERAGE PERCENT LARVAL VIABILITY (± SE)[a]

		Alkaloid concentration[b]				
Species	Replicate	0	5×	10×	15×	F_s
D. mettleri	3	50.0 ± 2.0	25.3 ± 8.2	10.7 ± 5.8	2.0 ± 2.0	10.186[c]
D. nigrospiracula	3	26.0 ± 2.0	2.0 ± 2.0	0.0	0.0	37.240[d]
D. mojavensis	3	50.7 ± 9.7	52.0 ± 11.7	34.7 ± 7.7	6.0 ± 3.1	7.641[c]

[a] Results of one-way ANOVA (F_s) on arcsine transformed data are also given.
[b] Times the concentration in fresh tissue (1.67 mg/ml).
[c] $P < 0.01$.
[d] $P < 0.001$.

perienced the viability decrease at the 5× concentration since all means were significantly different. The larval viability of *D. mojavensis* was only decreased in the 15× medium since the average viabilities in the 0, 5×, and 10× media were not significantly different.

The ability of *D. mojavensis* larvae to tolerate high concentrations of alkaloids was unexpected because this species does not utilize senita rots or saguaro-soaked soil, and its occasional utilization of saguaro rots would not expose it to alkaloid concentrations much greater than 1.67 mg/ml. The fact that the normal host plants of *D. mojavensis* contain toxic compounds, i.e., dihydroxy sterols and medium-chain fatty acids, provides a possible explanation of this phenomenon. Whatever mechanism, detoxification or exclusion, is responsible for the tolerance of *D. mojavensis* larvae to these compounds may also convey tolerance to the chemically simple saguaro alkaloids.

In previous studies on the toxicity of senita alkaloids to *Drosophila* larvae, the F_1 and F_2 generations of *D. mojavensis* were drastically reduced compared to controls when either senita cactus or medium containing 1% dry weight pilocereine was used (Kircher et al., 1967). Still, *D. mojavensis* produced more F_2 progeny under these conditions than most of the other eight species tested. Therefore, some tolerance to senita alkaloids has been demonstrated, but *D. mojavensis* larvae appear to be more tolerant of saguaro alkaloids than of senita alkaloids. Senita alkaloids in fly medium at 5% dry weight reduced the egg-to-adult viability of *D. nigrospiracula* to one third of the percent viability in control medium without alkaloids, while 10% alkaloids reduced the viability to zero (Fogleman et al., 1982). The viabilities of *D. mettleri* and *D. pachea* were not significantly reduced even in medium containing 10% senita alkaloids. Senita and saguaro alkaloids, then, have a similar effect on the larval viability of *D. nigrospiracula*. On the other hand, *D. mettleri* appears to be less affected by the alkaloids present in senita than by those in saguaro-soaked soils since the 5× treatment (Table 3) caused a significant reduction in larval viability.

TABLE 4. EFFECT OF SAGUARO ALKALOID CONCENTRATION ON LARVAL DEVELOPMENT TIME (AVERAGE NUMBER OF DAYS TO ECLOSION ± SE)[a]

	Alkaloid concentration[b]				
Species	0	5×	10×	15×	F_s
D. mettleri	18.6 ± 0.1	19.2 ± 0.2	19.2 ± 0.3	20.7 ± 0.3	5.891[c]
Number of flies	(75)	(38)	(16)	(3)	
D. nigrospiracula	15.2 ± 0.3	15.0 ± 0.0	—	—	—
Number of flies	(39)	(3)			
D. mojavensis	14.2 ± 0.1	14.6 ± 0.1	16.1 ± 0.1	16.4 ± 0.3	75.214[d]
Number of flies	(76)	(78)	(52)	(9)	

[a] Results of one-way ANOVA (F_s) are also given.
[b] Times the concentration in fresh tissue (1.67 mg/ml).
[c] $P < 0.01$.
[d] $P < 0.001$.

The average number of days to eclosion for each species in the different alkaloid concentration media are reported in Table 4. The data for *D. nigrospiracula* were not statistically analyzed due to the low number of flies that eclosed. Both *D. mettleri* and *D. mojavensis* showed a significant increase in development time with increasing concentration of alkaloids in the medium. Although the increase in development time for both species was approximately two days, the value of the F statistic for *D. mojavensis* was much greater than that for *D. mettleri*. This is probably due to the greater number of *D. mojavensis* adults which eclosed. The significance of the change in development time may be magnified as a consequence of the larger sample size. In comparison, a significant increase in the developmental rate of *D. mettleri* was not observed in response to senita alkaloids (Fogleman et al., 1982). The effect of senita alkaloids on the developmental rate of *D. nigrospiracula* was minor compared to the loss of larval viability, and their effect on *D. mojavensis* has not been tested.

In summary, the results show that saguaro alkaloids are, in fact, significantly more concentrated in samples of soaked soil than in the cactus tissue. The negative effect of elevated alkaloid concentration on fitness parameters of cactophilic *Drosophila* has also been demonstrated. The intolerance of *D. nigrospiracula* to elevated alkaloid concentration partly explains the inability of this species to utilize saguaro-soaked soil. Both *D. mettleri* and *D. mojavensis* are much more tolerant of high saguaro alkaloid concentration than *D. nigrospiracula*. The primary difference between *D. mettleri* and *D. mojavensis* with respect to saguaro alkaloids is the greater tolerance of *D. mettleri* adults. The viability of *D. mojavensis* larvae in saguaro-soaked soil has not been tested, but adults have never been reared from this substrate in nature. Other factors, such

as soil abrasiveness, tolerance to desiccation, or absence of chemicals associated with adult attraction, may also be involved in the exclusion of *D. mojavensis* from saguaro-soaked soil (Fogleman and Heed, 1987).

Little is known about the action of dietary alkaloids on insects (Fogleman et al., 1982). Suggestions on this subject include the idea that alkaloids may block steroid metabolism or phytosterol assimilation (Schreiber, 1958; Harley and Thorsteinson, 1967). The physiologic mechanism exhibited by *D. mettleri* and *D. mojavensis* which conveys tolerance to the toxic compounds in cacti is also unknown. However, a group of enzymes, collectively called mixed-function oxidases (MFOs), have well-established roles in the metabolism of insecticides and the protection of insect herbivores against chemical stress from secondary plant substances (Agosin and Perry, 1974; Brattsten et al., 1977). MFO activity in the midgut of southern armyworm moth larvae was induced to 270% that of control larvae when the *N*-heterocyclic compound, quinoline, was added to the diet at 0.1% (Brattsten et al., 1977). Other secondary plant compounds also induced MFO activity in this insect, but quinoline has the same chemical skeleton as both saguaro and senita alkaloids. A considerable amount of research has been recently reported on MFO activity in *Drosophila melanogaster*. In general, these reports demonstrate that MFO activity is induced by a variety of insecticides and mutagenic chemicals, most of which are nitrogen-containing compounds (Baars et al., 1980; Hällström et al., 1981; Hällström and Grafström, 1981; Zijlstra et al., 1984). MFO activity in this species of *Drosophila* may be genetically related to chemical resistance in that resistant strains have higher MFO activity than susceptible strains (Morton and Holwerda, 1985). In one study, the gene responsible for the MFO activity pattern in an insecticide-resistant strain of *D. melanogaster* was mapped near to, and thought to be identical with, a gene known to impart insecticide resistance (Hällström et al., 1982).

It is likely, therefore, that MFO enzymes are also involved in the ability of certain cactophilic *Drosophila* to tolerate the toxic compounds present in cacti. At present, our hypothesis is that *D. mettleri* and *D. mojavensis* have either a higher basal level of MFO activity than *D. nigrospiracula* or that MFO activity can be induced to higher levels in these species. Our results also lead us to expect a difference in MFO activity level between adults of *D. mettleri* and *D. mojavensis*.

The evolutionary history of the cactophilic *Drosophila* was recently reviewed by Heed and Mangan (1986). *Drosophila mettleri* is now thought to be a marginal isolate of a once widespread species. Many of the present-day adaptations of the four species associated with the Sonoran Desert probably were initiated along with the ecological shift of the region from thorn forest to existing desert conditions (Heed and Mangan, 1986). Unfortunately, it is not possible to determine when, in evolutionary time, *D. mettleri* developed the adaptations necessary to colonize soaked soil as a breeding substrate. The results

reported herein suggest that one adaptation that is necessary in order to utilize the saguaro-soil niche is the ability to tolerate toxic alkaloids. Given the chemical relatedness of saguaro and senita alkaloids, tolerance to the latter is probably derived from the same mechanism as tolerance to the former. The ecological significance of alkaloid tolerance, as previously mentioned, is that *D. mettleri* adults can and do use senita rots as alternate feeding substrates. This gives *D. mettleri* an ecological advantage over other, more restricted species. The observational rarity of senita-soaked soil in nature is an additional indication that the major advantage of the tolerance of *D. mettleri* to senita alkaloids is to the adult rather than the larval stage of the life cycle.

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PERSPECTIVES ON ALLELOPATHY IN MEXICAN TRADITIONAL AGROECOSYSTEMS: A Case Study in Tlaxcala

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Abstract—Agroecosystems in Tlaxcala, Mexico, are surrounded by trees and water channels and have a great variety of cultivated and noncultivated plants. The main results of a study carried out on a traditional agroecosystem in Santa Inés, Tlaxcala are presented. Some ecological aspects of polycultures, plant covers (dry leaves of *Alnus firmifolia*, *Berula erecta*, and *Juncus* sp.), and the allelopathic potential of crops and noncultivated plants (fresh and dry material) were analyzed. The main plants (trees, shrubs, and herbs) present in the agroecosystem were identified. The total number of weeds in plots where plant covers were added was reduced. The number of nodules of *Rhizobium phaseoli* and the production of bean and squash increased with plant covers. Corn, beans, and squash showed a clear allelopathic effect, as well as *Chenopodium murale, Tradescantia crassifolia, Melilotus indicus,* and *Amaranthus hybridus*, among other weeds. The contribution of allelopathy in studies of traditional agroecosystems is of great importance for the management of species in space and time. Allelopathy can be the basis of biological control of pests and weeds and of the discovery of new useful substances.

Key Words—Allelopathy, traditional agroecosystems, corn, bean, squash, *Alnus firmifolia, Berula erecta,* green manures, *Rhizobium phaseoli*, agroecology, Tlaxcala, "camellones."

INTRODUCTION

The need for an holistic view of the ecology of agroecosystems, especially of the traditional ones, is greater than ever, because the damage to natural re-

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sources and the demographic explosion endanger the future survival of humanity. The study of the traditional management of the land offers new alternatives to understand the structure and function of the ecological systems managed by man and to find appropriate technologies to get a sustainable production with minimum deterioration of the environment. The study of allelopathy, within this context, mainly contributes to the general management of the species in time and space, since it can be the basis for the biologic control of pests and weeds, and for the discovery of new drugs, herbicides, pesticides, and growth regulators (Farnsworth, 1977; Rice, 1984; Waller, 1982).

In Mexico (Figure 1), we find a traditional use of land in the deserts in the north; the "chinampas," "terrazas," and "camellones" in the center; and the corn fields and home gardens of the Mayas in the southeast. There exists much information related in some way to allelopathy, which should be carefully studied and applied to benefit the production of food, wood, and other raw materials.

In parts of Mexico, peasants deliberately permit the growth of some weeds in the crop fields. These are named "buen monte" in the southeast, as opposed to the bad weeds ("mal monte") that are commonly eliminated (Chacon and Gliessman, 1982). In certain shaded coffee plantations in Xalapa, Veracruz, some species of Commelinaceae from the herbaceous stratum are favored, cultivated, and managed by peasants together with the coffee crop, to protect the soil, to produce organic matter, to retain humidity, and to eliminate other weeds (Anaya et al., 1982; Ramos et al., 1983). In the chinampas of the Valley of Mexico, peasants use water hyacinth to improve the soil, because it can prevent the growth of some weeds, stop salt deposition by evaporation on the soil surface, and increase the organic matter content (Anaya et al., 1987).

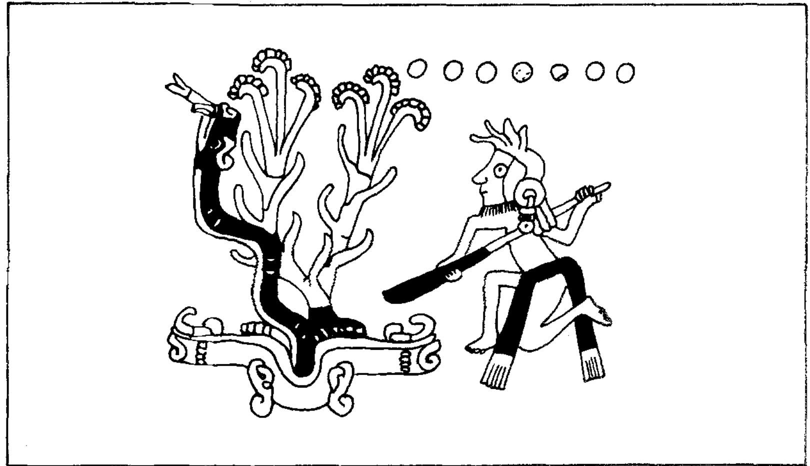

FIG. 1. Antique Nahua illustration representing the traditional cultivation of corn.

The traditional agroecosystems (camellones) in Tlaxcala, Mexico, include the management of trees, shrubs, and herbs that are useful in many ways for the local people. Fields are surrounded by water and trees, mainly *Alnus firmifolia* (Figure 2), and these constitute the main components in the agricultural landscape. Peasants cultivate a great variety of corn, bean, squash, oat, onion, lettuce, radish, barley, garlic, different spice herbs, lucerne and other forage plants; and fruit trees such as apple, appricot, peach, avocado, capulin, and tejocote (sloelike fruit), using different intercropping and rotation practices. They also use different plant and animal manures: for example, leaves of maguey, capulin, sabine, and alder or the decomposing organic matter from the leaves of plants accumulated in the water channels that surround some of the fields (González-Jácome, 1984, 1985).

Importantly, from an agricultural point of view, Tlaxcala is the most efficient and autosufficient state, as the consumption of fertilizers, pesticides, and herbicides is the lowest in Mexico (Dr. José Nieto de Pascual, personal communication).

In recent years, there have been studies of mixed-crop systems with the specific intention of looking for the effects of allelopathic compounds actively being added to the environment by the different members of the mixture (Ama-

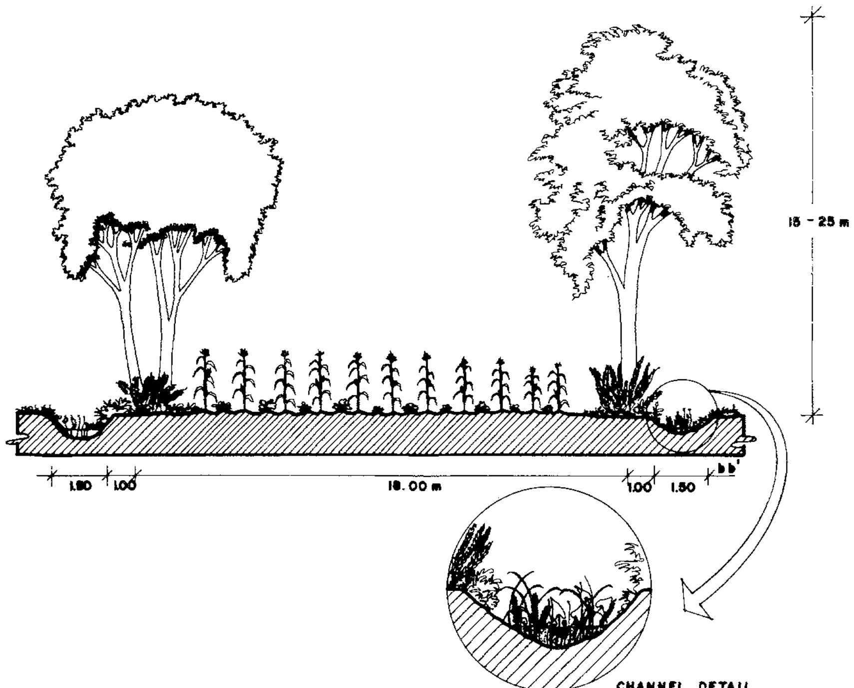

FIG. 2. Transverse view of a camellón in Santa Inés Tecoexcomac, Tlaxcala, México.

dor and Gliessman, 1982; Jiménez-Osornio et al., 1983; Dzyubenko and Petrenko, 1971). In order to better understand the way in which cultivated and wild plants in a traditional agroecosystem (camellón) of Tlaxcala interact with their neighbors, this study analyzes the use of some of the cultural practices of peasants in this zone, like the use of native selected seeds, the combination of different crops and varieties, the different purposes of the use of wild and cultivated trees and shrubs along the border of the camellones, the use of water deposits where aquatic plants and organic matter are accumulated, the rotation of crops, the control of weeds, the use of natural manures, the use of noncrop plants as fodder, food, or medicines. In relation to the use of green manures or plant covers, we studied the use of two different kinds: leaves of *Alnus firmifolia*, *Berula erecta* and *Juncus* sp. and their effect on the growth of weeds, and *Rhizobium* sp. inoculations. We studied also the results of different bioassays designed to detect the allelopathic potential of crops and noncultivated plants.

METHODS AND MATERIALS

Field Experiment. A field experiment was carried out in Santa Inés Tecoexcomac, Tlaxcala, Mexico, during 1985. This place is located in a valley between the Atoyac and the Zahuapan rivers, with a temperate, subhumid climate. Rainfall is concentrated during the period from May to October, with an annual average of 1000 mm, and into an irrigated agricultural zone, where the traditional cultivation plots are called camellones. These are strips of land surrounded on two or three sides by water channels, similar to the chinampas.

A typical camellón of 273 × 18 m, cultivated for the last three years with three varieties of corn, was selected. Some years before it had been cultivated in rotation with different kinds of beans, lucerne, and squash. The three varieties of native corn usually sowed in here are: spotted corn, red corn, and blue corn. The former is the most productive and the "slowest" of these, as it matures one or more months later than blue or red corn and it is used commonly to make tortillas. Red corn is used to make atole (hot drink made with milled dried corn and milk) and tamales (cooked corn dough with meat or fruit inside a dry corn leaf) because it is very sweet. Blue corn is also used to make tortillas, and its special flavor is appreciated by all the people.

Soil samples were collected at 0–40 cm for chemical and physical analysis. Plowing and sowing were accomplished in the traditional way with animal traction and manual work. The following crops were placed in a complete randomized block design: (1) native spotted corn in monoculture, (2) native spotted corn plus "canary" beans, (3) native spotted corn plus Italian squash.

Corn was sowed on April 30 by making a hole every 90 cm along the furrow with a shovel and putting four seeds in it. Beans and squash were sowed on May 15 in the same way, next to the furrows of corn. Three treatments with

four replications were applied to each culture: (1) control (without plant cover), (2) cover with *Alnus* leaves, and (3) cover with *Berula erecta* and *Juncus* sp. leaves.

Each plot was 3 × 21 m in size and had three longitudinal furrows, where 50 kg of leaves were spread during the first week of June and 50 kg more during the first week of July. The weeds in the different plots were identified and quantified on June 30, August 31, and September 30. The plots were weeded the last week of July. Squash was harvested in August and beans in September. Corn was cut in the last days of September and left drying in the field until October, when it was harvested. The total number of weeds was analyzed as a split–split plot design. Corn, bean, and squash production data were analyzed as a randomized complete block design.

Experiment with Rhizobium *in the field.* A plot 18 × 30 m was selected and corn was interplanted with canary bean. Before sowing, beans were divided into two groups: one group was inoculated with a mixture of three strains of *Rhizobium phaseoli* (C MexI, C MexII, and C Mex 22); the other group was not inoculated.

Following a randomized complete block design, four treatments with three replications were applied to each group: (1) cover with *Alnus* leaves; (2) cover with *Berula* and *Juncus* leaves; (3) without cover, weeding the plot; and (4) without cover, without weeding.

Each plot was 3 × 4 m in size. Corn was sowed on April 30, and beans were sowed every 50 cm next to the furrows of corn on June 15. The treatments were applied twice, on July 1 and August 5, spreading 30 kg of leaves uniformly along the furrows of corn and bean in each plot.

To quantify the number of nodules of *Rhizobium* in the roots, in each treatment two samples of bean plants were collected 30 and 60 days after sowing. The results were analyzed by analysis of variance with two factors.

Laboratory Bioassays. Four kinds of bioassays were carried out to detect the allelopathic potential of crops and weeds, testing leachates of fresh leaves, roots, and dry leaves as shown in Table 1. Aqueous leachates of 10 g of aerial parts and roots of fresh plants and 1 g of dry leaves were used, with the osmotic pressure being measured and controlled. Ten milliliters of each leachate were mixed with 10 ml of agar (2%) in Petri dishes where seeds were sowed. Controls were made with agar (1%). Petri dishes were placed in the dark at 25°C. All experiments included three replications. At the end of each experiment, the radicle length of the seedlings was measured. All the results were analyzed as a complete randomized block design.

Seeds of the following species were tested: cultivated seeds—native spotted corn, Italian squash, and canary bean; weed seeds—*Amaranthus hybridus, Plantago major, Hordeum vulgare, Echinochloa crusgalli, Portulaca oleracea, Amaranthus leucocarpus*, and *Brassica campestris*.

TABLE 1.

Leachates	vs.	Test seeds
Wild plants		Weed seeds
Wild plants		Cultivated seeds
Cultivated plants		Weed seeds
Cultivated plants		Cultivated seeds

RESULTS AND DISCUSSION

Field Experiments. Soil analysis showed that the plot is characterized by a slimy sand soil with an alkaline pH (8.3) and a low electrical conductivity (0.57 mmho/cm), without salinity problems. The plot is fertilized with small quantities of cow manure once a year, before sowing the crops, so it is fairly rich in organic matter (2.3%), nitrogen (0.15%), and phosphorus (14 kg/hectare); this soil has a TICC[1] of 19.5 meq[2]/100 g. Ca^{2+}, Mg^{2+}, and K^+ are the most abundant cations, characteristic of calcareous soils.

Table 2 lists the main species and families of weeds found in the plots. The Compositae was the most abundant family; the species *Bidens aurea, Melampodium perforatum, Galinsoga parviflora,* and *Simsia amplexicaulis* were very conspicuous in all the plots. These shared their dominance mainly with Chenopodiaceae (*Chenopodium album* and *Ch. murale*), Amaranthaceae

TABLE 2. LIST OF FAMILIES AND WEEDS IN THE PLOTS

Compositae	*Bidens aurea, Melampodium perforatum, Simsia amplexicaulis,* and seven more species
Amaranthaceae	*Amaranthus hybridus*
Chenopodiaceae	*Chenopodium album, Ch. murale*
Convolvulaceae	*Ipomea* sp.
Cucurbitaceae	*Sicyos deppei*
Leguminosae	*Melilotus indicus, Medicago lenticulata, Dalea leporina*
Polygonaceae	*Polygonum aviculare*
Commelinaceae	*Tradescantia crassifolia, Commelina erecta*
Oxalidaceae	*Oxalis* sp.
Solanaceae	*Physalis* sp., *Solanum nigrum*
Labiatae	*Salvia tiliaefolia, Marrubium* sp.
Onagraceae	*Lopezia racemosa*
Cruciferae	*Brassica camperstris, Raphanus raphanistrum, Lepidium* sp.
Portulacaceae	*Portulaca oleracea*
Cyperaceae	*Cyperus* sp.

[1]TICC = total interchange cations capacity.
[2]meq = milliequivalent.

(*Amaranthus hybridus*), Commelinaceae (*Tradescantia crassifolia*), Cucurbitaceae (*Sicyos deppei*), Convolvulaceae (*Ipomea* sp.), Leguminosae (*Melilotus indicus* and *M. lenticulata*), and Solanaceae (*Physalis* sp. and *Solanum nigrum*).

On the border of camellones under the shade of the trees, the herb stratum was dominated by Gramineae and the shrub stratum by *Baccharis glutinosa* (jarilla) (Figure 3). The latter is cut in winter and used by peasants as firewood. In the water channels, the most abundant aquatic plant is *Berula erecta* (Umbelliferae) and various species of *Juncus, Lemna minor, Hydrocotile* sp., and *Typha* sp., among others.

The total numbers of weeds in the plots when they were quantified on June 30, August 31, and September 30 (hereinafter termed "times") were significantly different between times (Table 3), since the entire parcel was hand weeded at the end of July (Figure 4). However, the interaction between times and managements (treatments) was significantly different (Table 3). In June, August, and September, the number of weeds in the plots with *Alnus* and *Berula* and *Juncus* was significantly reduced compared with the number of weeds in plots without cover. Also, the total number of weeds in plots with *Berula* and *Juncus* cover was significantly smaller than with *Alnus* cover. This suggests that the decomposition of both kinds of cover (especially *Berula* and *Juncus*) may release some allelopathic compounds that contribute to the control of weeds.

The total number of weeds in the corn–squash plots was significantly smaller than in the corn–bean and monoculture corn plots (Figure 4). This is due probably to the allelopathic potential of squash observed in many kinds of agroecosystems (Chacon and Gliessman, 1982).

The interaction between crops and managements (Table 4) was also significant; the total number of weeds in the three crops with plant cover was lower than in the plots without cover. *Alnus* and especially *Berula* and *Juncus* contributed to reduce the growth of weeds in all plots.

The production of corn was similar in the three treatments whether in monoculture or in polyculture (Table 5). The production of beans was significantly higher in the plots with both covers and that of squash was higher only in the *Berula* and *Juncus* treatment. It is observed that this kind of cover had the most inhibitory effect on the growth of weeds, but, on the other hand, it was the only one that improved squash production. Finally, squash seemed to be the only crop with an inhibitory effect on weed growth in this field experiment.

It is necessary to conduct more studies during the annual cycles to determine which are the soil properties that change with the incorporation of plant covers and improve bean and squash production, and to detect if there are some stimulating compounds released into the soil through the decomposition of plant covers. On the other hand, it is a fact that peasants receive many benefits using polyculture, because they obtain a diversified production in the same time and space.

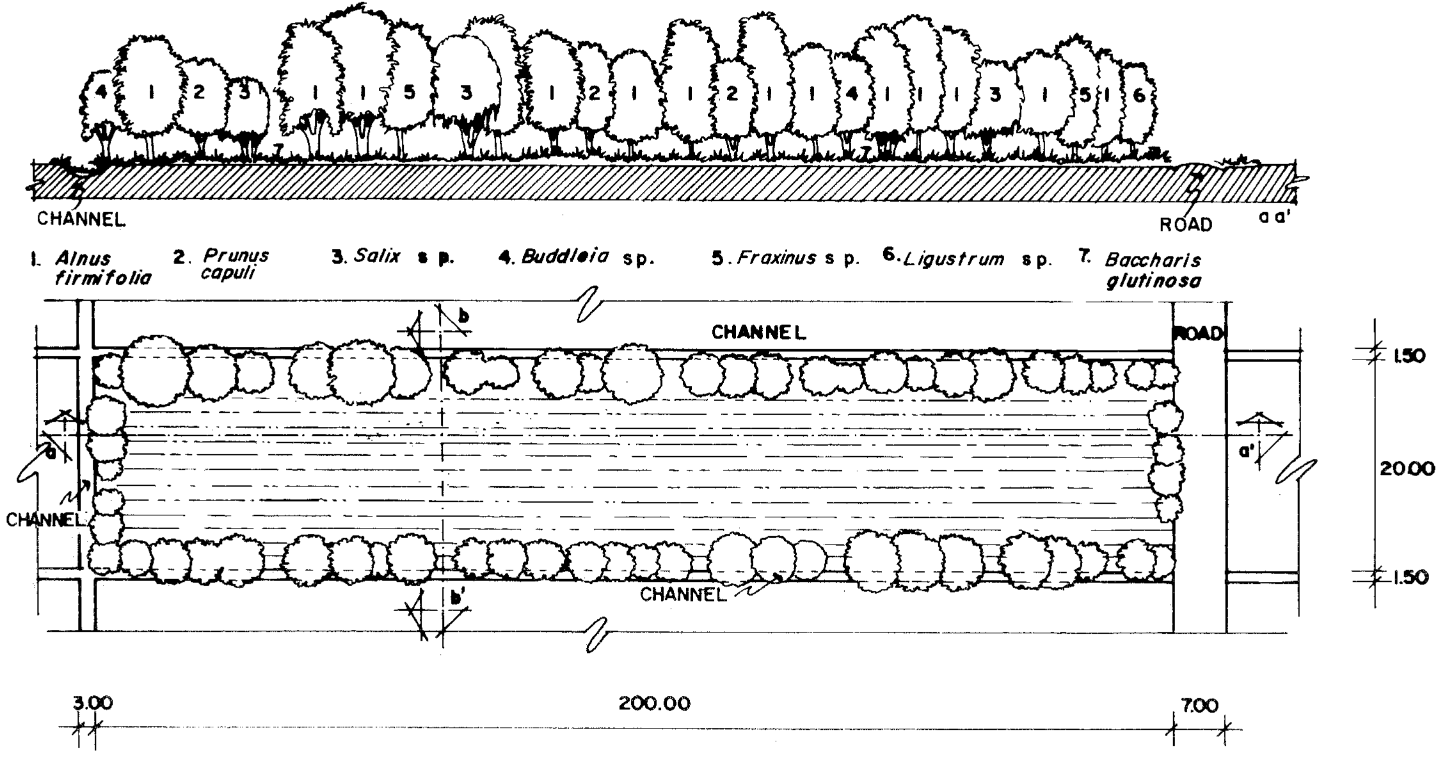

FIG. 3. Profile and scheme of the camellón, showing the main species in the border.

TABLE 3. NUMBER OF WEEDS: WHEN COUNTED AND UNDER DIFFERENT TREATMENTS (MANAGEMENTS)

Time	Management			Total
	Without cover	*Alnus*	*Berula* and *Juncus*	
June 30	3202	2996	2166	8364[a]
August 31	1291	963	1003	3257
September 30	1052	637	887	2576
Total	5545	4596[a]	4056[a]	

[a] Significant at 1% level (*F* test).

Experiment with Rhizobium. The numbers of nodules on the roots of beans were significantly different between inoculated and noninoculated beans at 60 days after they were sowed (Table 6). There is also a significant difference within each management. Both covers (*Alnus* and *Berula*) caused an increase of the number of nodules, especially *Berula* and *Juncus*. In the plots without weeding, the number of nodules in inoculated beans was also increased. Pena-Cabriales and Alexander (1983), suggested that appreciable *Rhizobium* growth in unamended soil occurs only in the presence of germinating seeds, growing roots, and decomposing nodules. In this case, it is possible that the presence of a great variety of compounds in the rich rhizosphere of these plots can be a factor that increases the development of *Rhizobium*. It is also possible that these compounds are inhibitory to other microorganisms or even green plants, because the effects are different depending on the receptor organism. This is a general rule in allelopathy.

Nevertheless, in the case of camellones, there exist various nitrogen-fixing microorganisms in the different species of cultivated and noncultivated plants whose dynamics are determined to a great degree by the special management the peasants give to the agroecosystems. Winter crops are preferentially legumes such as lucerne and broad bean. Several nitrogen-supplying species of noncultivated plants exist in the fields of Tlaxcala where *Alnus* is one of the most important ones. Through its root nodules, it releases significant amounts of nitrogen to the soil. This fact has been mentioned by many researchers on other species of *Alnus* (Atkinson and Hamilton, 1978; Berg and Doerksen, 1975). For this reason *Alnus* is an important element in the agroecosystem, but it is also a tree of increasing value for its wood products. Moreover, in the summer, *Macrodactylus* sp. (frailecillo), one of the main insect pests of corn and other cultivars, is trapped by the leaves of *Alnus* and of *Baccharis glutinosa* (jarilla), located at the borders of the plots, preventing its attack on the crops. Farrell et al. (1987) mentioned the same beneficial role of other "trap plants,"

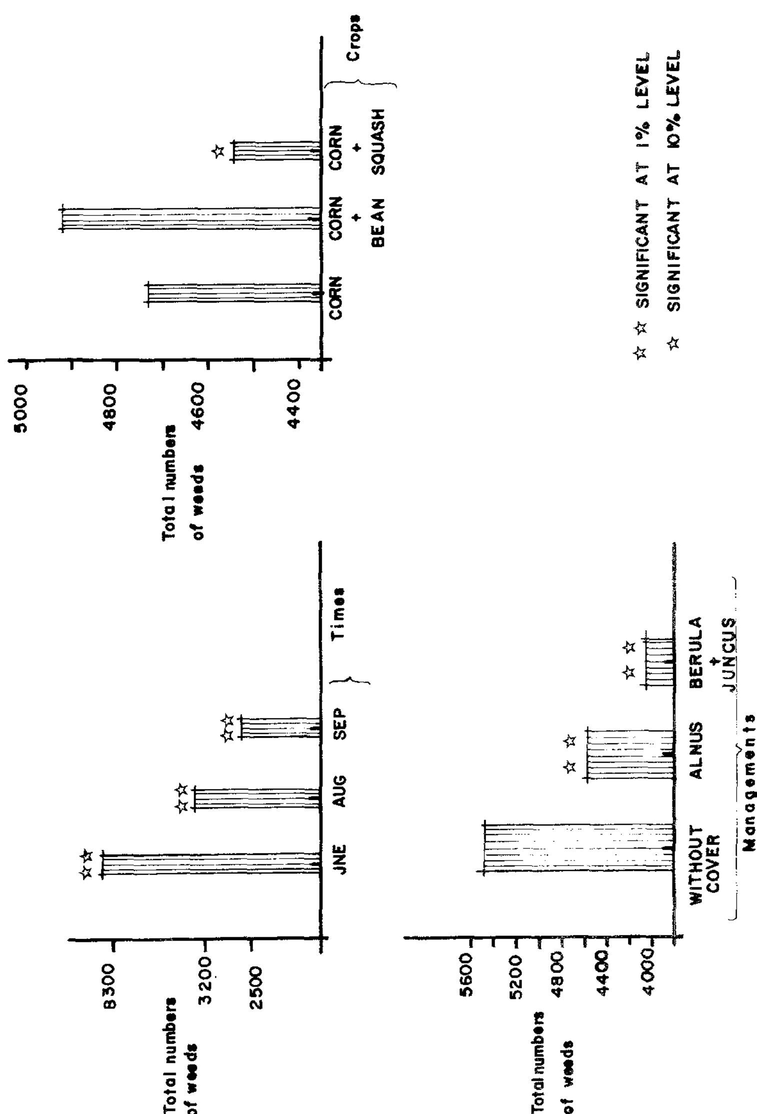

FIG. 4. Total number of weeds in times (months), crops, and managements.

TABLE 4. NUMBER OF WEEDS WITH CROPS UNDER DIFFERENT MANAGEMENT

Crop	Management			Total
	Without cover	*Alnus*	*Berula* and *Juncus*	
Corn	1782	1723	1215	4720
Corn–bean	1976	1527	1430	4933
Corn–squash	1787	1346	1411	4544[a]
Total	5545	4596[b]	4056[b]	

[a] Significant at 10% level (*F* test).
[b] Significant at 1% level (*F* test).

TABLE 5. PRODUCTION (kg/m^2, DRY WEIGHT) OF CROPS UNDER DIFFERENT TREATMENTS

Treatment	Crop			Total
	Corn	Bean	Squash	
Without cover	198	1.7	11.9	211.6
Alnus	203	2.6[a]	14.8	220.4
Berula and *Juncus*	202	2.7[a]	25.5[b]	230.2
Total	603	7.0	52.5	

[a] Significant at 5% level (*F* test).
[b] Significant at 10% level (*F* test).

TABLE 6. NUMBER OF ROOT NODULES ON BEANS INOCULATED WITH *Rhizobium*

Managements	Number of nodules					
	Beans inoculated		Beans not inoculated		Total	
	30 days	60 days	30 days	60 days	30 days	60 days
Without weeding	133	134[a]	119	72[a]	252	206[a]
Weeding	87	68[a]	112	78[a]	199	146[a]
Alnus	173	180[a]	131	111[a]	304	291[a]
Berula and *Juncus*	109	273[a]	96	96[a]	205	369[a]
Total	502	655[a]	458	357[a]	960	1012[a]

[a] Significant at 1% level (*F* test).

such as *Lupinus* spp. and *Brassica campestris*. *Alnus*, on the other hand, is a soil builder, because its litter production constantly adds organic matter to soils; the soil can be covered in some periods of the year, especially in winter, with a great amount of leaves of this tree. This fact is important for good development of *Rhizobium* (Table 6) and also for the control of some weeds such as *Brassica*, *Amaranthus*, *Echinochloa*, and *Portulaca* (Tables 3 and 7). This fact points out again the various responses of the receptor organisms to the allelochemicals. Some isoflavonoids, well known as phytoalexins, do not have inhibitory effects on *Rhizobium phaseoli*, as shown by Pankhurst and Biggs (1980).

Laboratory Bioassays. The detection of the allelopathic potential of leachates carried out for almost all the weeds present in the plots. Because of the great number of bioassays and results, we include only those where the results were significant (*F* test at the 1% level). The leachates of fresh and dry leaves and roots of *Alnus* and the leaves of *Berula* and *Juncus* did not have any significant effect on the growth of corn, squash, and bean, as is shown in Table 7. The roots of *Alnus* stimulated one of the crops, squash, and inhibited weed growth. Dried leaves were more inhibitory than fresh ones.

Table 8 shows the effects of the leachates of fresh weeds on the radicle growth of corn, bean, and squash. *Bidens aurea, Melilotus indicus, Simsia amplexicaulis, Chenopodium murale, Ipomea* sp., *Melampodium perforatum, Cyperus* sp., and *Portulaca oleracea* produced high inhibition of radicle growth of test species. Leachates from dried weeds produced the greatest radicle growth inhibition, not only on the crop species but also upon other weeds (Table 9). *Chenopodium murale, Ipomea* sp., *Sicyos deppei, Lopezia racemosa, Simsia amplexicaulis, Physalis* sp., *Melilotus indicus, Amaranthus hybridus, Brassica campestris*, and *Portulaca oleracea*, among others, produced more than 50% inhibition. Beans and squash were highly affected by the leachates. *Sicyos deppei*, which is considered by peasants as one of the worst weeds, was one of the most inhibiting species to corn (73%), followed by *Lopezia racemosa* (74%). *Chenopodium album* and *Melilotus indicus* also markedly inhibited the growth of corn (65% and 68%).

The weed species most affected by leachates from dry material were: *Brassica campestris, Portulaca oleracea, Echinochloa crusgalli,* and *Amaranthus hybridus*. Only *Brassica campestris, Tradescantia crassifolia, Chenopodium murale*, and *Oxalis* sp. inhibited *Hordeum*. It is important to note that the most abundant weeds in the plots were the ones which produced the greatest inhibitions. *Sicyos deppei* and *Physalis* sp. (green tomato) greatly inhibited bean and squash. *Physalis* sp. is a cultivated species, but in the crop field it was found in its wild state. Probably *Physalis* sp. cannot be interplanted with bean, squash, and corn because of its allelopathic effect on these three crops.

TABLE 7. PERCENT RADICLE GROWTH INHIBITION (STIMULATION) OF TEST SPECIES BY VARIOUS LEACHATES[a]

	Test species								
	Crops			Weed					
Leachates	Corn	Bean	Squash	*Brassica campestris*	*Portulaca oleracea*	*Hordeum vulgare*	*Echinochloa crusgalli*	*Amaranthus leucocarpus*	*Plantago major*
Fresh									
Leaves of *Alnus*	NS	NS	NS	72	NS	NS	21	14	NS
Roots of *Alnus*	NS	NS	(48)	NS	NS	NS	27	(20)	(32)
Leaves of *Berula* and *Juncus*	NS	NS	NS	28	NS	NS	NS	NS	(34)
Dry									
Leaves of *Alnus*	NS	NS	NS	29	48	(21)	21	50	NS
Leaves of *Berula* and *Juncus*	NS	NS	NS	41	47	25	39	61	24

[a] All numbers are significant at the 1% level (*F* test). The numbers in parenthesis are stimulations. NS, nonsignificant effect.

TABLE 8. PERCENT INHIBITION OF CORN, BEANS, AND SQUASH BY LEACHATES OF FRESH WEEDS[a]

Test species

	Bidens aurea	*Bidens odorata*	*Ambrosia cumanensis*	*Galinsoga parviflora*	*Simsia amplexicaulis*	*Melampodium perforatum*	*Chenopodium album*	*Chenopodium murale*	*Ipomea* sp.	*Sicyos deppei*	*Melilotus indicus*
Corn	42	21	NS	NS	31	21	NS	33	30	NS	39
Beans	30	29	25	NS	32	NS	NS	33	22	NS	NS
Squash	29	23	NS	NS	23	40	22	NS	34	30	23

	Medicago lenticulata	*Tradescantia crassifolia*	*Cyperus* sp.	*Physalis* sp.	*Acalypha* sp.	*Lopezia racemosa*	*Brassica campestris*	*Oxalis* sp.	*Salvia tiliaefolia*	*Amaranthus hybridus*	*Portulaca oleracea*
Corn	NS	30	NS	NS	NS	NS	NS	NS	26	NS	NS
Beans	NS	NS	NS	NS	NS	33	NS	27	NS	NS	NS
Squash	NS	34	38	NS	NS	20	NS	NS	NS	27	29

[a] All numbers are significant at 1% level (F test). NS, nonsignificant effect.

TBLE 9. PERCENT INHIBITION OF RADICLE GROWTH OF CROPS AND WEEDS BY LEACHATES OF DRIED WEEDS[a]

	Test Species																		
	Bidens aurea	*Bidens odorata*	*Galinsoga parviflora*	*Simsia amplexicaulis*	*Melampodium perforatum*	*Chenopodium album*	*Chenopodium murale*	*Amaranthus hybridus*	*Ipomea* sp.	*Sicyos deppei*	*Melilotus indicus*	*Medicago lenticulata*	*Tradescantia crassifolia*	*Physalis* sp.	*Lopezia racemosa*	*Brassica campestris*	*Portulaca oleracea*	*Oxalis* sp.	*Cyperus* sp.
Crop																			
Corn	NS	NS	23	NS	NS	65	46	57	50	73	68	NS	21	26	74	49	44	46	NS
Bean	48	NS	48	69	62	57	38	39	57	62	NS	NS	52	71	40	41	54	30	35
Squash	43	NS	43	51	46	55	57	40	59	68	NS	NS	48	68	54	24	57	33	42
Weed																			
Brassica campestris	50	NS	50	80	32	78	74	69	80	80	86	47	46	56	75	81	85	34	27
Portulaca oleracea	—	27	36	—	45	—	82	—	29	83	53	35	43	73	NS	85	64	85	27
Hordeum vulgare	—	NS	NS	—	NS	—	47	—	NS	NS	NS	NS	NS	20	NS	68	NS	44	NS
Echinochloa crusgalli	—	46	34	—	41	—	69	—	23	NS	57	NS	41	56	NS	95	67	75	NS
Amaranthus leucocarpus	—	NS	59	—	35	—	81	—	NS	66	71	59	68	55	61	81	38	60	20
Plantago major	—	32	33	—	NS	—	NS	—	NS	NS	NS	27	28	NS	39	NS	61	35	NS

[a] All numbers are significant at 1% level (F test). NS, nonsignificant effect.

Some of the weeds present in the plots and tested for their allelopathic potential had already been reported as allelopathic: *Ambrosia cumanensis, Portulaca oleracea, Chenopodium album, Cyperus* sp., *Amaranthus* sp., *Brassica campestris, Melilotus indicus,* and *Medicago* sp. (Anaya and del Amo, 1978; Rice, 1984).

The leachates of fresh leaves of cultivated plants had no inhibitory effect on the growth of corn; however, the root of beans stimulated its growth 31% (Table 10). All the leachates of dry leaves caused an inhibitory effect, not only on corn but also on bean and squash. Leachates of dried crops (Table 10) produced a stronger inhibitory effect on weed growth than leachates from fresh leaves. In general, *P. oleracea* and *E. crusgalli* were the species most affected by the leachates of crops. *Hordeum vulgare* was the only species not affected by the leachates of crops, either fresh or dry.

In nature, the allelopathic interaction between crops and weeds is carried out as a dynamic active liberation process by aerial parts or roots and by decomposition, especially of dead leaves. The allelopathic substances can inhibit and stimulate other organisms in the environment, and the specific knowledge of their effects could allow us to interplant some wild plants with crops, to use a particular plant cover, to fertilize the soil with dried weeds or crops, and to mix two or more crops, among other managements. Pronin and Yakovlev reported in 1970 (stated by Gliessman, 1985) that yields of corn and fodder beans increased in mixed plantations and that this is associated with a favorable increase of root excretions of each plant on the other, rather than merely an improvement of nitrogen nutrition by the legume. Gliessman (1983) reported the use of *Stizolobium deeringeanum* planted in rotation with corn in Tabasco, Mexico, to control weed growth. The interplanting of squash (*Cucurbita pepo*) in corn–bean polycultures by peasants in southeastern Mexico helps to control weeds due to the combined effects of shade and phytotoxins (Amador and Gliessman, 1982).

In another study, Jiménez-Osornio and Schultz (1981) found that corn in monoculture in unweeded plots produced 20% less than corn interplanted with bottle gourd also in unweeded plots. This suggests that the allelopathic potential of this member of the Cucurbitaceae can interfere with weeds and permit a better growth of corn. The biomass and density of *Polygonum* sp., one of the main weeds of the zone of this study, was significantly reduced in the plots where bottle gourd was planted. They demonstrated the important influence that cultivated plants have on weeds, especially corn. Dzyubenko and Petrenko (1971) demonstrated that root excretions of maize inhibit the growth of some weeds such as *Amaranthus retroflexus*. Jiménez-Osornio et al. (1983) found that corn pollen severely affects the growth of some weeds.

If we gain knowledge about the ecological and cultural fundamentals of the traditional management of the land, we can make sure that a sustainable

TABLE 10. PERCENT INHIBITION (STIMULATION) OF RADICLE GROWTH BY LEACHATES OF PLANT PARTS AND CROP PLANTS[a]

Leachates species	Test species								
	Corn	Bean	Squash	*Brassica campestris*	*Portulaca oleracea*	*Hordeum vulgare*	*Echinochloa crusgalli*	*Amaranthus leucocarpus*	*Plantago major*
Fresh:									
Corn									
Aerial part	NS	NS	NS	NS	37	NS	34	NS	NS
Root	NS	NS	NS	NS	21	NS	28	23	41
Bean									
Aerial part	NS	20	26	43	33	NS	25	23	34
Root	(31)	NS	NS	NS	28	NS	NS	NS	NS
Squash									
Aerial part	NS	22	20	NS	35	NS	30	29	22
Root	NS	(27)	23	NS	30	NS	26	30	43
Dry									
Corn	42	36	47	73	23	NS	29	NS	(29)
Bean	73	57	64	82	24	NS	34	49	NS
Squash	51	43	64	88	43	NS	44	70	NS

[a] All numbers are significant to 1% level (F test). NS, nonsignificant.

agriculture suitable to the needs and particular resources of the people of any locality will be available. Also we can improve this management by combining appropriately new technologies with the old ones, introducing more varieties of crops, and increasing the use of the noncrop plants as organic matter producers, nitrogen or soil fixers, insect or weed controllers, food or drug producers, etc. All these activities open new alternatives to improve the quality of life of peasants and country people, and help to conserve the germplasm of our different geographic areas.

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ANALYSIS OF PEACH BARK VOLATILES AND THEIR ELECTROANTENNOGRAM ACTIVITY WITH LESSER PEACHTREE BORER, *Synanthedon pictipes* (GROTE AND ROBINSON)[1,2]

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Abstract—Bark volatiles from two peach cultivars (Bisco and Redskin) were obtained by vacuum steam distillation and fractionated by preparative gas chromatography. The fractions were then assayed with the electroantennogram (EAG) method on antennae of female lesser peachtree borer [*Synanthedon pictipes* (Grote and Robinson)]. With both cultivars, two fractions elicited the largest responses. Analysis of this material by GC-MS revealed a complex mixture made up of aromatic alcohols, esters, ketones, and acids, as well as phenols, aliphatic aldehydes, and aliphatic acids. EAG responses to pure samples of all identified components were recorded, and many of these compounds were found to be quite active. Among the most stimulatory were guaiacol, methyl benzoate, and 1-phenyl-1,2-propanedione. Also tested were six-carbon aliphatic aldehydes and alcohols which are components of the foliar tissue of most plants. Of these, 1-hexanol showed moderate activity, while the aldehydes and unsaturated alcohols were only weakly active.

Key Words—*Synanthedon pictipes*, Lepidoptera, Sesiidae, host volatiles, electroantennogram, olfaction, oviposition, benzaldehyde, methyl benzoate, guaiacol, phenol.

INTRODUCTION

The lesser peachtree borer, *Synanthedon pictipes* (Grote and Robinson) feeds as a larva under the bark on the limbs and trunk of its host. This predation

[1] Lepidoptera: Sesiidae.
[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.
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causes severe damage, which may result in the girdling and death of the limb. Numerous studies have dealt with the female-produced pheromones and associated male behavior of this and related sesiids (Tumlinson et al., 1974). However, few of the details of the postmating ovipositional behavior of females are known.

Female *S. pictipes* oviposit in crevices in the bark, particularly around wounds and cankers. Investigations with the congeneric peachtree borer [*S. exitosa* (Say)] indicate that nonvolatile substances found in peach bark promote oviposition after arrival at a potential site (Gentry and Wells, 1982). In an early study (Snapp and Swingle, 1929), several volatile materials, including a steam distillate of peach bark, were found to attract *S. pictipes* (sex not specified) to field traps. In the present study, the composition of the peach bark volatile mixture is determined and the influence of certain constituent compounds on the olfactory system of female *S. pictipes* is evaluated.

METHODS AND MATERIALS

Steam Distillation of Bark. Bark was removed from the Bisco and Redskin cultivars of peach and ground to a powder in a Wiley mill. The ground bark was added (300 g/distillation) to a 5.0-liter flask containing 3.0 liter of distilled water. A slight flow of nitrogen was bled into the flask during the course of the distillation. The mixture was distilled under reduced pressure at 40–50°C until ca. 1.6 liter of distillate was collected in a cooled 2.0-liter flask. A dry ice-cooled trap was placed in the system to prevent any volatile material from escaping the distillate. The aqueous distillate and cold-trap contents were combined and extracted with diethyl ether for 16 hr in a continuous liquid–liquid extractor.

The ether extracts were then passed through anhydrous sodium sulfate and concentrated to a volume of 20 ml by distillation through a Vigreux column. Samples were further concentrated to a volume of 1.0 ml under a gentle stream of nitrogen.

Fractionation and Electroantennography. All samples of steam distillate were fractionated by preparative GC on a 1.8-m × 4.0-mm ID glass column packed with 3% OV-17 on Gas-Chrom Q (60/80). A glass-lined splitter directed 10% of the effluent to a hydrogen flame-ionization detector, while the remaining 90% was directed to a thermal gradient fraction collector cooled by a Dry Ice–methanol bath (Brownlee and Silverstein, 1968). The column temperature was programmed from 40 to 200°C at 8°C/min with a 1-min hold at 40°C, and fractions were collected in 305-mm capillary tubes which could be flame sealed after collection or immediately rinsed out with a small volume of diethyl ether. The carrier gas (helium) flow rate at 40°C was 20 ml/min, and the injector and detector temperatures were 230°C.

Electroantennogram (EAG) signals were amplified 100-fold with a Grass P-18 DC preamplifier and recorded on a Fisher Recordall chart recorder (Andersen and Wilkin, 1986). The insects used were laboratory reared by the method of Reed and Tromley (1985), and females could be stored for up to five days in a refrigerator in Petri dishes lined with moist filter paper. A female antenna was prepared by removal of the distal few segments, followed by excision of the antenna at its base. It could then be mounted between two glass capillary tubes containing 0.7% aqueous NaCl. Silver–silver chloride wires were inserted into each capillary and connected to the active or ground inputs of the preamplifier. The active electrode was shielded with aluminum foil as described by Roelofs (1977). Fractions were assayed by breaking the ends of a sealed capillary and injecting 0.4 ml of air through the tube and into a filtered airstream (100 ml/min) which flowed over the antenna (Roelofs, 1977).

GC-MS and Verification of Structure. Electron impact mass spectrometry (70 eV) was carried out on a Kratos MS-30 instrument coupled with a gas chromatograph. Fused silica capillary columns (J & W Scientific, Inc.) coated with DB-1701 (25 m × 0.32 mm) and DB-Wax (30 m × 0.32 mm) were used for analysis. When necessary, proton NMR data were obtained from isolated compounds in $CDCl_3$ by means of a Bruker WM-300 spectrometer.

Identifications were made by comparison of sample mass spectra with authentic reference spectra and structural verification was accomplished by comparing GLC retention data of unknowns with those of authentic standards on the columns described above. The temperature program for the DB-1701 column consisted of a 5-min hold at 40°C followed by an increase of 6°C/min to a temperature of 200°C. For DB-Wax, an initial hold at 40°C for 1 min was followed by an increase of 6°C/min to 200°C. In both cases, the carrier gas was helium and the injector and detector temperatures were held at 230°C. Splitless injection was used to introduce all samples to the columns.

All standards obtained from commercial sources were found to be at least 95% pure by GC analysis. *p*-Cymene-8-ol was obtained from R. Bos, Groningen, The Netherlands, and was 95% pure. *p*-Vinylphenol was prepared from *p*-coumaric acid by the method of Hashimoto et al. (1976) and was purified to 90% by recrystallization from hexane. Retention indices were calculated from the homologous *n*-alkane series (Van den Dool and Kratz, 1963).

Electroantennography of Synthetic Compounds. Numerous synthetic compounds, including those identified in the peach volatile mixture, were screened individually for activity at the 100-μg dosage level. Test substances in 10 μl of ether solution were placed on small filter paper triangles. After evaporation of the solvent, the triangles were placed in disposable Pasteur pipets which were closed at the large end with rubber septa (Arthur Thomas Co.). Stimuli were delivered to female *S. pictipes* antennae as described above, except that 1.0-ml quantities of air were pumped through the pipet and into the airstream. Since the magnitudes of the responses decreased over time, the response to each test

compound is expressed as a proportion of the response to a standard compound (Visser, 1979; Dickens, 1984). The standard (10 μg of guaiacol) was presented approximately 30 sec prior to and following each test stimulus. The values of the two responses were averaged and then divided into the response value for the intervening test stimulus. In most instances, each test compound was presented to three female antennae. However, due to a shortage of insects, some compounds were tested on only two antennae. Where appropriate, responses were compared statistically by a *t* test for two means.

Selected compounds were also tested in dosage-dependence studies. In this case, 1, 10, 100, and 1000 μg of each test substance were applied to filter paper and presented in the same manner as above, and 10 μg of guaicol was used as a standard. Dosage–response data were obtained on two female antennae.

RESULTS AND DISCUSSION

Analysis and EAG of Peach Bark Steam Distillates. Capillary GC analysis of the steam distillates showed that the volatile mixture released by ground peach bark was dominated by a few components (Figure 1). One in particular was very prominent, comprising 78.5% of the Bisco steam distillate, and 84.8%

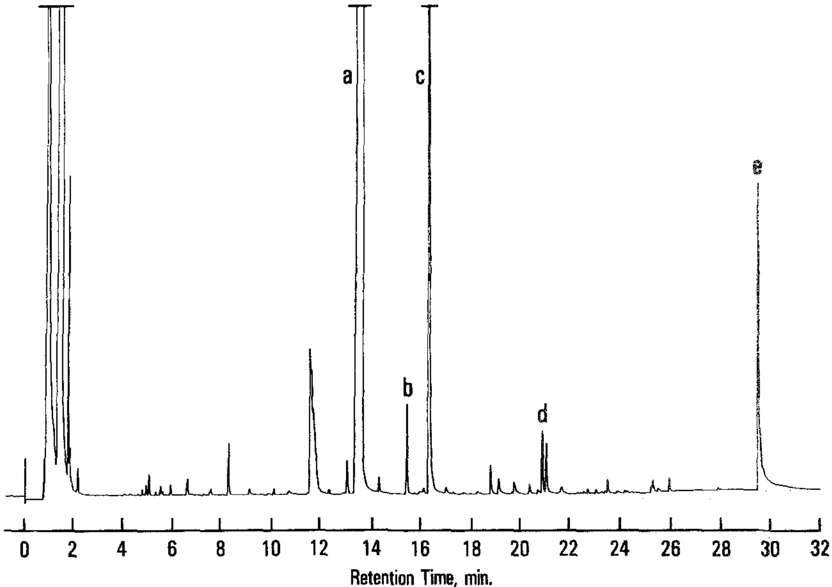

FIG. 1. Gas chromatogram of the steam distillate from Bisco peach bark on DB-Wax capillary: (a) benzaldehyde; (b) methyl benzoate; (c) 2-methylbutanoic acid plus ethyl benzoate; (d) 2-phenylethanol; (e) benzoic acid.

of the Redskin mixture. This was identified by GC-MS as benzaldehyde. Two other components, 2-methylbutanoic acid and benzoic acid, together with benzaldehyde, constituted over 90% of the steam distillates of both cultivars. 2-Methylbutanoic acid was present in larger quantities in the Bisco cultivar (ca. 10%), and benzoic acid occurred at approximately 3% in both. At these levels, benzoic acid is likely to be an artifact formed by the oxidation of benzaldehyde during sample preparation.

The steam distillate mixtures from both cultivars were fractionated by GLC on 3% OV-17 and the fractions tested in EAG assays to determine the antennal sensitivity of *S. pictipes*. EAG activity was found to be distributed over a number of fractions (Table 1), but two fractions eluting at 10–14 min gave consistantly stronger responses than all others with both cultivars. Examination of the GC recordings showed that a very small proportion (<2%) of the volatile material was associated with these fractions, suggesting that highly active compounds were present. Benzaldehyde, the dominant mixture component, was not eluted in this temperature range but was largely contained in the 8 to 10-min fraction (Table 1).

A larger quantity of the active 10 to 14-min Bisco material was collected as one fraction, concentrated 50-fold over the original steam distillate, and analyzed by GC-MS. The pertinent spectral and chromatographic data are given in Table 2. Identified components in the concentrated active material constituted 97% of the peak area measured from the DB-Wax column. Of the 25 com-

TABLE 1. RELATIVE RESPONSES OF FEMALE *S. pictipes* ANTENNAE TO GC FRACTIONS OF PEACH BARK STEAM DISTILLATES FROM TWO CULTIVARS

	Relative response[b]	
Retention (min)[a]	Bisco	Redskin
0–2	9.7	13.9
2–4	25.8	18.8
4–6	45.2	37.8
6–8	35.5	54.1
8–10	67.7	82.8
10–12	100.0 (1.55 mV)[c]	100.0 (1.22 mV)[c]
12–14	90.3	95.9
14–16	64.5	82.8
16–18	67.7	65.6
18–20	64.5	82.8
20–22	54.8	60.7

[a] On preparative OV-17 column.
[b] Percentage of the largest response. Mean of two individuals.
[c] Mean absolute response to most active fraction.

TABLE 2. MASS SPECTRAL AND CHROMATOGRAPHIC DATA FOR COMPOUNDS IDENTIFIED IN 10 TO 14-MIN OV-17 FRACTION OF BISCO AND REDSKIN STEAM DISTILLATES

Compound	Prominent ions (m/e)[a]	Retention index DB-Wax	Percent in Bisco[b]	Percent in Redskin[b]
Nonanal[c]	43, 57, 70, 98, 82	1403	0.4	
Benzaldehyde	77, *106*, 105, 51	1500	0.8	14.6
Decanal[c]	41, 43, 55, 70, 83	1504	1.3	
(*E*)-2-Nonenal[c]	41, 43, 55, 70, 83	1539	tr	2.9
Methyl benzoate	105, 77, *136*, 51	1633	17.6	3.7
Ethyl benzoate	105, 77, 122 *150*	1675	tr	7.2
2-Methylbutanoic acid	74, 57, 41, 87, *102*	1676	10.6	
α-Terpineol[c,d]	81, 59, 41 93, 121	1707	1.2	1.0
p-Dimethoxy-benzene[c]	123, *138*, 95	1750	tr	0.8
Methyl salicylate	120, *152*, 92, 65	1782	0.5	tr
Senecioic acid[c]	*100*, 39, 55, 82	1804	4.2	1.7
1-Phenyl-1,2-propanedione[c]	105, 77, 43, *148*	1817	0.7	
1-Phenylethanol	107, 79, *122*, 77	1825	tr	
Carveol	109, 41, 55, 69, 84	1846	tr	
Hexanoic acid	60, 73, 41, 100, 87	1855	4.2	1.2
p-Cymene-8-ol	43, 135, 60, 90, 117, *150*	1859	0.6	0.6
Guaiacol	81, 109, *124*, 53	1863	tr	
Benzyl alcohol	79, *108*, 77, 107, 51	1879	2.5	1.2
2-Phenylethanol	91, 92, *122*, 65, 77	1920	14.7	
Heptanoic acid	60, 73, 97, 43, 107	1963	1.0	1.5
Phenol	*93*, 39, 66, 65	2004		7.4
Octanoic acid	60, 73 43, 85, 101, 115	2072	3.6	4.5
p-Ethylphenol	107, *122*, 77, 91	2188	0.6	
p-Vinylphenol[e]	*120*, 91, 65, 39, 77	2386	tr	
Benzoic acid	105, 77, *122*, 51, 50	2400	32.7	40.0

[a] In order of decreasing abundance. Molecular ion italicized when present.
[b] Measured by using DB-Wax column and flame ionization detection.
[c] Chromatographic verification performed on DB-Wax column only. All other compounds verified on both DB-Wax and DB-1701.
[d] Peak contains a second unidentified compound, consequently the measure of relative percentage is an estimate.
[e] Structure verified by [^{1}H]NMR: δ 7.29, m, 2H; 6.78, m, 2H; 6.64, dd, 1H; 5.56, d, 1 H; 5.11, d, 1H. Material for NMR isolated by preparative GC of a diethyl ether extract obtained by continuous extraction of an aqueous homogenate of ground Bisco bark.

pounds identified, 15 were aromatics, and the remainder were divided between aliphatic aldehydes, aliphatic acids, and monoterpene alcohols. Eleven components comprised over 1% each of the 10 to 14-min mixture, of which four constituted at least 10% each (Table 2). Together, the latter made up ca. 75% of the active fraction (Table 2).

Benzaldehyde and 2-methylbutanoic acid were largely eluted prior to 10 min. The quantities of these two components in the active fraction are due to peak tailing and represent only a small fraction of the quantities found in the whole steam distillate.

Most major components of the Redskin 10 to 14-min fraction could be identified by their gas chromatographic retention times on DB-Wax. The components determined in this manner made up over 80% of the fraction peak area (Table 2). One major component could not be accounted for by chromatographic comparison with the Bisco mixture and was consequently identified as phenol by GC-MS (Table 2). While the composition of Redskin was generally similar to Bisco, the relative quantities of many major components were drastically reduced (Table 2). Methyl benzoate comprised less than 4% of the Redskin mixture, while neither 2-phenylethanol nor 2-methylbutanoic acid were detected. These facts may explain the slightly smaller responses to the 10 to 12- and 12 to 14-min Redskin fractions in EAG tests when compared to the Bisco results (Table 1).

EAG Activity of Identified Components. Since the active fractions proved to be complex mixtures, individual components were screened for EAG activity at the 100-μg dosage level. Selected compounds were also tested at various dosages in order to give an indication of maximum responses and response threshold levels. Differences in volatility between compounds make it difficult to directly compare EAG responses of structurally dissimilar stimuli. However, large differences in response to structurally similar compounds were taken to indicate inequalities in the number of stimulated antennal receptors. Guaiacol was found to be quite active in preliminary experiments, so it was chosen as a standard that was presented to the antennae at the 10 μg level. Responses to all other test compounds are reported as proportions of the response to guaiacol.

Table 3 illustrates that all compounds tested produced measurable EAG responses. However, large variations in response magnitude were observed. Aromatic alcohols varied substantially in the magnitude of responses elicited. Those with secondary or tertiary hydroxyl groups, such as *p*-cymene-8-ol and 1-phenylethanol were significantly more active than the primary aromatic alcohols, benzyl alcohol and 2-phenylethanol ($P < 0.02$, Table 3). This difference was also apparent in dosage–response studies (Figure 2) where secondary and tertiary alcohols showed a larger increase in response than primary alcohols at dosages between 1 and 100 μg.

Two nonaromatic cyclic alcohols were also tested; α-terpineol proved to

TABLE 3. EAG OF FEMALE *S. pictipes* TO COMPONENTS OF THE 10 TO 14-MIN OV-17 FRACTION OF PEACH BARK STEAM DISTILLATES (100 μg EACH)[a]

Compound	Relative response ± SE	Compound	Relative response ± SE
Acids		Esters	
2-Methylbutanoic[b]	1.14 ± 0.02	Methyl benzoate	2.70 ± 0.25
Senecioic	1.15 ± 0.04	Ethyl benzoate	2.50 ± 0.19
Hexanoic[b]	2.42 ± 0.34	Methyl salicylate	1.90 ± 0.16
Heptanoic[b]	2.17 ± 0.17	Ketones	
Octanoic[b]	1.66 ± 0.20	1-Phenyl-1,2-propanedione	3.00 ± 0.22
Benzoic[b]	0.36 ± 0.03		
Alcohols		Phenols	
Benzyl	0.96 ± 0.10	Phenol	0.51 ± 0.12
2-Phenylethanol	1.01 ± 0.09	*p*-Ethylphenol	1.40 ± 0.09
1-Phenylethanol	1.78 ± 0.01	*p*-Vinylphenol	0.75 ± 0.05
p-Cymene-8-ol	2.23 ± 0.20	*p*-Dimethoxybenzene[b]	1.36 ± 0.06
Carveol	1.30 ± 0.03	Guaiacol	2.62 ± 0.22
α-Terpineol[b]	2.73 ± 0.29	Control	
Aldehydes		Diethyl ether	0.18 ± 0.04
Nonanal[b]	2.48 ± 0.46		
Decanal[b]	2.33 ± 0.12		
(*E*)-2-Nonenal[b]	2.69 ± 0.10		
Benzaldehyde	1.14 ± 0.10		

[a]Responses are a proportion of the response to 10 μg guaiacol (relative response).
[b]Compounds tested on antennae from two females.

be highly active, but carveol was not. The former bears similarity to *p*-cymene-8-ol in that an isopropanol substituent is attached to the six-membered ring, while in the latter a secondary hydroxyl group is situated directly on the cyclohexane ring. Since none of the chiral alcohols tested in this study (1-phenylethanol, α-terpineol, and carveol) were optically pure, the effect of chirality on the responses to alcohols is not known.

Like the alcohols, phenolic compounds showed variation in activity. Phenol elicited only very small responses, while responses to guaiacol (*o*-methoxyphenol) were significantly larger ($P < 0.01$) than those to phenol and 4-ethylphenol. Dosage–response curves for phenol and *p*-ethylphenol (Figure 2) reveal similar maximum responses. However, *p*-ethylphenol appears to saturate the receptor near 100 μg while phenol elicits a lower response at 100 μg and shows a continued increase between 100 and 1000 μg. The dosage–response curve for guaiacol had a completely different appearance than those for other phenolics, with both a lower threshold value and a larger maximum response magnitude (Figure 2).

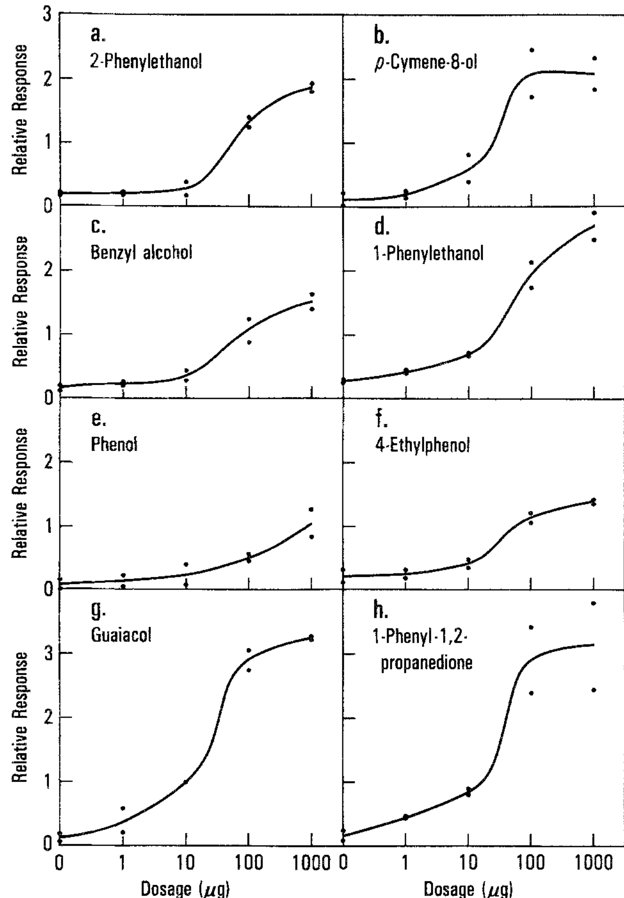

FIG. 2. Dosage–response curves for various peach bark volatile compounds; each point represents the relative response of a single female at a particular dosage.

Several aliphatic acids were major constituents of the volatile mixture. With the linear forms, maximum response magnitudes were seen at six carbons, and the responses decreased with increasing chain length. Neither of the two branched five-carbon acids, 2-methylbutanoic and senecioic, produced large responses, and the aromatic benzoic acid elicited the lowest responses of any compound (Table 3).

Responses to aliphatic aldehydes were all quite strong, but benzaldehyde was substantially less active than the aliphatic compounds (Table 3). Recall that the latter was the dominant component of the steam distillates, comprising over 75% of both mixtures.

The three identified aromatic esters showed strong activity, with methyl

benzoate being one of the most active compounds tested. Ethyl benzoate was significantly less stimulatory than methyl benzoate ($P < 0.05$), and methyl salicylate was even less active (Table 3).

The only ketone found in the peach volatile mixture was 1-phenyl-1,2-propanedione. This compound occurred in small quantities in the volatile mixture, but it elicited the largest responses of any compound in the 100-μg experiment (Table 3) and was also quite active at 1 μg (Figure 2).

The data discussed above indicate that *S. pictipes* female antennae are responsive to the volatile components of peach bark and that they are sensitive to relatively minor differences in structure. To further test the selectivity of the *S. pictipes* olfactory system, a numbr of six-carbon aliphatic alcohols and aldehydes were examined by EAG. These are widely distributed components of the foliar tissue of higher plants, and many leaf-associated insect species have been found to respond strongly to them in EAG tests (Visser, 1979; Guerin and Visser, 1980; Dickens, 1984; Dickens and Boldt, 1985). Being a wood-feeding insect, *S. pictipes* does not associate with foliar tissue and may be expected to respond to a different complex of volatiles than leaf feeders.

Of the six-carbon compounds tested, only 1-hexanol showed activity comparable to the more active of the peach volatile compounds (Table 4). Responses to hexanal and (*E*)-2-hexenal are much smaller than those to 1-hexanol, and substantially smaller than many of the peach volatile compounds (Table 3 and 4). It is interesting to note that the nine- and ten-carbon aldehydes found in the peach bark mixture elicited somewhat larger responses than their six-carbon counterparts (Table 3 and 4). Previous studies have indicated that receptors sensitive to the six-carbon green leaf volatiles are the most abundant receptor types on the antennae of many leaf-feeding insect species (Visser, 1979; Guerin and Visser, 1980; Kozlowski and Visser, 1981).

The Colorado potato beetle, *Leptinotarsa decimlineata*, and the oak flea weevil, *Rhynchaenus quercus*, are most responsive to saturated and unsaturated six-carbon alcohols (Visser, 1979; Kozlowski and Visser, 1981) while the carrot fly is most responsive to hexanol and (*E*)-2-hexenal. With the chrysomelid

TABLE 4. EAG RESPONSES TO SIX-CARBON ALDEHYDES AND ALCOHOLS[a]

Compound	Relative response ± SE
Hexanol	0.82 ± 0.08
(*E*)-2-Hexenal	1.21 ± 0.01
1-Hexanol	2.28 ± 0.29
(*E*)-2-Hexen-1-ol	1.65 ± 0.18
(*Z*)-3-Hexen-1-ol	1.44 ± 0.02

[a]Responses are expressed as a proportion of the response to 10 μg guaiacol. 100 μg dosage level.

beetle *Trirhabda bacharides*, 1-pentanol and 1-hexanol were both quite active, while heptanal, octanal, and nonanal were all more active than hexanal (Dickens and Boldt, 1985). However, (*E*)-2-hexenal gave the largest responses of any compound tested with this species (Dickens and Boldt, 1985). A similar situation exists with antennae of the boll weevil, *Anthronomus grandis*, which are very responsive to saturated and unsaturated six-carbon alcohols (Dickens, 1984). Boll weevil response to saturated aldehydes is maximal at seven carbons, but again (*E*)-2-hexenal is highly active.

The reduced sensitivity of *S. pictipes* to six-carbon "green leaf volatiles" in comparison to many of the peach bark volatile compounds appears to be unusual among phytophagous insects. This may represent an adaptation by this species to the complex of compounds characteristic of its host plant.

CONCLUSIONS

The volatile mixture obtained from ground peach bark is dominated by a single component identified as benzaldehyde. The olfactory system of *S. pictipes*, however, does not respond maximally to the benzaldehyde-containing fraction, but rather to a fraction containing less than 2% of the total mixture. This fraction contained numerous simple aromatic compounds as well as volatile aliphatic acids and aldehydes. Female *S. pictipes* antennae were responsive to many of these compounds but showed some selectivity. For example, aromatic esters generally elicited larger responses than simple phenols. Also, the six-carbon "green leaf volatiles" showed low activity in comparison to many of the peach volatile compounds (e.g., aromatic esters and ketones).

Only one published study exists concerning the responsiveness of *S. pictipes* to plant-derived volatiles and it contains only descriptive results (Snapp and Swingle, 1929). However, of 122 materials tested in field trapping experiments, only the steam distillates of green peaches, and peach bark as well as ethyl benzoate, phenol, salicylaldehyde, malate plus acetaldehyde and sodium bisulfite were found to be "slightly attractive" (Snapp and Swingle, 1929). It is interesting to note that two peach volatile compounds ethyl benzoate and phenol, were mentioned as being attractive and that several bark volatile constituents are structurally similar to the reported attractants. These include methyl benzoate, methyl salicylate, *p*-ethylphenol, *p*-vinylphenol, guaiacol, and benzaldehyde. It is notable that the compounds mentioned above elicit EAG responses of variable magnitude. The relationship of EAG response with behavioral activity for plant volatiles is not well understood, and only further behavioral testing will fully determine the role of bark volatiles in the location of oviposition sites by *S. pictipes*.

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DIGESTION AND ABSORPTION OF *Eucalyptus* ESSENTIAL OILS IN GREATER GLIDER (*Petauroides volans*) AND BRUSHTAIL POSSUM (*Trichosurus vulpecula*)

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Abstract—Measurements were made of the quantity and composition of the steam-volatile essential oils in gastrointestinal tract contents of greater gliders fed *Eucalyptus radiata* foliage and brushtail possums fed *E. melliodora* foliage. In both species, there was less oil in the stomach contents than in an equivalent mass of foliage. Only minor losses of leaf oils occurred during mastication by greater gliders, and absorption from the stomach appeared to be the major reason for the difference in the oil content of ingested leaves and of stomach contents. The apparent digestibility of oils over the whole gut was 96–97%, although oils from the cecum and feces of both species contained compounds not present in the original leaf oils. Absorption of oils before they reach the hindgut should reduce the severity of antimicrobial effects but may involve a metabolic cost to the animal in detoxification and excretion.

Key Words—Folivores, marsupials, allelochemicals, transformation, detoxification.

INTRODUCTION

The greater glider (*Petauroides volans*) and the brushtail possum (*Trichosurus vulpecula*) are folivorous marsupials. Greater gliders feed almost exclusively

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on eucalypt leaf (Marples, 1973). Although the leaves of eucalypts form an important part of the diet of the brushtail possum in southeastern Australia, they are usually supplemented with foliage from other species of trees and shrubs, fruits, flowers, and herbage (Kerle, 1984). The importance of noneucalypt foods in the diet of the brushtail possum has led several authors (e.g., Freeland and Winter, 1975) to speculate that the consumption of eucalypt foliage is limited by the presence of "toxic" allelochemicals such as essential oils.

Although there has long been speculation about the effects that essential oils of *Eucalyptus* spp. might have on phytophagous animals (Pratt, 1937; Fleay, 1937; Betts, 1978), recent studies have failed to find relationships between the level and/or composition of leaf oils and the feeding perferences of some mammals (e.g., koala: Southwell, 1978; and insects (*Paropsis*): Morrow and Fox, 1980).

Irrespective of the effects of oils on gross food preference, their ingestion results in a metabolic cost for detoxification (Cleland, 1946; Hinks and Bollinger, 1957a, b), and their biological actions have the potential to affect populations of microbes in the digestive tracts of animals (Freeland and Janzen, 1974). Several studies have demonstrated a deleterious effect of mono- and sesquiterpenes on ruminal fermentation (Nagy et al., 1964; Nagy and Tengerdy, 1968; Oh et al., 1967, 1968). However, all these studies were performed in vitro, consequently no allowance was made for absorption of the oils, and in some cases the concentrations of oils used were unrealistically high. In contrast to ruminants, essential oils in hindgut fermenters may be absorbed and detoxified before they reach the site of microbial activity.

This paper describes the pattern of absorption of essential oils from the gut of the greater glider and brushtail possum. Volatile material extracted from digesta at five points along the gut was separated into its component compounds by gas–liquid chromatography. Also, the hypothesis that leaf oils may be lost during mastication was tested in greater gliders.

METHODS AND MATERIALS

Animals. Greater gliders were caught by hand during logging operations in a forest dominated by New England blackbutt (*E. andrewsii* ssp. *campanulata*) (Forest Type 161: Forestry Commission of New South Wales, 1965) in northern New South Wales. Brushtail possums were caught in wire cage traps in eucalypt woodland dominated by *E. melliodora*, *E. blakelyi*, *E. viminalis*, and *E. caliginosa* near Armidale, NSW. Both species were housed in metabolism cages in an air-conditioned room (20 ± 3°C) on a 12 : 12 light–dark regime for at least three weeks prior to each experiment. The greater gliders were fed *E. radiata* foliage and the brushtail possums fed *E. melliodora* foliage which was collected fresh each week and stored in plastic bags with the stems in water

at 8°C. Further details of these procedures and those used in the sampling of foliage and the collection of feces and urine are given by Foley and Hume (1987).

Experiment 1: Digestion of Essential Oils. Six greater gliders (one male, five females) were fed foliage from one *E. radiata* tree and three male brushtail possums foliage from one *E. melliodora* tree for 14 days. Samples of the leaves offered and feces collected for the last five days were stored in plastic bags at −15°C. These samples were steam distilled and the distillates analyzed by gas–liquid chromatography (GLC) and mass spectrometry (GLC-MS) as described below.

Experiment 2: Sites of Oil Absorption. Three female greater gliders were fed foliage from one *E. radiata* tree, and three male brushtail possums were fed foliage from one *E. melliodora* tree for 10 days. Samples of the diet and feces were collected for the last five days. The animals were then killed by an overdose of sodium pentabarbitone at 1200 hr, five hours after foliage was last available. The digestive tract was quickly excised, and the contents of the stomach, small intestine, cecum, proximal colon, distal colon, and rectum were removed, bulked within each species, frozen in liquid nitrogen, and stored at −15°C. These samples were steam distilled, and the distillate was analyzed by GLC as described below.

No marker substance was used in this experiment since in preliminary experiments only traces of oil could be recovered from gut contents of greater gliders that had received the marker Cr-EDTA in the drinking water (0.28 mg/ml). The oil appeared to be polymerized and oxidized and would not pass through the GLC column. The chelated chromium may have catalyzed the autoxidation of essential oil components (Garnier and Gaiffe, 1967).

Experiment 3: Oil Losses during Mastication. The amount of terpene lost during mastication of leaf by greater gliders was measured after conversion of the respirometers described by Foley (1984) to an open-flow system. Expired air was bubbled through two flasks containing cyclohexane which had been shown in a preliminary experiment to trap expired terpenes. At 0600 hr on day 1, leaves from one *E. radiata* tree were placed in the chamber and the pump started. At 1800 hr, a greater glider was placed in the chamber and allowed to feed normally. Fresh cyclohexane was placed in the traps. At 0600 hr on day 2, uneaten leaves, feces, and urine were removed from the chamber, fresh cyclohexane was placed in the traps, and the animal was left until 1800 hr when the experiment was terminated. This procedure was replicated three times with different animals. Samples for the two controls (leaf only, animal only) and the experimental treatment were bulked over the three replicates. Two runs in which a known volume of *E. radiata* essential oil was evaporated in the chamber were conducted to estimate recoveries. The cyclohexane was removed from each sample by fractional distillation on a series of Vigreaux and packed columns.

The remaining material was analyzed by GLC-MS after addition of *n*-dodecane as an internal standard.

Analytical. Essential oils were extracted from wet leaves, feces, and gut contents by steam distillation with cohobation in an all-glass apparatus (Hughes, 1970). Eucalypt leaves were distilled for 8–12 hr; gut samples were distilled for 12–24 hr. All oil samples were stored in air-tight glass bottles over sodium sulfate at −20°C.

Analytical GLC was carried out on a Perkin-Elmer 900 instrument using a quartz-silica SCOT column (50 m × 0.5 mm ID) coated with FFAP (free fatty acid phase polyethylene glycol reacted with nitroterephthalic acid) and with helium as the carrier gas. A Hewlett Packard 3370A Integrator was used to determine peak areas.

Combined GLC–mass spectrometry (GLC-MS) was performed on a Shimadzu GC6-AMP instrument with a SCOT column (70 m × 0.5 mm) coated with FFAP and programmed from 80°C to 225°C at 3°C/min. This system was connected to an AEI MS12 mass spectrometer via an all-glass straight split. Mass spectra were recorded at 70 eV ionization voltage with an ion source temperature of 150°C. Spectra were recorded every 6 sec on a VG Digispec Display data system which produced standard bar graphs for direct comparison with published spectra. Chemical ionization mass spectrometry was performed on an AEI MS902 mass spectrometer fitted with a Chemspect source. Ammonia was used as reagent gas at a pressure of 0.5 torr. High-resolution mass spectrometry was performed on this instrument under the same conditions using perfluorokerosene as reference and a peak timing method (Brophy et al., 1979). Identification of compounds was based on comparison of mass spectra with those of known compounds and coinjection with authentic compounds.

The oil distilled from the feces of greater gliders (20 mg in 1 ml methanol) was added to 4 ml of 1 M aqueous sodium hydroxide, and the mixture was heated at reflux for 4 hr. The basic solution was extracted with pentane (3 × 2 ml), the aqueous layer acidified with conc. hydrochloric acid and then reextracted with methylene chloride (3 × 2 ml). Both solutions were dried over sodium sulfate and the solvent removed under a stream of nitrogen. The residue resulting from the methylene chloride solution was taken up in 1 ml ether and treated with diazomethane.

RESULTS

The yield of steam-volatile oils from foliage, gut contents, and feces in all experiments is given in Table 1. The percentage composition of the major components of the oils from *E. radiata* leaves and the corresponding greater glider feces and from *E. melliodora* leaves and brushtail possum feces is given in Tables 2 and 3. GLC traces of the oil from leaf and feces of each species are shown in Figure 1.

TABLE 1. YIELD OF STEAM-VOLATILE ESSENTIAL OILS FROM EUCALYPT FOLIAGE AND FROM DIFFERENT PARTS OF THE GUT OF THE GREATER GLIDER AND BRUSHTAIL POSSUM

			Yield (ml/100 g dry matter)					
Experiment	Species		Leaf	Stomach	Small intestine	Cecum/Proximal colon	Distal colon	Feces
1	Greater glider	*E. radiata*	7.45	—	—	—	—	0.10[a]
	Brushtail possum	*E. melliodora*	0.82	—	—	—	—	0.02[b]
2	Greater glider[b]	*E. radiata*	11.05	6.59	Trace	0.32	Trace	0.09
	Brushtail possum[b]	*E. melliodora*	1.35	0.66	Trace	0.28	0.04	0.03

[a] $N = 6$.
[b] $N = 3$.

TABLE 2. MAJOR COMPONENTS (>1.0%) OF STEAM-VOLATILE OIL FROM *E. radiata* AND CONCENTRATION IN DIGESTA FROM DIFFERENT PARTS OF THE GUT OF THE GREATER GLIDER

		Experiment 1		Experiment 2			
					Percentage leaf		
Peak number (Figure 1A, B)	Identification	Leaf (% composition)	Feces (% leaf)	Leaf (% composition)	Stomach	Cecum	Feces
1	α-pinene	4.9	23	6.6	81	34	5
5	α-phellandrene	9.3	40	16.5	103	8	40
6	α-terpinene	4.4	42	6.8	101	?[a]	74
8	1,8-cineole + β-phellandrene	4.3	43	5.1	100	36	192
9	γ-terpinene	7.5	30	14.6	105	?[a]	33
10	*p*-cymene	10.5	23	3.4	55	147	89
11	terpinolene	2.2	41	4.3	105	37	49
15	*trans*-*p*-menth-2-en-1-ol	4.5	107	2.6	124	157	190
16	terpinen-4-ol + caryophyllene + aromadendrene	19.8	17	15.6	146	14	38
17	*cis*-*p*-menth-2-en-1-ol	3.3	79	1.8	127	?[a]	200
18	*cis*-piperitol	1.4	77	—	—	—	—
19	α-terpineol + viridiflorene	2.1	42	1.5	117	10	19
20	piperitone	—	—	1.3	172	121	58
21	*trans*-piperitol	2.6	111	1.2	111	181	247
22	δ-cadinene	1.0	32	1.8	69	?[a]	98
23	4-phenylbutanone	1.5	12	—	—	—	—
24	$C_{15}H_{26}O$	1.1	63	—	—	—	—
26	γ-eudesmol	1.2	84	2.3	44	18	129
27	α-eudesmol	1.1	85	1.2	63	49	215
28	β-eudesmol	1.6	84	1.2	79	60	333

[a] Solvents comprised >60% of area and peaks could not be accurately defined.

TABLE 3. MAJOR COMPONENTS (>1.0%) OF STEAM-VOLATILE OIL FROM *E. melliodora* AND CONCENTRATION IN DIGESTA FROM DIFFERENT PARTS OF THE GUT OF THE BRUSHTAIL POSSUM

Peak number (Figure 1C, D)	Identification	Experiment 1: Leaf (% composition)	Experiment 1: Feces (% leaf)	Experiment 2: Leaf (% composition)	Experiment 2: Percentage leaf: Stomach	Experiment 2: Percentage leaf: Cecum	Experiment 2: Percentage leaf: Feces
2	*iso*valeraldehyde	2.0	124	—	—	—	—
3	α-pinene	7.5	101	3.8	96	83	19
5	limonene	4.9	64	—	—	—	—
6	1,8-cineole	63.1	5	56.7	99	29	10
8	*p*-cymene	2.2	70	1.2	153	143	48
9	terpinolene	—	—	4.2	69	144	95
13	α-terpineol	1.7	62	2.1	180	143	120
—	unknown	—	—	1.1	130	281	220
—	unknown	—	—	2.0	—	—	—
19	$C_{15}H_{26}O$	1.8	87	5.1	—	—	—
—	unknown	—	—	1.6	24	28	—

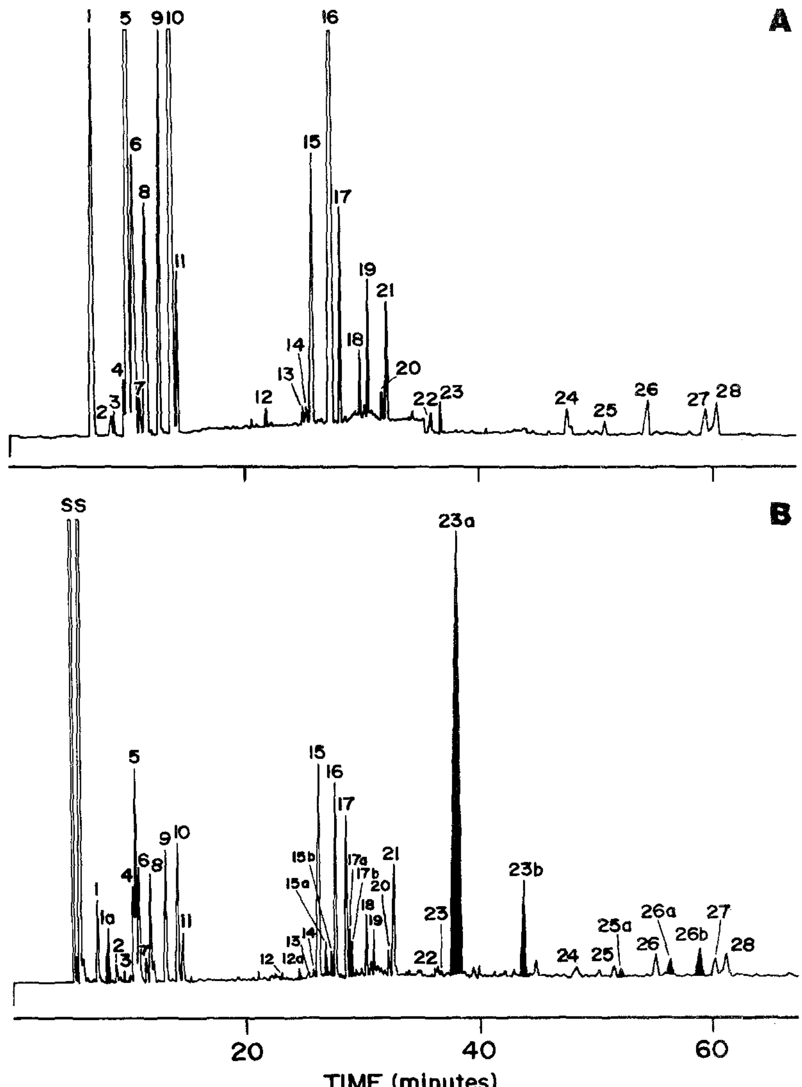

FIG. 1. GLC trace of steam-volatile essential oils from (A) *E. radiata* foliage, (B) feces from greater gliders fed *E. radiata* foliage, (C) *E. melliodora* foliage, and (D) feces from brushtail possums fed *E. melliodora* foliage. Shaded peaks represent feces oil components not present in leaf oils. Peaks not identified in Tables 2 and 3 are as follows: *E. radiata*/greater glider: 2, β-pinene; 3, sabinene; 4, myrcene; 7, limonene; 12, $C_{10}H_{18}O$; 12a, menthone; 13, linalool; 14, unknown; 15a, menthyl acetate; 15b, unknown; 17a, menthol, 17b, alloaromadendrene; 23a, an octane diol dibutyrate; 23b, globulol; 25, $C_{15}H_{26}O$; 25a, $C_{15}H_{24}O$; 26a, $C_{15}H_{28}O$; 26b, thymol. *E. melliodora*/brushtail possum: 4, myrcene; 7, γ-terpinene; 10, terpinen-4-ol; 11, caryophyllene; 11a, b, unknown; 12, pinocarveol; 14, bicyclogermacrene; 15, *cis*-mentha-1(7),8-dien-2-ol; 16, *trans*-mentha-1(7),8-dien-2-ol; 16a, an octane diol dibutyrate; 16b, unknown; 16c, 17, 18, 19a, $C_{15}H_{26}O$; 20, $C_{15}H_{24}O$. "S" represents solvent.

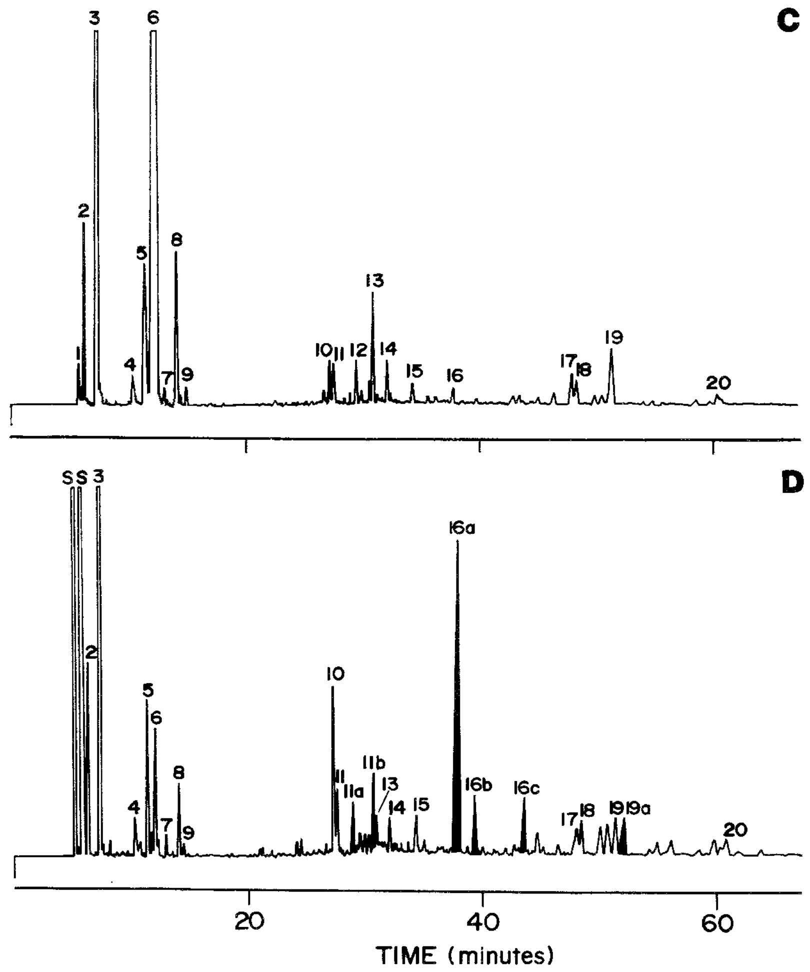

FIG. 1. Continued.

Experiment 1. E. radiata oil was complex, consisting primarily of terpinen-4-ol (20%), *p*-cymene (11%), α-phellandrene (9%) and γ-terpinene (8%). *E. melliodora* oil was much simpler, being dominated by 1,8-cineole (63%) with smaller amounts of α-pinene (7%), and limonene (4%). Both oils contained only small amounts of sesquiterpenes (3–5%).

Only minor amounts of oil were recovered from the feces of both species. However, these oils were more complex than those from the corresponding leaves. The oil from the brushtail possum feces was notable for the almost complete absence of 1,8-cineole (3% vs. 63% in the leaf). In the oil from the feces of the greater glider, the peaks representing terpinen-4-ol, *p*-cymene, α-

pinene, and α-phellandrene were greatly reduced, and no oil component passed through the gut without some apparent digestion.

In both species, some peaks appeared in the oil from the feces but did not occur in the oil from the leaves. While many of these were minor (e.g., Figure 1B, peaks 12a, 15a, 17a), peak 23a was the largest component (21%) of the oil from the greater glider feces. The same component appeared in the oil of the brushtail possum feces (Figure 1D, peak 16a) as 14% of the total oil. Infrared spectra of the two feces oils showed large absorptions at 1740 cm^{-1} and 1160 cm^{-1}, characteristic of an ester function. The strength of these bands, absent in the leaf oils, suggested that they belonged to the large extra peaks in the GLC traces of both feces oils. The mass spectra of these GLC peaks indicated that the highest mass ion was at m/z 243; however, chemical ionization mass spectrometry showed a (MH^+) ion at m/z 287, and accurate mass measurement of this ion resulted in a mass of 287.2236, indicating a formula of $C_{16}H_{31}O_4$ (287.2220) for the (MH^+) ion.

Alkaline hydrolysis of a sample of the oil from the greater glider feces resulted in the elimination of this significant extra GLC peak, lending support to the suggestion that it was an ester. From the base-soluble fraction, after acidification, solvent extraction, and methylation, a GLC trace was obtained which contained a peak, the mass spectrum of which is suggestive of an octane diol, which fits the solubility characteristics of the compound. The mass spectrum was similar (but not identical) to that of 2-ethylhexane-1,3-diol. A small amount of methyl butyrate was also detected in this fraction.

It appears from these results that the unknown peak in the feces oil from both folivores is a dibutyrate ester of an octane diol. In fact, the mass spectrum of 2-ethylhexane-1,3-dibutyrate was similar (but not identical) to that of the natural material. The natural material had a shorter retention time (on FFAP) than either 2-ethylhexane-1,3-dibutyrate or diisobutyrate, suggesting greater branching in the natural material.

Experiment 2. Details of the yield and percentage composition of the major components of the steam-volatile oils recovered from different parts of the digestive tracts of the two species are given in Tables 1–3. Although the yields of oils from the leaves were notably higher in this experiment than in experiment 1, the yield of oil from the feces was similar. On the other hand, while the percentage composition of the oils from leaves was similar to that in experiment 1, those isolated from the feces were different. For example, the octane diol dibutyrate found in experiment 1, although present in this sample, comprised only 9% of the oil from greater glider feces and 3% of that from brushtail possum feces.

Experiment 3. The essential oil peaks on the GLC trace of cyclohexane-soluble material from expired air of greater gliders in preliminary experiments were identified by their mass spectra. This also indicated that some of the other

peaks represented aliphatic straight-chain hydrocarbons resulting from impurities in the cyclohexane solvent.

No peaks representing essential oils were apparent in the GLC traces of the air samples from the leaf alone in the chamber, from greater gliders feeding on the leaf in the chamber, or from the greater gliders alone in the chamber. Recoveries of evaporated terpenes in the two runs with *E. radiata* essential oils were 28% and 35%.

DISCUSSION

Some workers (e.g., Von Rudloff, 1975) have criticized steam distillation as a means of extracting essential oils because of the possibility of inducing artifactual rearrangements of components of the oils. This was unlikely to have been a serious problem in the present study. Lassak (unpublished) has shown that the steam-volatile essential oil of the leaves of *E. dives*, a close relative of *E. radiata* (Ladiges et al., 1983) is chromatographically identical to that extracted from individual oil glands with a fine capillary needle.

Using the mean intake and dry-matter digestibility figures for greater gliders and brushtail possums fed *E. radiata* and *E. melliodora*, respectively (44 g/kg body mass$^{0.75}$/day and 58% in greater gliders and 36 g and 51% in brushtail possums; Foley, 1984), it can be calculated that greater gliders apparently digested 97% of the essential oils of *E. radiata* while brushtail possums apparently digested 96% of *E. melliodora* essential oils. Using similar techniques, Eberhard et al. (1975) found that koalas apparently digested 70–97% of the essential oils of the leaves of *E. punctata*. Similarly, Southwell et al. (1980) found only traces of essential oil in the feces of brushtail possums dosed with 5 ml of purified oil components (*p*-cymene and 1,8-cineole) daily for five days. Igimi et al. (1974) detected only 10% of the ^{14}C label in the feces of rabbits fed [^{14}C]*d*-limonene. That components of these essential oils are readily absorbed is not surprising in view of their low molecular weight and high lipid solubility. The important question is where are they absorbed?

The apparent interaction between Cr-EDTA and essential oils in the gut, discovered in preliminary experiments, meant that the site of absorption could not be accurately ascertained. However, analysis of oils from different parts of the gut showed that the quantity of oil in the stomach contents was only 49% of what would be expected, on the basis of digesta mass, in brushtail possums and 59% in greater gliders. Similar discrepancies have recently been observed in the rumen contents of mule deer (*Odocoileus hemionus*) (Cluff et al., 1982) and stomach ingesta of pygmy rabbits (*Brachylagus idahoensis*) (White et al., 1982). There are two possible explanations for this. First, lipid-soluble material such as essential oils could be rapidly absorbed across the mucosa of the stomach of both ruminants and hindgut fermenters (Cook et al., 1952, Alexander

and Chowdhury, 1958). Igimi et al. (1974) have shown that there is rapid disappearance of [^{14}C]*d*-limonene from the rat stomach after dosing by stomach tube. Similarly, Narjisse (1981) was unable to detect monoterpenes in the rumen contents of goats 3 hr after direct infusions.

Alternatively, volatile oils may be lost during mastication of the leaf. If this is the case, it is surprising that the percentage loss from stomach contents was greater in the brushtail possum than in the greater glider, since mastication in the greater glider produces finer particles (Gipps, 1980; Foley, 1984). However, the coarse grinding action of brushtail possum teeth may be more effective in disrupting oil glands than the fine cutting action of greater gliders (Gipps, 1980).

The results of experiments designed to measure losses of essential oils during mastication by greater gliders suggested that this was of only minor importance. Although preliminary qualitative experiments had detected terpenes arising from expired breath, no traces of oils were detected in the quantitative experiment. This was unexpected since several steps were taken to maximize the recovery of oil components. This involved decreasing the rate of air flow through the chamber, bulking samples from three animals, and distilling the cyclohexane through longer packed columns. Although recoveries of standards evaporated in the chamber averaged only 32%, the losses measured during mastication cannot explain the low concentration of oil in the stomach contents of the greater gliders relative to that ingested.

Using a similar collection system (but with diethyl ether), White et al. (1982) found that twice as much monoterpene was trapped when *Artemisia tridentata* foliage was in a chamber with pygmy rabbits compared with *Artemisia* alone. Nevertheless, this represented only a minor proportion (0.5%) of the total fraction "missing" from the stomach contents. No measurements of the efficiency of the collecting apparatus were made in the experiments of White et al. (1982), but even assuming that this was only 10%, losses during mastication of *Artemisia* would still account for only 5% of the volume of oil missing from the stomach. It would seem that in both the White et al. (1982) and the present study, although losses through mastication can occur, they are of minor quantitative importance, and absorption from the stomach must be the principal avenue of loss.

Further absorption must take place in the small intestine, since the amount of terpene reaching the hindgut was of the order of only 1% of that ingested by both greater gliders and brushtail possums. There would thus seem to be little chance of major interaction with the microbial ecosystem in the hindgut. On the other hand, it is interesting that a major unknown feces peak was found in both the greater glider and brushtail possum. No examination was made of the feces of animals fed noneucalypt diets in the present experiments, but Southwell et al. (1980) did not detect any similar metabolite in the feces of brushtail pos-

sums fed a diet of fruit or fruit supplemented with 1,8-cineole, *p*-cymene, or α-pinene.

The fact that the major unknown feces peak was detected only in or distal to the cecum suggests that it is a product of microbial metabolism. Although the unknown compound was nonterpenoid and most likely a dibutyryl ester of an octane diol, it may have resulted from microbial fermentation of terpenes. For example, Joglekar and Dhavalikar (1969) isolated the 10-carbon compound 3,7-dimethyl-1,7-octane-diol from the fermentation of citral by a soil pseudomonad. Similarly, Bhattacharyya and Dhavalikar (1965) isolated the nonterpenoid anhydride of 2-nonene-2,3-dicarboxylic acid from the *Aspergillus niger* fermentation of a number of different terpenes such as camphene, β-santalene, longifolene, caryophyllene, and δ-cadinene. They suggested that the anhydride was formed by a metabolic shift in which the normal oxidative process in the mold would have been impaired to such a degree that excess pyruvate and acetate were channeled into the formation of the anhydride.

New feces peaks could also arise by absorption and subsequent biliary excretion. Eberhard et al. (1975) suggested that biliary excretion would be important, together with urinary excretion, in dealing with those compounds greater than mol wt 150 (i.e., monoterpenoids and sesquiterpenoids). The amount of digesta in the small intestine of both species was too small to recover any oil, and the gallbladders of both species contained only a minor amount of bile. Igimi et al. (1974) found that 25% of ^{14}C from ingested [^{14}C] *d*-limonene in rats was excreted in the bile within 14 hr. However, since only 5% of the dose was eventually excreted in the feces, much of the biliary excretion must have been fermented or reabsorbed lower in the gut and excreted in the urine. In the present study, it is likely that the majority of oil ingested by both species was absorbed, detoxified, and excreted in the urine. Southwell et al. (1980) found several novel products in the urine of brushtail possums fed fruit and isolated terpenes. Future studies using labeled terpenes would be necessary to identify the pathways of detoxification and to quantify the routes of excretion of ingested essential oils.

The possibility that dietary essential oils could have deleterious effects on gut microorganisms has been raised by several authors (e.g., Freeland and Janzen, 1974; Bryant and Kuropat, 1980). This possibility is based on the work of Nagy et al. (1964), Nagy and Tengerdy (1968), and Oh et al. (1967, 1968), who found that some sagebrush *Artemisia* and Douglas fir terpenes could inhibit fermentation in the rumen of deer. However, this work has been challenged (Welch et al., 1981, 1982; Welch and McArthur, 1979) on the ground that the volumes of oil used to demonstrate microbial inhibition were unrealistically high in relation to the amounts normally expected to be ingested. Also, the in vitro systems did not allow for absorption of the oil. Oh et al. (1967) found that microbial inhibition occurred at an essential oil concentration of 1.2% of

deer rumen fluid. This is about 20 times greater than the concentration of oil found in the hindgut of the greater glider and the brushtail possum in this study.

On the other hand, Sadler (1983) found that pure compounds and ether dilutions down to 10^{-4} of 1,8-cineole, *d*-limonene, terpinen-4-ol, and α-terpineol inhibited the growth of cellulolytic bacteria which had been previously cultured on *Eucalyptus viminalis* leaf in vitro. Similarly, while ether extracts of *E. viminalis* and *E. blakelyi* inhibited both "adapted" and "nonadapted" cellulolytic bacteria, extracts of *E. radiata* did not differ from controls even though these leaves (from the same batch as those used in experiment 3) contained substantial proportions of terpinen-4-ol and α-terpineol. Thus antimicrobial effects of essential oils may be due to synergistic effects of particular components (see also Akimov et al., 1977). Andrews et al. (1980) suggested that the antimicrobial action of terpenes results from disruption of cytoplasmic membranes and that gram-negative organisms are more resistant than gram-positive microbes. Nothing is known of the occurence of each of these groups in the hindgut of greater gliders and brushtail possums, although London (1981) found the cecal flora of the koala to be predominantly gram-positive.

The results from the present study indicate that in both the greater glider and the brushtail possum the microbial population in the hindgut is largely, although not completely, protected from the deleterious effects of *Eucalyptus* essential oils by their absorption mainly from the stomach and small intestine. However, the metabolic cost of detoxifying absorbed oils in the liver may limit the range of eucalypt species that these folivorous marsupials can utilize as a sole source of nutrients. It is suggested that future studies of leaf choice by arboreal folivores should take account of the levels of primary nutrients as well as allelochemicals in selected and rejected plant species.

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1,8-CINEOLE (EUCALYPTOL), A MOSQUITO FEEDING AND OVIPOSITIONAL REPELLENT FROM VOLATILE OIL OF *Hemizonia fitchii* (ASTERACEAE)[1]

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Abstract—The mosquito feeding and ovipositional repellency of the major monoterpenoid present in the volatile oil of *Hemizonia fitchii* (Asteraceae), i.e., 1,8-cineole, was investigated. Although 1,8-cineole did not exhibit any significant mosquito larvicidal activity, it was moderately effective as a feeding repellent and highly effective as an ovipositional repellent against adult *Aedes aegypti* (yellow fever mosquito). The ovipositional repellency of 1,8-cineole, coupled with the presence of several *Hemizonia* chromenes previously shown to possess mosquito larvicidal activity, may therefore account in large part for the observed suppression of local mosquito populations which was associated with *H. fitchii* plants in northern California.

Key Words—*Hemizonia fitchii*, Asteraceae, Compositae, 1,8-cineole, eucalyptol, mosquito repellent, *Aedes aegypti*, Diptera, Culicidae.

INTRODUCTION

In a systematic search for naturally occurring compounds that may be useful as sources and/or models of new insect control agents, we have been screening extracts from over 800 species of xeric higher plants, from the arid and semiarid regions of the western United States, for activity against several species of economically important pest insects. Xerophytes represent an important resource in the search for potentially useful bioactive compounds because such plants

[1] Part III in the series "Biologically Active Constituents of North American Plants." For Part II, see Klocke et al., 1986.

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are native to stressful environments where biochemical interactions among organisms via their secondary metabolites (antibiosis, allelopathy, etc.) may play a key role in survival, coevolution, and the maintenance of ecological balances (Harborne, 1982; Rodriguez, 1983; Myers, 1984). For example, we have isolated and identified insecticidal linear furanocoumarins from *Thamnosma montana* Torr. & Frem. (Rutaceae) (Klocke and Balandrin, 1984), insect growth-inhibiting polyphenolics (hydrolyzable tannins) from a variety of semiarid land plants (Klocke et al., 1986), and mosquito larvicidal chromenes from *Hemizonia fitchii* A. Gray (Asteraceae) (Klocke et al., 1985).

Our interest in *H. fitchii* (Fitch's spikeweed, tarweed) stems from field observations made in Butte County near Oroville, California, that this plant species was associated with reduced populations of the mosquito *Aedes melanimon* Dyar. Previous work (Klocke et al., 1985) established that these plants accumulate a series of volatile bioactive chromenes (Proksch and Rodriguez, 1983) that possess moderate larvicidal activity against *Culex pipiens* L. (the common house mosquito) and *Oncopeltus fasciatus* (Dallas) (the large milkweed bug). However, other constituents of the steam-distilled volatile oil of *H. fitchii*, including several volatile fatty acids and 1,8-cineole, were found to be essentially devoid of larvicidal activity against *C. pipiens* (Klocke et al., 1985).

In this communication, we have extended our investigation of the biologically active chemical constituents of *H. fitchii* to include the major monoterpenoid present in its volatile oil, i.e., 1,8-cineole, as a feeding and ovipositional repellent against the yellow fever mosquito, *Aedes aegypti* (L.).

METHODS AND MATERIALS

Collection and Extraction of Plant Material. Whole aerial parts of *H. fitchii* were collected in August, 1981, in Butte County near Oroville, California, by Mr. Elmer J. Kingsford (formerly with the Butte County Mosquito Abatement District, Oroville, California). A voucher specimen representing this collection has been deposited in the herbarium of NPI in Salt Lake City, Utah. Samples of the volatile oil of *H. fitchii* were prepared as described previously (Klocke et al., 1985) by subjecting the plant parts to steam distillation for 24 hr using a modified Clevenger-type circulatory steam-distillation apparatus (von Rudloff, 1969). Steam distillates were collected into diethyl ether, concentrated under a stream of nitrogen, and stored in tightly capped glass vials at −20°C.

Thin-Layer Chromatography (TLC) of Volatile Oil. Analytical and preparative silica gel TLC was carried out as described previously (Klocke et al., 1985). However, an additional method of visualization, involving spraying the TLC plates with 50% v/v aqueous sulfuric acid spray reagent followed by oven heating (15 min), was utilized.

Direct Insertion Probe Mass Spectrometry (MS). Electron impact mass spectral (EI-MS) data of the major constituents isolated from the volatile oil of *H. fitchii* were acquired using a Varian MAT (Florham Park, New Jersey) 112S double-focusing mass spectrometer operating at an ionization voltage of 70 eV.

For comparative purposes, an authentic sample of 1,8-cineole (eucalyptol) was purchased from Sigma Chemical Co. (St. Louis, Missouri) and was analyzed by TLC, GC, and GC-MS (Klocke et al., 1985).

Rearing of Aedes aegypti *Mosquitoes.* A colony of *Aedes aegypti* (L.) (Culicidae) Rockefeller strain was started using eggs supplied by Dr. George B. Craig, Jr., from the Vector Biology Laboratories of the University of Notre Dame, Notre Dame, Indiana. All stages of *A. aegypti* were reared at approximately 26°C in ambient humidity and photoperiod. Larvae were fed ground dried dog food. Adults were fed fresh orange slices and, after mating, the females took blood meals from anesthetized gerbils. Eggs were oviposited just above the water line on moist paper towels lining plastic 1-qt bowls filled with tap water. Eggs were collected, slowly dried, and used as needed (Smith, 1962).

Mosquito Antifeeding/Repellency Bioassay. To test the antifeeding/repellency properties of 1,8-cineole against adult female mosquitoes, two gerbils (in each of three replicate trials) were anesthetized [Vetalar (ketamine HCl, Parke, Davis & Co., Detroit, Michigan), 35–45 mg/kg intramuscular] and placed inside a piece of tubular stockinette, leaving only the hind legs and tail of each gerbil exposed. One gram of Vaseline (Chesebrough, New York, New York) was applied topically over the exposed areas of each control gerbil. One hundred milligrams of 1,8-cineole were incorporated into 1 g of Vaseline (i.e., 10% w/w), and this preparation was applied topically over the exposed areas of each experimental gerbil. Each gerbil was proffered to 1000–2000 starved (72 hr) adult mosquitoes of both sexes (from the same cohort) inside a BioQuip (Santa Monica, California) (18″ × 18″ × 18″) cage. The gerbils were removed after 25–30 min, and the number of engorged females in each cage was counted.

Mosquito Ovipositional Bioassays. To test the ovipositional repellency properties of 1,8-cineole against gravid female mosquitoes, both "choice" and "no-choice" ovipositional bioassays were conducted. The "choice" bioassay provided the gravid female mosquitoes with a choice of ovipositing on paper toweling lining glass crystallizing dishes (70 × 50 mm) containing either 1,8-cineole in tap water (at a concentration of 0.8, 0.4, or 0.2% w/v) or tap water only. The numbers of eggs laid during the course of the bioassay (9–11 hr) were counted in the dishes containing 0.8% and 0.4% w/v 1,8-cineole. The numbers of eggs laid in the dishes containing either 0.2% w/v 1,8-cineole or tap water only were too numerous to be counted and were therefore estimated gravimetrically (dry weight).

In the "no-choice" bioassay, the gravid females in each BioQuip cage were presented with a glass crystallizing dish containing either 1% w/v 1,8-

cineole in tap water or tap water only. The 1,8-cineole mixture and water were replaced with fresh 1,8-cineole mixture or water, respectively, every 9–11 hr until the conclusion of the experiment (approximately three to five days).

Egg Viability Assay. The viability of eggs oviposited in dishes containing 1% w/v 1,8-cineole in tap water was determined. The 1,8-cineole mixture was replaced daily with fresh 1% w/v 1,8-cineole in tap water during the period of egg laying, and for three days after the dishes had been removed from the cages, to ensure embryonation of all eggs. Groups of 100–150 eggs were then transferred to dishes containing tap water only, and the extent of hatching was recorded. Eggs oviposited in dishes containing tap water only served as the controls. The development of mosquitoes hatching from the 1,8-cineole-treated eggs and the control eggs was monitored throughout all stages of their life cycle.

RESULTS

Mosquito Antifeeding/Repellency Bioassay. The results of the antifeeding/repellency bioassay indicated that in all three trials, two to three times as many adult female mosquitoes fed upon the control gerbils (Vaseline only) as did those that were presented with the experimental gerbils (10% w/w 1,8-cineole in Vaseline) (Table 1). Female mosquitoes that landed on the control gerbils began probing the exposed areas with their proboscises within 2 min after the gerbils were presented to them, whereas the female mosquitoes alighting on the treated gerbils were repelled for approximately 16 min. After this time, the female mosquitoes began to feed upon the experimental gerbils, but they were observed to keep their heads as far from the gerbils as possible while feeding.

Mosquito Ovipositional Bioassays. The results of the "choice" ovipositional bioassay indicated that the gravid females were deterred from ovipositing in dishes containing 0.2% w/v or greater amounts of 1,8-cineole (Table 2). The numbers of eggs laid in the experimental dishes decreased with increasing concentrations of 1,8-cineole (Table 2). In all trials at the highest concentration of 1,8-cineole tested (i.e., 0.8% w/v), the gravid females laid very few eggs (an

TABLE 1. MOSQUITO ANTIFEEDING/REPELLENCY BIOASSAY

	No. satiated females in trial		
	1	2	3
Experimental (10% w/w 1,8-cineole)	12	14	56
Control	34	34	97

TABLE 2. MOSQUITO "CHOICE" OVIPOSITIONAL BIOASSAY

	1,8-Cineole (%, w/v)	No. eggs laid in trial		
		1	2	3
Experimental	0.8	19	6	64
Control	—	>1500	>1500	>1500
Experimental	0.4	51	789	338
Control	—	>1500	>3000	>3000
Experimental	0.2	10.1 mg[a]	22.9 mg	37.4 mg
Control	—	23.7 mg	58.0 mg	84.4 mg

[a] Eggs laid were too numerous to count. Therefore, results were determined gravimetrically (dry weight).

average of 30), whereas they consistently laid more than 1500 eggs in the control dishes (Table 2). At the lowest concentration of 1,8-cineole tested (i.e., 0.2% w/v), oviposition was still notably deterred. On average, 24 mg of eggs were oviposited in the experimental dishes containing 0.2% w/v 1,8-cineole compared to an average of 55 mg in the control dishes (Table 2).

In the "no-choice" ovipositional bioassay, oviposition by the gravid females was completely inhibited by 1% w/v 1,8-cineole in tap water and was delayed for as long as 24–36 hr compared to controls. After this delay, only a small number of eggs were laid in the experimental dishes. The eggs that were laid in the presence of 1,8-cineole were generally oviposited on the paper toweling along the outer rim of the experimental dishes, i.e., the point farthest from the main concentration of 1,8-cineole. This differed from the control eggs, which were typically laid just above the water line in the control dishes. However, normal oviposition resumed shortly after the dishes containing 1% w/v 1,8-cineole were replaced with dishes filled with tap water only.

Egg Viability Assay. There was no significant difference between the numbers of control and 1,8-cineole-treated eggs that hatched. Furthermore, the mosquitoes that hatched from the 1,8-cineole-treated eggs developed normally throughout all stages of their life cycle.

DISCUSSION

In our previous work (Klocke et al., 1985), we reported that compounds produced by *H. fitchii* plants appeared to suppress local mosquito populations in Butte County, in northern California. The three major chromenes produced and accumulated by *H. fitchii* were isolated and were shown by bioassay to be moderately toxic to *C. pipiens* (common house mosquito) larvae (Klocke et al.,

1985). The other major volatile constituents of *H. fitchii*, namely, the three volatile fatty acids (isobutyric, isovaleric, and 2-methylbutyric acids) and 1,8-cineole were found to be inactive as mosquito larvicides. However, we postulated that some of these volatile compounds might act as ovipositional repellents to mosquito adults (Klocke et al., 1985). In the present report, we provide evidence that 1,8-cineole, the major monoterpenoid in the volatile oil of *H. fitchii*, acts both as a feeding repellent and as an ovipositional repellent against *A. aegypti*.

Our results (Tables 1 and 2) show that 1,8-cineole is a stronger deterrent of oviposition than of feeding for female *A. aegypti*. The topically applied preparation of 10% w/w 1,8-cineole in Vaseline provided some measure of protection to the treated gerbils against female mosquitoes seeking a blood meal (Table 1). However, the feeding repellency afforded by 1,8-cineole lasted for only approximately 16 min. On the other hand, lower concentrations of 1,8-cineole (i.e., 0.2–0.8% w/v) resulted in a more pronounced and much longer lasting effect (9–11 hr) as an ovipositional repellent (Table 2).

The decreasing effectiveness of 1,8-cineole as a feeding repellent over time, and its concentration dependency as an ovipositional repellent (Table 2), may be due to losses incurred because of the volatility of the compound. The amount of 1,8-cineole vapor present in any given area naturally decreases over the time course of the experiment and in a concentration gradient over distance (space), perhaps to a point below a threshold level that deters female mosquitoes from feeding and ovipositing. For example, in the "no-choice" ovipositional bioassay, the female mosquitoes generally oviposited their eggs along the outer rim of the experimental dishes (i.e., the point farthest from the main concentration of 1,8-cineole), whereas the control eggs were typically oviposited just above the water line.

1,8-Cineole has previously been reported to repel American cockroaches (Verma and Meloan, 1981; Maugh, 1982; Scriven and Meloan, 1984) and Colorado potato beetles (Schearer, 1984; J.A. Klocke, K.J. Brown, and M.F. Balandrin, unpublished results). Against the leafhopper *Amrasca devastans* (Distant), 1,8-cineole was found to inhibit mating (Saxena and Kumar, 1984), but not oviposition (Saxena and Basit, 1982). Other insects are attracted by 1,8-cineole. For example, 1,8-cineole, as a constituent of many floral scents, may serve as a pollinator (and sex) attractant for certain male euglossine bees (Rodriguez and Levin, 1976; Harborne, 1982; Janzen et al., 1982; Romero and Nelson, 1986).

1,8-Cineole is present in certain essential oils isolated from plants in the families Asteraceae (Compositae), Magnoliaceae, and Rutaceae, and in certain arthropod defense secretions, all of which have been shown to be effective insect repellents (Rodriguez and Levin, 1976; Moore and Brown, 1981; Olagbemiro and Staddon, 1983; Schearer, 1984). In addition, other plant parts and

extracts containing significant amounts of 1,8-cineole have previously been reported to repel insects. For example, the Tasmanian blue gum tree (*Eucalyptus globulus* Labill., a native of Australasia) produces a pharmaceutical grade volatile oil (eucalyptus oil) that contains at least 70–85% 1,8-cineole (eucalyptol) (Swinyard and Lowenthal, 1975; Leung, 1980; Tyler et al., 1981). Eucalyptus oil enjoys a wide reputation as an insect repellent and insecticide in Australia and elsewhere (Cribb and Cribb, 1983). It has also been reported to be useful in insect (especially mosquito) repellent formulations (Jacobson, 1966; Der Marderosian, 1975; APhA, 1979), and it is reportedly effective as a mosquito repellent on human skin (Wilen and Wilen, 1984). *Eucalyptus* (i.e., *E. globulus*) leaves have been reported to repel fleas and other insects (Lust, 1974) and to possess insecticidal properties (Cribb and Cribb, 1983). Similarly, the leaves of the sweet bay laurel tree (*Laurus nobilis* L.), which contain an essential oil consisting of 30–50% 1,8-cineole (Leung, 1980), have been reported to be effective general insect repellents, with specific activity against moths, fleas (Lust, 1974), and cockroaches (Verma and Meloan, 1981; Maugh, 1982; Scriven and Meloan, 1984). Laurel leaves and berries, and their volatile oils, are also reportedly useful as weak insecticides (Stuart, 1982). Other notable sources of 1,8-cineole include cajeput (or cajuput) oil (50–65% 1,8-cineole), spike lavender oil (40–60% 1,8-cineole and camphor), and rosemary oil (ca. 47% 1,8-cineole) (Grieve, 1971; Leung, 1980; Tyler et al., 1981; Stuart, 1982; Duke, 1985). Cajeput oil and leaves exhibit mosquito and general insect repellent properties (e.g., against fleas and lice) (Jacobson, 1966; Stuart, 1982; Duke, 1985), and lavender and rosemary oils are sometimes used as moth repellents and externally as insect repellents (Morton, 1976; Leung, 1980; Stuart, 1982; Duke, 1985).

1,8-Cineole has a camphoraceous (camphor-like) odor, and volatile oils containing large quantities of this compound (e.g., eucalyptus oil, cajeput oil) have been used externally as ectoparasiticides and antiseptics (Swinyard and Lowenthal, 1975; Windholz et al., 1976, 1983; Leung, 1980; Tyler et al., 1981). The general insect repellent activity of 1,8-cineole may be mediated by a mechanism of action similar to that of camphor, a widely used moth repellent. Wright (1975) has theorized that mosquito repellents act by blocking, "plugging up," or "jamming" the olfactory receptors on the mosquitoes' antennae [see Dethier (1956) and Rodriguez and Levin (1976) for brief discussions of this topic]. If this is the case, then a number of volatile terpenoids that exhibit a camphoraceous odor and vapor may possess an insect repellent activity similar to that of 1,8-cineole and camphor. The similarities in chemical structure and physicochemical properties between 1,8-cineole and camphor are illustrated in Figure 1.

It is noteworthy that Australian/Tasmanian *Eucalyptus* trees have long been reported to be useful in the elimination of mosquito breeding places. In the past,

1,8-CINEOLE
(eucalyptol)

MW = 154 ($C_{10}H_{18}O$)
Colorless liquid,
bp 176-177°C (mp 1.5°C)
Density, 0.92-0.93

d-CAMPHOR

MW = 152 ($C_{10}H_{16}O$)
Translucent mass with
crystalline fracture,
mp 179.8°C, bp 204°C
(Sublimes appreciably at
room temp. and pressure)
Density, 0.99

FIG. 1. Comparison of the stereostructure and physicochemical properties of 1,8-cineole (eucalyptol) with those of the widely used moth repellent, camphor.

Tasmanian blue gum trees were introduced into the coastal and wet regions of southern and western Europe (especially in the Mediterranean regions of Italy and Spain), Africa, the Middle East, India, and many other parts of the world that serve as breeding places for malarial mosquitoes. These very large and rapidly growing trees, which have large water requirements, have been found to be extremely useful in draining marshy areas and in reclaiming swampy lands, making them mosquito- and malaria-free areas within a few years. In Spain and elsewhere, where *E. globulus* has been associated with malaria reduction, it is known as the "fever tree." In the past, widely publicized successes in malaria-ridden areas have been attributed almost exclusively to the soil drainage effects caused by the large water requirements of the *Eucalyptus* trees (Grieve, 1971; Stuart, 1982; Cribb and Cribb, 1983; Bianchini et al., 1985). The ability of these trees to dry up wet lands is indisputable.

In light of our new findings, the successes achieved in destroying mosquito breeding places with the planting of *Eucalyptus* trees may also be at least partly attributable to the effects of 1,8-cineole and related (camphor-like) volatile terpenoids. Local saturation of water, air, and possibly soil particles with 1,8-cineole might have a significant ecological effect in reducing mosquito populations by virtue of the effectiveness of the compound as an ovipositional mosquito repellent. It has previously been shown that ecologically significant concentrations of 1,8-cineole are produced under natural conditions by certain aromatic plants (including *Eucalyptus* spp.) in California and elsewhere. Eco-

logically significant concentrations of 1,8-cineole are present in the atmosphere surrounding these plants and adsorbed onto colloidal soil particles in their immediate surroundings (Harborne, 1982). In the case of *H. fitchii*, it is possible that the nearby pond water and local atmosphere (where these plants were found growing) were saturated with 1,8-cineole. Thus, the ovipositional repellency of 1,8-cineole, coupled with the larvicidal activity of the *Hemizonia* chromenes (Klocke et al., 1985), may explain in large part the observed suppression of local mosquito populations which was associated with *H. fitchii* in northern California.

1,8-Cineole (eucalyptol) has been widely used (either as a pure compound or as the major constituent of eucalyptus oil) as an antiseptic, external analgesic, inhalational expectorant and antitussive, and as a nasal and bronchial decongestant in well-known over-the-counter proprietary pharmaceutical formulations, and as a flavoring agent in foods and toothpastes (Swinyard and Lowenthal, 1975; Windholz et al., 1976, 1983; APhA, 1979; Leung, 1980; Tyler et al., 1981). Since 1,8-cineole is approved and is currently being used commercially in products for inhalational, oral, and external use, the compound may be safe for use in insect repellency products (e.g., as a mosquito feeding repellent for humans and as a flea repellent for pet cats and dogs) and/or as an ovipositional repellent in potential mosquito breeding places. However, before widespread commercial use of the insect repellent properties of 1,8-cineole can be realized, certain questions concerning its safety and effectiveness need to be addressed, particularly its potential attractiveness to stinging insects, its potential for excessive percutaneous skin absorption, its extreme volatility, and its potential toxicity towards nontarget organisms. Careful product formulation may help to overcome such difficulties.

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INTERACTIONS OF PHEROMONE COMPONENT ODOR PLUMES OF WESTERN PINE BEETLE[1,2]

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Abstract—The relationships between catch of *D. brevicomis* LeC. at sources of the synergistic pheromone components, *exo*-brevicomin (E) and frontalin (F), and increasing distance of separation of sources were investigated in the forest. The two components were each released with the host monoterpene, myrcene (M), in trap pairs. The traps of each pair were spaced apart at various distances (0–16 m) in either horizontal or vertical lines that were perpendicular to the mean wind direction. Both sexes were most strongly attracted when the two components were released from the same source, and increasing distance of separation between components caused exponential decreases in trap catch for all trap configurations. Males were significantly more attracted to traps with E, M alone than to corresponding traps with F, M alone, while females exhibited a preference for F, M. The theoretical relationships and properties of two coalescing plumes of individual components and their intersecting "active space" are presented and discussed. It is proposed that "confusion" or "communication disruption" techniques for insect control may be more successful if components are released individually from many points rather than released similarly in blends.

Key Words—*Dendroctonus brevicomis*, Coleoptera, Scolytidae, pheromone, synergism, plumes, *exo*-brevicomin, frontalin, myrcene.

[1] *Dendroctonus brevicomis* LeC. (Coleoptera: Scolytidae).
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INTRODUCTION

To date there has been very little research on the theoretical and biological consequences of physically separating the release points of individual pheromone components that normally would be released together. There are several reasons why research on the interactions of pheromone component odor plumes is of interest to chemical ecologists. From a practical standpoint, it may be that "confusion methods," which disrupt pheromone communication, are more effective if synergistic components are released separately in disjointed blends than if full blends are released. From a field test design standpoint, it is of importance to understand how far apart traps of different component blends must be placed in order to minimize interactions and yet take advantage of "homogeneous" populations levels found in a particular area. Finally, it is of basic interest to determine the physical dimensions of synergistic component interaction for various species and their sexes.

There are two major ways that a semiochemical treatment in a trap can interfere with the catches of insects in nearby traps containing different treatments. In the first way, semiochemicals can affect the responses of insects at distances from the source that include the adjacent traps. For instance, traps releasing high concentrations of attractants may cause high numbers of insects to enter the vicinity and some could be caught incidentally on nearby traps and thus artificially increase their catches. Schlyter et al. (1987a) found that high-releasing pheromone traps did increase the catch of the bark beetle *Ips typographus* on nearby blank traps as well as lower-releasing traps, presumably by attracting large numbers of beetles into the area. They also found that the catch on the adjacent traps became increasingly greater as the distances between traps were decreased. The second way that semiochemical treatments can affect the catches on adjacent traps is by synergistic component odor plume interactions. This has not been studied with insects in the field and is the focus of this paper.

The Western pine beetle *Dendroctonus brevicomis* LeConte (Coleoptera: Scolytidae), provides an appropriate system for investigations into the interactions of pheromone component odor plumes because of its sex-specifically-produced synergistic components. The female begins the colonization of ponderosa pine (*Pinus ponderosa* Doug. ex. Laws.) when she bores through the outer bark and excavates a gallery in the phloem layer. She produces a pheromone synergist, *exo*-brevicomin (E), which accumulates in her hindgut upon feeding (Pitman et al., 1969; Hughes and Renwick, 1977; Byers et al., 1984) and is released with the fecal pellets in the frass (Silverstein et al., 1968). Although Libbey et al. (1974) found that females in glass tubes could release small amounts of E without feeding, it appears most E is released during feeding. A male attracted to the entrance tunnel soon releases frontalin (F) (Kinzer et al., 1969; Libbey et al., 1974; Browne et al., 1979), and it appears he is capable

of doing so immediately upon defecation (Byers et al., 1984). F, together with E, synergistically enhances the attraction of both sexes, with a small further increase due to the host resin monoterpene, myrcene (M) (Wood et al., 1976; Bedard et al., 1980). This synergism results in a dramatic increase in beetle visitation and cooperative "mass attack" which functions to overcome the resinous defense mechanisms of the host tree (Wood, 1982).

In several earlier reports, the female-produced E was found to attract primarily males, while the male-produced F attracted mostly females (Bedard et al., 1969; Vité and Pitman, 1969a, b; Pitman and Vité, 1971; Hughes and Pitman, 1970). However, it must be understood that release of F alone probably does not occur in nature because males always join females in their galleries. On the other hand, it is possible for males to release F upon landing on the tree as large quantities are present in their hindguts (Byers et al., 1984). Vité et al. (1972) proposed that *D. brevicomis* could release pheromone upon landing with a "contact pheromone" as opposed to the feeding-dependent pheromone components of many *Ips* species ("frass pheromone"). Wood et al. (1976) determined that only the (+)-E and the (−)-F enantiomers were bioactive. Furthermore, the presence of inactive enantiomers in the racemic mixtures did not cause inhibition or inactivation.

The objectives investigated here are: (1) What are the relationships between the distance of separation, either horizontal or vertical, of release points of the synergists, E and F, and the attraction of each sex to these sources? (2) What are the theoretical mathematical relationships among the ratios of the pheromone component concentrations at various places in the intersecting odor plumes for varying degrees of intersection?

METHODS AND MATERIALS

Interacting Exo-*brevicomin (E) and Frontalin (F) Odor Plumes.* All experiments were conducted at two sites (A and B) in the Sierra National Forest near Bass Lake, Madera County, California, at about 1000 m elevation. In September 1978, a test was conducted at site A with pairs of tubular sticky traps (19 cm diam. × 30.5 cm high, 6.3-mm wire mesh coated with Stickem Special), each supported on a pipe standard 1.2 m above the ground. One trap of a pair contained a release device (Byers and Wood, 1980) that had a glass tube (52 mm × 3.5 mm ID) with E (>95% and no F by GLC) and another with myrcene (M) (>97%), while the other trap contained M and a glass tube (62 mm × 2.2 mm ID) with F (>95% with no E by GLC). The chemicals from Chem. Samples Co., Cleveland, Ohio, were each released at about 2 mg/day under field conditions (Browne, 1978) or somewhat less at about 1.5 mg/day according to Tilden and Bedard (1985). Eleven 1-day-long, replicates were ob-

tained on each of five trap pairs horizontally spaced apart at distances of 0, 2, 4, 8, and 16 m. The trap pairs were oriented perpendicular to the mean wind direction and were randomized in different areas, which were at least 25 m apart, each day. Beetles were collected each day, cleaned in solvent, and sex was determined.

In August 1985, a similar test at site B was conducted for six, 1-day-long, replicates but at distances of 0, 0.5, 1.5, and 3 m between traps in a pair. At the same time, at site B, a test of trap pairs (baited the same way) vertically spaced apart at distances of 0, 0.5, 1.5, and 3 m was conducted. The "bottom" traps were placed at 1.2 m height with the "top" trap at the respective distances above the bottom trap. Trap pairs were switched each day (1-day-long replicates) so that top and bottom placements occurred equally among the treatments, while randomization of trap pairs between areas occurred every second day, three times.

Treatment effects within pairs were analyzed by Wilcoxon matched-pair tests both for total catch and male–female numbers. The sex ratios of catch on treatments within and between trap pairs were determined as were 95% binomial confidence limits (Byers and Wood, 1980), and chi-square tests were performed to test for differences. A nonlinear relationship between trap catch and distance of component separation may be expected based on the nature of the wind–plume interactions over time. Therefore, several linear and nonlinear regressions were performed to determine which curve best fits the data (linear, logarithmic, exponential, power, $Y = a + bX^2$, and $Y = a + b/X$). This was done to be able to predict catches at various distances not tested here. Exponential regression was found to fit the data best (highest r^2 values) and was used to draw the lines in Figures 1–3.

Theoretical Model of Interactions of Odor Plumes. Elkinton and Cardé (1984) show the Gaussian distribution of the "active space" of pheromone concentration for a 3-min time-averaged plume as modified from Slade (1968). In Figure 4, two such plumes have been intersected. Based on the understanding of this figure, one can theorize about the concentrations of each component when the component odor plumes have specified standard deviations and distances of separation. It can be assumed that both E and F, which have similar molecular weights and volatilities, will have similar Gaussian plumes. Thus, the shape of the intersection area of the plumes where both compounds are present (Figure 5) and the intersection midline where the ratio is 1:1 (H) can be calculated iteratively by computer from the normal probability density function:

$$\int \text{rel} = 1/(\sigma\sqrt{2\pi})e^{-(x-\mu)^2/(2\sigma^2)}$$

where σ is the standard deviation (1), μ is the mean (4.5 or 7.5), and x is any point on the x axis. The above equation can be used to solve for $H = (\int \text{rel})$ at

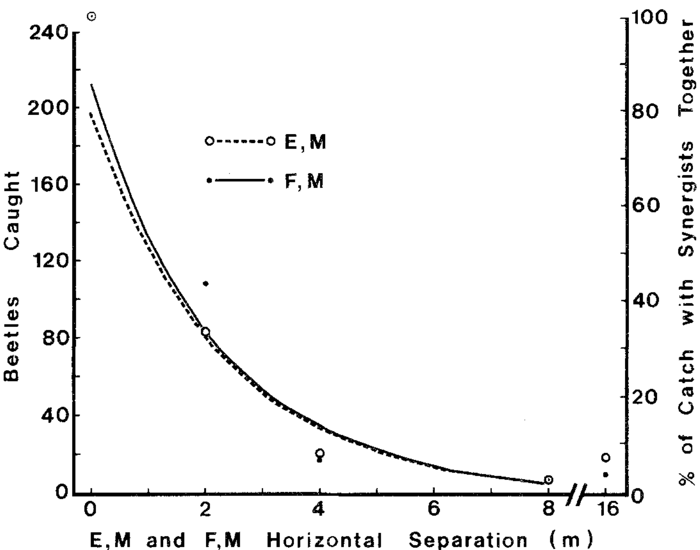

FIG. 1. Reduction in catch of *D. brevicomis* at sources of the pheromone synergists *exo*-brevicomin (E) and frontalin (F) with increasing horizontal distance of separation between sources. Each point represents a total of 11 one-day replicates. The pheromone components, each with myrcene (M), were each released at about 1.5 mg/day from sticky traps at 1.5 m height in the Sierra National Forest, California (September 17–28, 1978).

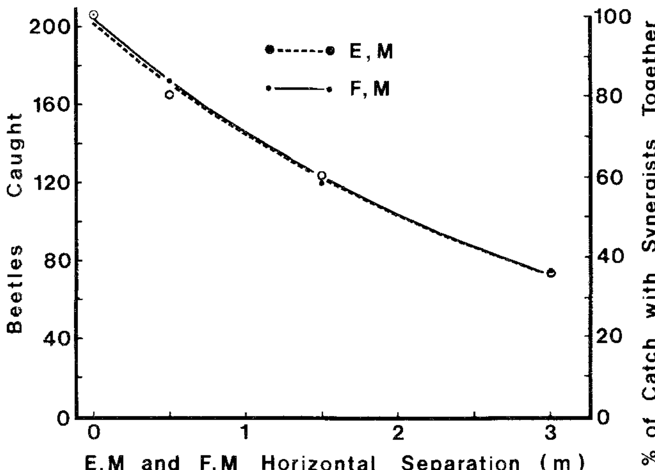

FIG. 2. Reduction in catch of *D. brevicomis* at sources of the pheromone synergists *exo*-brevicomin (E) and frontalin (F) with increasing horizontal distance of separation between sources. Each point represents a total of six one-day replicates. The pheromone components, each with myrcene (M), were each released at about 1.5 mg/day from sticky traps at 1.5 m height in the Sierra National Forest, California (August 20–26, 1985).

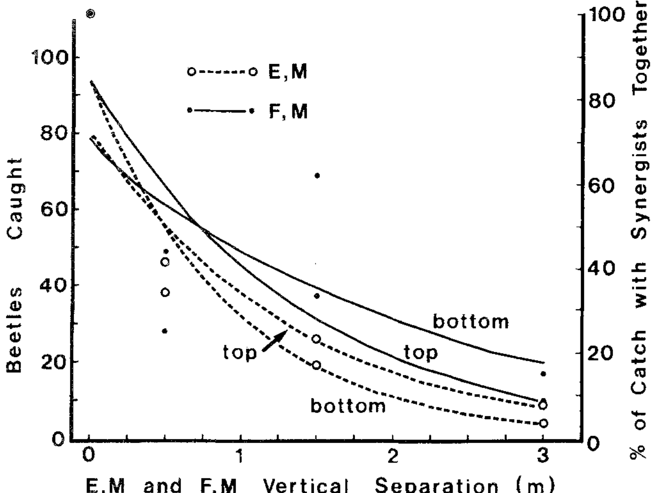

FIG. 3. Reduction in catch of *D. brevicomis* at sources of the pheromone synergists *exo*-brevicomin (E) and frontalin (F) with increasing vertical distance of separation between sources. Each point represents a total of three 1-day replicates. The pheromone components, each with myrcene (M), were each released at about 1.5 mg/day from sticky traps at 1.5 m (bottom trap) or more height (top trap) in the Sierra National Forest, California (August 20–26, 1985).

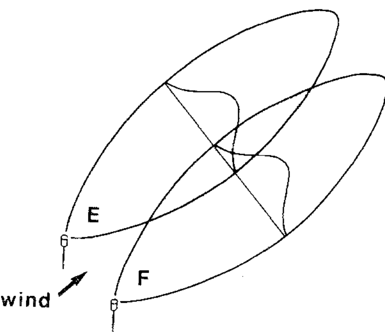

FIG. 4. Diagrammatic representation of the "active space" intersection area (shaded area) of two synergistic Gaussian plumes with 3-min averaging times (modified from Elkinton and Cardé after Slade, 1968).

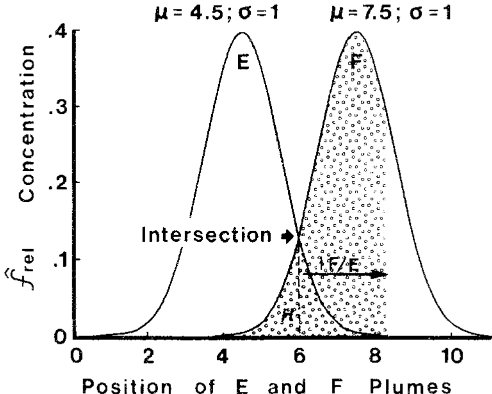

FIG. 5. Graphic representation of the cross-section of the concentration distributions of two Gaussian plumes of the pheromone synergists (E and F). The "active space" is related to the height of the dashed line (H) and the width of the shaded area, and it does not have a Gaussian distribution as do E or F with means of 4.5 or 7.5 and standard deviations of 1. Flight-path deviations along the x axis, for example to the right, would increase the ratio of F/E dramatically and unnaturally compared to natural pheromone plumes.

$x = 6$. The height of the intersection or point where both components are equal (H) can be iteratively calculated for varying degrees of plume intersection (Figure 6) with the following BASIC program that utilizes the above equation:

```
FOR W = I + M TO I + M + S STEP SS:
    Y = 1/(SD*SQR(2*PI))*EXP (1)^(-((I - W)^2)/(2*SD^2)):
    X = 2*M + (W - (I + M))*2:PRINT X, Y:NEXT W
```

where I is the intersection point (e.g., 6), M is the starting distance of either mean to intersection, S is the maximum distance means will be separated, SS is the step size on x axis for each calculation, SD is the standard deviation of the plumes (equal for both plumes), X is the x axis value or separation of component plumes, and Y is the y axis value or H of the intersection midline.

The ratio of F to E that an insect would encounter as it traverses laterally over the plume intersection area is depicted in Figure 5. The precise relationship beginning at a ratio of 1 at the intersection point (H) and moving to the right is shown in Figure 7. The results were calculated iteratively by computer assuming that the E and F plumes have equal standard deviations but the following equation will also work on unequal variances:

$$\text{Ratio F/E} = [1/(\sigma_F\sqrt{2\pi})e^{-(x-\mu_F)^2/(2\sigma_F^2)}]/[1/(\sigma_E\sqrt{2\pi})e^{-(x-\mu_E)^2/(2\sigma_E^2)}]$$

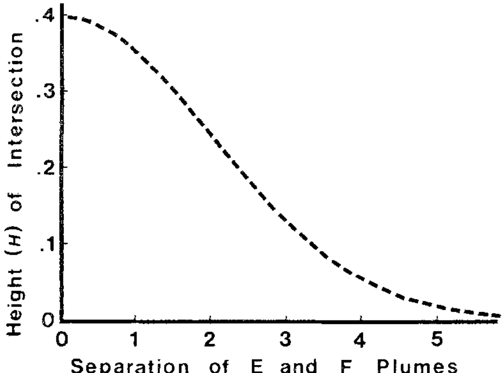

FIG. 6. Gaussian relationship between height of the intersection midline (concentration) of two separated synergist plumes (dashed line in Figure 5) and the separation distance between the longitudinal axes of the plumes.

where σ_F and σ_E are the standard deviations of F and E, respectively, μ_F and μ_E are the means of F and E, respectively, and x is any point on the x axis. Exponential regression was then performed on the results to obtain the equation of the curve in Figure 7.

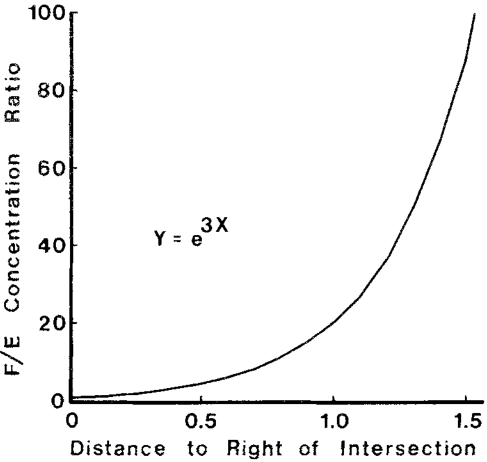

FIG. 7. Exponential relationship between the ratio of F/E concentrations (Figure 5) within two intersecting synergist plumes and the distance to the right of the longitudinal intersection midline (dashed line in Figure 5) of the plumes.

RESULTS

Interacting Exo-*brevicomin (E) and Frontalin (F) Odor Plumes.* The catches of *D. brevicomis* on E, M and F, M trap pairs spaced apart horizontally at various distances from 0 to 16 m in 1978 are shown in Figure 1. The points at 16 m were not used in calculating the exponential regression curves because these values were not appreciably different from those at 8 m, indicating that interaction effects were negligible at distances >8 m. It can be seen that trap catches on E, M ($Y(\%) = 79.62e^{-0.448X}$, $r^2 = 0.95$) were similar to corresponding catches on F, M ($Y(\%) = 85.64e^{-0.461X}$, $r^2 = 0.92$) within a pair at any separation distance. The interactions between the E and F synergists were insignificant when the distance of their separation was increased farther than 4 m. When the horizontal separation test was repeated in 1985 at distances from 0 to 3 m, a similar relationship occurred (Figure 2). Again the trap catches on E, M ($Y(\%) = 97.91e^{-0.334X}$, $r^2 = 1.00$) were almost identical to corresponding catches on F, M ($Y(\%) = 98.83e^{-0.337X}$, $r^2 = 1.00$).

In the 1985 test of E, M and F, M trap pairs spaced apart vertically at distances from 0 to 3 m, the results have been presented with respect to the special symmetry of "top" and "bottom" (Figure 3). The catches on top and bottom traps with either E, M or F, M are similar, although F, M appears to have attracted more beetles. The relationships between percent of catch when synergists were together and the vertical separation distance for the respective traps are given by the following exponential equations: top F, M ($Y(\%) = 85.25e^{-0.738X}$, $r^2 = 0.95$); bottom F, M ($Y(\%) = 69.13e^{-0.452X}$, $r^2 = 0.49$); top E, M ($Y(\%) = 72.75e^{-0.754X}$, $r^2 = 0.92$); bottom E, M ($Y(\%) = 85.14e^{-1.067X}$, $r^2 = 0.99$).

Although the total (male + female) catches appear similar on E, M and F, M traps in the experiments (Figures 1–3), there are significant, but no dramatic, male–female differences as reflected in the Yates chi-square test and Wilcoxon matched-pair test (Table 1). In most cases, the F, M trap had a lower sex ratio (proportionately less males) than the E, M trap within a pair, both for horizontally and vertically placed traps (Table 1). The Wilcoxon tests also showed, in many cases, that the distribution of male, or female, catches on the replicates within a trap pair were significantly different (Table 1). A comparison of E, M and F, M vertical pairs with the Wilcoxon test, without regard to position, showed that at 1.5 m separation the female as well as the total catches had significantly different distributions on the synergists. Also at 3 m separation, the male, female, and total catches had significantly different distributions ($P < 0.05$, Wilcoxon). The sex ratios and (95% binomial confidence limits) for the vertical pairs are: E, M (≥0.5 m) = 0.84 (0.64–1.10) and F, M (≥0.5 m) = 1.54 (1.10–2.15), which were different at $P < 0.05$, Yates χ^2.

Furthermore, when the total catch on F, M traps is compared to E, M traps

for all horizontally or vertically paired traps (separated by at least 0.5 m), there are significant differences in the sex ratios in all cases except the "top" vertical traps (Table 1). In two of the four cases, the sex ratio on F, M was significantly different from the control, while in the other two cases, the sex ratio on E, M was significantly different. From the sex ratios alone it is impossible to determine if males or females or both prefer one of the synergists, but the Wilcoxon tests indicate that males prefer E, M while females prefer F, M. The sex ratios were virtually identical on the top and bottom traps, 1.10 (0.83–1.47) and 1.04 (0.77–1.40), respectively. However, these ratios were significantly different from the control where the synergists were placed together ($P < 0.05$), although they were not different from the "horizontal control" which was placed in the same area at the same time (Table 1).

Temnochila chlorodia (Mannerheim), a predator of *D. brevicomis* and known to be attracted to E (Bedard et al., 1969), was caught in 1978 only on the traps containing E, M (11 beetles) except for one caught on F, M at the 2-m spacing. In 1985 on the horizontal traps, *T. chlorodia* were caught only on the E, M-containing traps (16 males, 6 females) except for two males and one female on the F, M traps at 1.5 m. The vertical traps caught these predatory beetles again only on traps with E, M (18 males, 6 females) except for one male and two females on F, M at 1.5 m and one female on F, M at 0.5 m.

Theoretical Model of Interactions of Odor Plumes. In Figure 4, two 3-min time-averaged plumes are shown coalescing when the mean wind direction is perpendicular to the two sources. The "active space" (cf. Elkinton and Cardé, 1984) where the two components (E and F) are both above the threshold of response (cf. Roelofs, 1978) is shown in the shaded area (Figure 4). The two bell-shaped curves represent the Gaussian distribution of concentration for each component. The "active space" as shown in Figure 4 actually is fundamentally different from the normal active space in which both components vary similarly based on a Gaussian distribution. In Figure 5 the relative concentrations of each component along the latitudinal axis are shown and demonstrate the peculiar properties of the intersection area of the two plumes. The intersection midline where the two components are of equal concentration (presumed most favorable for orientation) is represented by *H* and the dashed line (Figure 5). If the two odor sources (shown in Figure 5) are placed together and then physically separated until the two plumes do not intersect, then the relative concentration at the intersection midline (*H*) decreases, as shown in Figure 6, according to a normal curve.

Referring to Figure 5, if an insect were flying upwind along the intersection midline and then veered more into the F odor plume, then the ratio of F/E would increase exponentially according to Figure 7 ($r^2 = 1$). The exponential increase in the ratio would continue even if the insect flew to the outside (right) of the F concentration peak. Exponential relationships result for all Gaussian distri-

TABLE 1. SEX RATIOS OF CATCH OF *D. brevicomis* ON HORIZONTAL OR VERTICAL TRAP PAIRS SPACED DIFFERENT DISTANCES APART AND CONTAINING PHEROMONE COMPONENTS F, M (FRONTALIN + MYRCENE) OR E, M (*exo*-BREVICOMIN + MYRCENE)

Trap, (separation distance, m)	Horizontal traps		Vertical traps—1985	
	1978	1985	Bottom	Top
	♂/♀ (95% BCL)[a]	♂/♀ (95% BCL)	♂/♀ (95% BCL)	♂/♀ (95% BCL)
E, M + F, M (0)	0.68 (0.57–0.82)	1.03 (0.85–1.25)	0.67 (0.56–0.81)[b]	0.67 (0.56–0.81)[b]
F, M (0.5)		1.05 (0.78–1.41)	1.00 (0.48–2.06)[d]	2.06 (1.14–3.72)
E, M (0.5)		1.23 (0.91–1.67)[2]	3.60 (1.81–7.16)[d,e]	1.11 (0.59–2.08)
F, M (1.5)		1.11 (0.77–1.58)[2]	0.57 (0.35–0.92)	0.68 (0.36–1.30)
E, M (1.5)		1.58 (1.11–2.27)[2,e]	1.71 (0.70–4.22)	1.17 (0.55–2.40)
F, M (2)	0.48 (0.32–0.72)			
E, M (2)	0.60 (0.38–0.93)			
F, M (3)		0.70 (0.45–1.11)[1,2,d]	0.55 (0.21–1.42)	0.43 (0.12–1.52)
E, M (3)		3.11 (1.84–5.26)[1,2,d,e]	3.00 (0.43–20.94)	0.12 (0.02–0.77)
F, M (4)	0.70 (0.28–1.78)			
E, M (4)	1.50 (0.63–3.57)			
F, M (8)	7♀[1,2]			
E, M (8)	2.50 (0.56–11.2)[1,2]			
F, M (16)	0.12 (0.02–0.77)[1]			
E, M (16)	1.57 (0.63–3.93)[1]			
Totals:				
F, M ≥ 0.5 m	0.44 (0.31–0.63)[d,e]	0.98 (0.80–1.21)[d]	0.65 (0.45–0.95)[d]	1.13 (0.76–1.69)[e]
E, M ≥ 0.5 m	0.85 (0.61–1.21)[d]	1.59 (1.29–1.97)[d,e]	2.83 (1.67–4.82)[d,e]	0.92 (0.58–1.45)
Both ≥ 0.5 m	0.61 (0.48–0.78)	1.25 (1.08–1.44)	1.10 (0.83–1.47)[e]	1.04 (0.77–1.40)[e]

[a]95% binomial confidence limits for sex ratios (Byers and Wood, 1980).
[b]Combined catches for top and bottom traps that were placed together.
[c]1, 2, and 3 indicate the treatments within a trap pair differed in their distributions of males (1), females (2), or total (3) caught, $P < 0.05$, Wilcoxon matched-pairs test.
[d]Indicates the sex ratios within a trap pair were significantly different, $P < 0.05$, chi square with Yates.
[e]Indicates the sex ratio was significantly different from E, M + F, M (0 m) control, $P < 0.05$, chi square with Yates.

butions with equal variances. Ratio changes also occur for distributions with unequal variances, but the relationships are more complicated (but can be calculated with the ratio equation).

DISCUSSION

The release of E, M and F, M from separate points situated either vertically or horizontally might appear to be possible in nature on standing or fallen trees. However, a "pioneer" female would release E, M in small amounts only until she was joined by other females and males during the initial stages of the mass attack. Based on the results presented here, she would most likely attract a male to the area. Once both attractants are released from the pioneering pair, then a "balanced" sex ratio occurs (Vité and Pitman, 1969a). As mentioned above, several earlier studies, and the present one, indicate that relatively more males are attracted to E, E, M, or E + oleoresin while more females are attracted to F, F, M, or F + oleoresin. However, it must be noted that the respective attraction rates to each component were much less than to the synergistic blend.

There are no apparent differences between the sexes in attraction to a concentration range of E, F, M (1 : 1 : 1) covering five orders of magnitude in the laboratory (Byers and Wood, 1981) and three orders in the field (Tilden and Bedard, 1985). Thus, it is difficult to find an explanation for the sexual preferences for the respective components. It would not seem adaptive for a male to release F unless he was with a female in her gallery, and males are not known to initiate attacks and "call" for females. Therefore, it would appear females never have the opportunity to respond to F alone. Although Libbey et al. (1974) did find F was released from male + male confinement in glass tubes, males never are found in galleries in this way (Byers et al., 1984). On the other hand, the male preference for E could possibly be adaptive if males use it to seek females after landing on the tree. Could it be that these sexual preferences are the "behavioral remnants" of a previous era when the sexes sought each other (true sex pheromones)? Later in evolution each sex could have relied on the opposite-sex component as the primary one that is synergized by the same-sex produced component. Analysis of the attraction response of each sex to various component ratios may indicate which component is synergizing which component or whether they are mutually synergistic (Byers, 1987).

Linn and Gaston (1981) have performed wind-tunnel tests with male cabbage looper moths in which two pheromone component plumes were allowed to intersect between 35 and 85 cm downwind of the sources. They found no effect on upwind orientation by separating the components 12 cm apart (85-cm downwind plume intersection), but when the separation was only 8 cm (35-cm downwind intersection) there were significantly less moths approaching the

sources. However, one of the components is attractive alone while the other is not, so the case is different than if neither component is significantly active alone, as in the Western pine beetle. The male moths were apparently "confused" as they flew out of the intersecting plumes (removal of the inactive-alone component 35 cm downwind) during their upwind flight to the active-alone component, as if they had suddenly passed by a female (full blend). When the plumes were further apart, the moths were essentially only flying up the active-alone plume, presumably in a long-range orientation mode.

In field studies with the Western pine beetle, the relationships between catch and component separation distance (Figures 1–3) have underlying causes which are apparently very complex. In nature the wind direction can vary widely and, although the trap pairs were placed perpendicular to the prevailing wind, sometimes the wind carried the odor of one component directly through the source of the other component (this error should not have occurred with the vertically placed trap pairs). Considering a constant perpendicular mean wind direction, however, there are several differences between the geometries of the active spaces of an idealized plume of a two-component blend and that of two coalescing single-component plumes. For the coalescing plumes (Figure 5), the active space is narrower and shorter than in a normal blend, and the "head" of the active space is far from either source and oriented between the sources compared to a normal blend with the "head" at the source. Another difference is due to the fact that the periphery of a plume is more variable in time with regard to concentration (Elkinton and Cardé, 1984), and it is the peripheries of plumes that comprise the active space of coalescing plumes. Finally, Figure 5 shows the concentration ratios of the two components for a particular degree of plume intersection. This is quite different from the case with the normal blend where both components have corresponding Gaussian distributions. Significant effects on behavior of various component ratios are well known in moths (Roelofs, 1978; Roelofs and Cardé, 1977; Baker and Cardé, 1979) and, more recently, in bark beetles (Schlyter et al., 1987b; Tilden and Bedard, 1985; Byers, 1987). The theoretical relationships have been considered for time-averaged plumes which are, in fact, filamentary in an instantaneous sense (Okubo, 1980), but the theories here would still generally apply to such plumes.

The concept of using multicomponent pheromones in a blend that is released from many scattered sources in the field for mating disruption and insect control is well established. However, the field and theoretical results presented here indicate that release of the individual components from a mosaic mixture of sources may be more disruptive of olfactory communication than use of full blends. It is hoped that the considerations presented here on interacting component plumes will inspire new types of experiments at both basic and applied levels.

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DETERMINATION OF CHIRALITY IN 5-HYDROXY-4-METHYL-3-HEPTANONE, THE AGGREGATION PHEROMONE OF *Sitophilus oryzae* (L.) AND *S. zeamais* Motschulsky[1]

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Abstract—The chirality of the pheromone of the rice weevil, *Sitophilus oryzae* (L.), and the maize weevil, *S. zeamais* (Motschulsky), 5-hydroxy-4-methyl-3-heptanone, was determined using an acetyl lactate derivatization procedure. Maize weevils were shown to produce >98% 4*S*,5*R*. Determination was more difficult with rice weevils due to a smaller quantity of insect extract, but they were shown to produce at least 92% 4*S*,5*R*.

The attractancy of the four synthetic stereoisomers of 5-hydroxy-4-methyl-3-heptanone was tested using rice and maize weevils. As expected, both species were most strongly attracted to the 4*S*,5*R* enantiomer. Maize weevils also showed low but significant responses ($P < 0.05$) to both 4*R*,5*R* and 4*S*,5*S*. Rice weevils showed a highly significant ($P < 0.01$) response to 4*R*,5*S*, although it was only about one third the response to 4*S*,5*R*. Thus, (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone is clearly the major component of the pheromone of both *S. zeamais* and *S. oryzae*.

[1]This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation for its use by USDA.

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Key Words—Chirality, enantiomers, 5-hyroxy-4-methyl-3-heptanone, stereoisomer, *Sitophilus oryzae*, *Sitophilus zeamais*, rice weevil, maize weevil, aggregation pheromone, Coleoptera, Curculionidae.

INTRODUCTION

Sitophilus spp. weevils cause severe damage to cereal grains throughout the world, through direct feeding on grain kernels. The male-produced aggregation pheromone of the rice weevil, *Sitophilus oryzae* (L.) and the maize weevil, *S. zeamais* Motschulsky, has been identified as (R^*,S^*)-5-hydroxy-4-methyl-3-heptanone,[2] of unknown enantiomeric composition (Schmuff et al., 1984; Phillips et al., 1985). Some response to the R^*,R^* diastereomer by both species was observed in the laboratory, but this compound was present at less than 0.5% in natural pheromone samples.

5-Hydroxy-4-methyl-3-heptanone possesses two asymmetric centers and thus can exist as four different stereoisomers (Figure 1). The asymmetric center at position four is adjacent to the ketone and as such is susceptible to racemization by either acid or especially base. The hydroxyl group beta to the ketone makes the molecule even more labile in that retro aldol cleavage or facile dehydration can occur with complete loss of stereochemistry. At the outset of this work we were unsure as to whether these properties would prevent the determination of the chirality through racemization or elimination of the required acetyl lactate diastereomeric ester. The objective of this work was to determine the natural enantiomeric ratios in maize and rice weevils, and to examine the ability of both species to distinguish between the four synthetic stereoisomers.

METHODS AND MATERIALS

Insects. Rice and maize weevils were obtained from laboratory stock cultures and were reared according to procedures previously described (Phillips and Burkholder, 1981; Walgenbach et al., 1983).

Weevils for experimental use were collected on day of emergence by sieving and were separated by sex according to dimorphic rostral characteristics (Halstead, 1963). Weevils were held on wheat at constant densities on a 16:8 light–dark regime and 27° ± 1°C and 65 ± 5% relative humidity.

Extracts. Collections of pheromone from individual virgin male maize and rice weevils were made as previously described (Phillips and Burkholder, 1981). Parallel collections from cracked wheat controls were made at the same time.

[2] R^*,S^* represents an unknown mixture of the $4R,5S$ and $4S,5R$ enantiomers. Similarly, R^*,R^* represents an unknown mixture of $4R,5R$ and $4S,5S$.

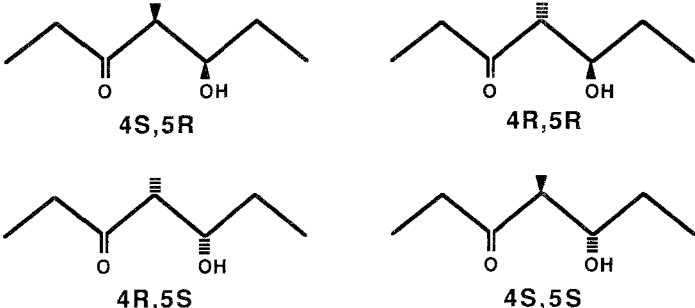

FIG. 1. Stereoisomers of 5-hydroxy-4-methyl-3-heptanone.

Glass wool-filtered hexane extracts containing 1152 insect-day equivalents (IDE) (two samples) for maize weevils, 1464 and 1500 IDE for rice weevils, and 1000 DE (two samples) for the cracked wheat control were each divided into two portions. Half the material was purified on Florisil by elution with hexane–diethyl ether (1 : 1) as previously described (Phillips et al., 1985). The samples were concentrated under N_2 to approximately 200 μl and placed in silanized screw-top vials fitted with a Teflon liner. The four samples for each species and cracked wheat control, plus the chiral standards, were shipped via air express to Vancouver, in a container maintained at −78.5°C by dry ice. On arrival, samples were transferred to a low-temperature freezer for continued maintenance at −78.5°C.

Synthetic Standards. A racemic sample of 5-hydroxy-4-methyl-3-heptanone containing approximately equal amounts of R^*,S^* and R^*,R^* diastereomers (Phillips et al., 1985) was used to establish reaction conditions, capillary gas chromatograph column selection, and the operating conditions. The four optically pure stereoisomers of 5-hydroxy-4-methyl-3-heptanone used as standards in this study were synthesized by Mori and Ebata (1986). Chemical purity for all four samples was >98%.

Analyses. Splitless capillary gas chromatography was carried out on a Hewlett Packard HP 5890 using a 30-m × 0.25-mm ID fused silica column with injector and detector temperatures of 250°C. The column, SP-2340 (Supelco Inc., Bellefonte, Pennsylvania), was temperature programmed as follows: 100°C for 2 min, 20°C/min to 150°C, and isothermal at 150°C. Flame ionization detection was employed with a helium carrier and makeup gas. All samples were chromatographed to ascertain concentrations and ensure that no underlying impurity existed at the retention time of the acetyl lactate derivatives. Splitless gas chromatography–mass spectroscopy (GC-MS) was carried out on a Hewlett Packard HP 5985B in which the same SP-2340 column had been

installed. The temperature program was identical, except the initial temperature of 100°C was held for only 1 min.

Derivatization of Samples. To the sample contained in 10 μl of hexane, a benzene solution (5 μl) containing dimethylaminopyridine (250 μg) was added followed immediately by the acetyl lactate reagent [8 μl of methylene dichloride solution containing 200 μg of the acetyl lactyl chloride prepared as previously described (Slessor et al., 1985)]. When larger or smaller aliquots were used, the reagent amounts were scaled proportionately. The reactions were sealed in micro vials and kept at room temperature for 24–48 hr. Work-up involved the addition of 10 μl hexane followed by washes with 1% aqueous HCl (1× 50 μl), 5% aqueous NaOH (4× 50 μl), 5% aqueous $NaHCO_3$ (1× 50 μl), and finally water (1× 50 μl).

Bioassay. The dual-choice pitfall bioassay developed by Phillips and Burkholder (1981) was used to test the attractancy of synthetic stereoisomers. Bioassays were conducted as previously described (Walgenbach et al., 1983) except that they ran for 30 min.

For the first experiment, (4*R*,5*S*), (4*S*,5*R*), and *RS*, a racemic mixture of the two enantiomers, were tested. In the second experiment, (4*R*,5*R*), (4*S*,5*S*), and *RR* (racemic mixture) were tested, with a 4*S*,5*R* series included as a positive control.

Test samples were diluted in UV-grade hexane such that 2 μl of solvent was applied. The same amount of solvent was used in the control vessel of each pitfall chamber. For rice weevils, 38 ng of each stereoisomer or mixture were tested. Maize weevils were tested using 100 ng.

Statistical Analysis. Response (treatment − control) was calculated and paired t-tests were used to determine the attractancy of each isomer. Isomers were compared using an analysis of variance (ANOVA). Significance was accepted at $P < 0.05$ for both tests. When the F test proved significant, means were compared using a Duncan's (1955) multiple-range test, also at $P < 0.05$. If male and female responses were statistically equivalent, results were pooled.

The sum of treatment and control was also calculated and was used as a measure of insect activity. Insect activities generated by the isomers were compared using ANOVA at $P < 0.05$.

RESULTS

Determination of Chirality. Concentrations of the pheromone samples ranged from 20 to 24 ng/μl for the maize weevil and 7 to 32 ng/μl for the rice weevil. All insect extracts showed the later eluting R^*,S^* diastereomer to be predominant, indicating that little, if any, epimerization had occurred on preparation and transfer of the samples.

Acetyl lactate formation and work-up of the dilute synthetic pheromone produced a sample which exhibited four baseline separated peaks when gas chromatographed on a 30-m SP-2340 capillary column. Other columns used gave varying degrees of separation, none of which were as good as the SP-2340. GC-MS showed virtually identical fragmentation patterns for all of the four diastereomers, with intense ions at *m/z* 115(71); 97(45); 87(75); 70(40); 57(100); 43(35).

Derivatization of the maize weevil pheromone in both purified and crude preparations showed 90–99% of the 4*S*,5*R* stereoisomer (Table 1), with a small amount of material running marginally faster than the 4*R*,5*R*. Examination of the GC-MS of maize weevil sample 2, Florisil acetyl lactate derivative (96%), revealed the latter peak to be primarily an impurity [*m/z* 133(100); 57(43)] and comprised of at most 50% 4*R*,5*R* isomer. The maize weevil pheromone is accordingly 98% or higher (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone.

The lower pheromone amounts produced by the rice weevil yielded samples with larger impurity concentrations for a given amount of pheromone. This led to much more varied (55–97%) and less accurate determinations, but all showed high 4*S*,5*R* chirality. Again, the marginally faster peak was present in the region of the 4*R*,5*R* isomer and in about the same absolute amount found in maize weevil samples. GC-MS of rice weevil sample 4, crude acetyl lactate derivative, indicated the same impurity containing no more than 50% 4*R*,5*R* isomer. These results indicate the chirality to be at least 92% 4*S*,5*R*, and rice weevil sample 2, Florisil acetyl lactate derivative, showed this value to be at least 97%. These results indicate that the rice weevil pheromone is greater than 92% (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone.

Weevil Response to Synthetic Stereoisomers. In the first experiment, maize weevils were most strongly attracted to 4*S*,5*R* with a mean response of 7.1 ($P < 0.001$; Figure 2a). The response to 4*R*,5*S* was not significant ($\bar{X} = 1.2$; $P > 0.05$). The response to the *RS* mixture fell between the responses to the two enantiomers and was highly significant ($\bar{X} = 4.3$; $P < 0.001$). ANOVA indicated that the responses to the *RS* mixture and 4*S*,5*R* were significantly higher than the response to 4*R*,5*S*. However, all activity values were high ($\bar{X}$ range 6.5–8.3) and were not significantly different from one another. The 4*R*,5*S* isomer is apparently close enough to the pheromone to get the insects moving, but not close enough to direct them, and they fall randomly into the treatment and control traps.

In experiment 2, 4*S*,5*R* again proved strongly attractive ($\bar{X} = 6.3$; $P < 0.001$) (Figure 2b). Low, but significant responses to 4*S*,5*S* ($\bar{X} = 1.7$; $P < 0.05$) and 4*R*,5*R* ($\bar{X} = 1.0$; $P < 0.05$) were observed. However, response to the *RR* mixture was not significant ($\bar{X} = 0.6$; $P > 0.05$). The activity values were correspondingly low. Responses to (4*R*,5*R*), (4*S*,5*S*), and *RR* were statistically equivalent, but 4*S*,5*R* was significantly more attractive than all others.

TABLE 1. RETENTION TIMES AND INTEGRATOR COUNTS FOR SITOPHINONE STEREOISOMERS AND THEIR *S*-(+)-ACETYL LACTATE DERIVATIVES[a]

	Underivatized		Acetyl lactate derivative	
Source and preparation	Time	Counts	Time	Counts
Synthetic 4*S*,5*R*	5.37		15.10	
4*R*,5*S*	5.37		15.39	
4*R*,5*R*	5.12		15.69	
4*S*,5*S*	5.13		16.05	
MW 1, Florisil	5.37	101	15.11	63
			15.59	<1
MW 2, Florisil	5.12	2	15.07	118
	5.37	119	15.59	5
MW 3, Crude	5.12	2	15.06	106
	5.37	107	15.57	5
MW 4, Crude	5.12	1	15.02	114
	5.36	114	15.52	8
RW 1, Florisil	5.37	35	15.13	160
			15.62	47
RW 2, Florisil	5.37	125	15.13	105
			15.64	3
RW 3, Crude	5.37	74	15.04	38
			15.55	10
RW 4, Crude	5.12	3	15.11	133
	5.37	162	15.61	14
CW 1, Florisil			15.59	4
CW 2, Florisil			15.61	4
CW 3, Crude	5.37	6	15.12	2
CW 4, Crude	5.36	4		

[a] MW = maize weevil; RW = rice weevil; CW = cracked wheat control. Integrator counts are proportional to peak area.

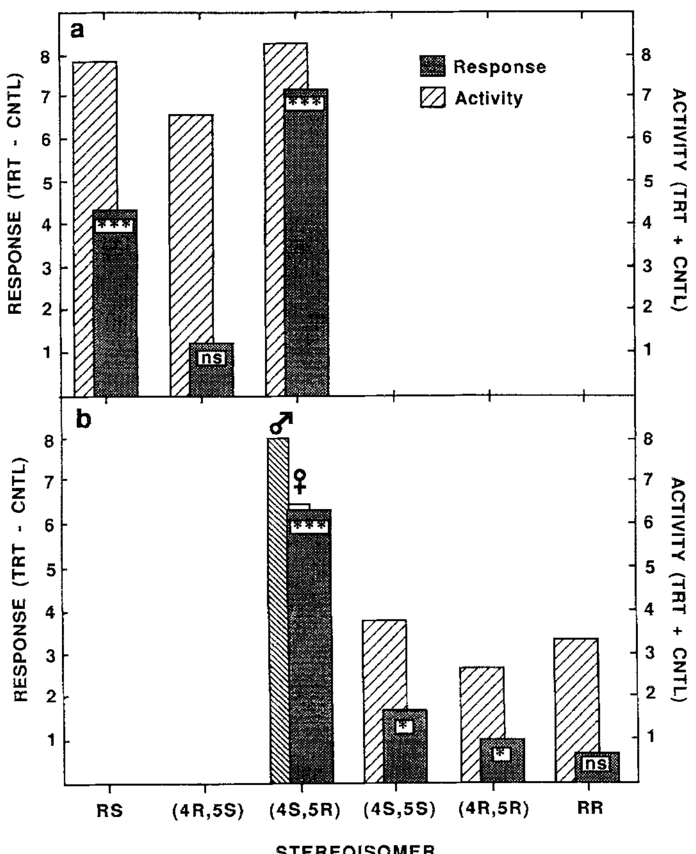

FIG. 2. Mean response (treatment − control) and activity (treatment + control) of maize weevils to stereoisomers of 5-hydroxy-4-methyl-3-heptanone. Male and female results combined for analysis, unless significantly different, as indicated. $^{***}P < 0.001$; $^{*}P < 0.05$; ns, not significant, Student's t test for paired data. (a) experiment 1; (b) experiment 2.

The rice weevil profile for both experiments was similar to that of the maize weevil (Figure 3). In experiment 1, response was strongest to 4*S*,5*R* ($\bar{X} = 7.5$; $P < 0.001$), followed closely by the *RS* mixture ($\bar{X} = 5.8$; $P < 0.001$). The 4*R*,5*S* enantiomer proved least attractive, but in contrast to the maize weevil, the lower response still proved highly significant ($\bar{X} = 2.6$; $P < 0.01$). The response to 4*R*,5*S* was significantly lower than the response to either the *RS* mixture or 4*S*,5*R*. This trend held true for activity as well. All activity values were high (>6.4), but activation by both *RS* and 4*S*,5*R* was significantly greater than for 4*R*,5*S*.

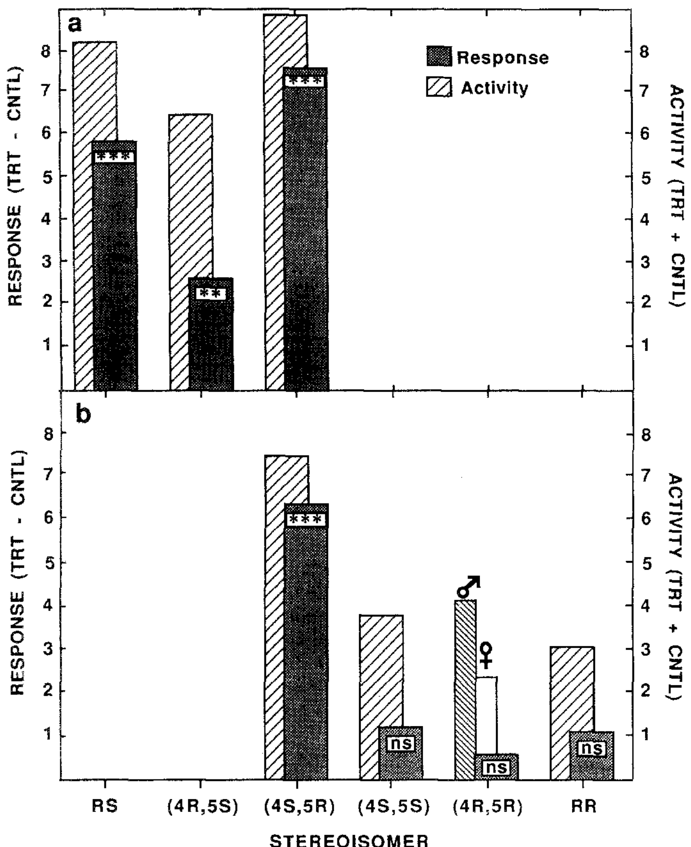

FIG. 3. Mean response (treatment − control) and activity (treatment + control) of rice weevils to stereoisomers of 5-hydroxy-4-methyl-3-heptanone. Male and female results combined for analysis, unless significantly different, as indicated. $^{***}P < 0.001$; $^{**}P < 0.01$; ns, not significant, Student's t test for paired data. (a) experiment 1; (b) experiment 2.

In experiment 2, rice weevil results were again similar to those of the maize weevil (Figure 3b). The 4*S*,5*R* isomer generated a mean response of 6.3 ($P < 0.001$). However, rice weevils did not respond significantly to 4*S*,5*S* ($\overline{X} = 1.2$; $P > 0.05$) nor 4*R*,5*R* ($\overline{X} = 0.6$; $P > 0.05$), as had maize weevils. Neither was there a significant response to the *RR* mixture ($\overline{X} = 1.1$; $P > 0.05$). These results were paralleled by low activity values. As with maize weevils, the response to 4*S*,5*R* was significantly higher than the responses to all other isomers.

DISCUSSION

It is clear that the pheromone of the *S. zeamais* and *S. oryzae* is defined by its optical as well as chemical properties. This is well-documented in other beetle species as well as in other groups of insects (Silverstein, 1979; Mori, 1984). Both maize and rice weevils responded most strongly to the 4*S*,5*R* isomer of 5-hydroxy-4-methyl-3-heptanone, and it has been proven to be the major component of the aggregation pheromone of both species. In addition, some interesting differences were observed in the specificity of the response of the two species to the other three stereoisomers.

In both species, the *RS* enantiomeric mixture produced somewhat lower responses than 4*S*,5*R* alone. However, only half as much 4*S*,5*R* was tested in the *RS* mixture, and thus the effect could simply be due to the lower concentration of this enantiomer.

The low responses observed by both species to the *RR* enantiomeric mixture contradict previous work (Phillips et al., 1985), where both species showed comparable responses to (R^*,S^*), (R^*,R^*), and a racemic mixture of the diastereomers. Since the present study indicated that optically pure 4*S*,5*S* and 4*R*,5*R* generated responses much lower than to the 4*S*,5*R* stereoisomer or the *RS* mixture, contamination of the mixtures used in the 1985 study is implied. Indeed, examination of gas chromatograph traces of R^*,R^* used in the previous study suggest the presence of 2–4% R^*,S^*. This represents $<$1–8 ng of 4*S*,5*R* in the 1985 work, but maize weevils are known to respond to amounts this low (Walgenbach et al., 1983).

Walgenbach and Burkholder (1986) showed that weevil activity (treatment + control) was a valuable additional tool in interpreting pitfall bioassay results. In the present study, activity appears to be a gauge of the relative "closeness" of the stereoisomer to 4*S*,5*R*. For instance, for these species, both response and activity to 4*S*,5*R* were high, indicating that many weevils were trapped in the treatment receptacle and few in the control receptacle. Similarly, low responses and activities were observed for the 4*R*,5*R* and 4*S*,5*S* stereoisomers, indicating that few insects were trapped in either receptacle. However, activities to 4*R*,5*S* were high compared to relatively low responses. This indicates that many insects left the pitfall chamber and were trapped, but they fell more or less randomly into both receptacles. Using this activity information in conjunction with response data may thus be helpful in interpreting which characteristics of the molecule are important in receptor specificity. When response and activity are both high, it is an indication that the weevils are not only activated, but their response is directional, since few incorrect (i.e., control) choices are made. Almost all responses are to the treatment, so response (T − C) and activity (T + C) are very close. For compounds where the receptor fit is interpreted to be nearly correct, high activities, but low responses are observed. The compound

is able to "activate" the insects, but is not able to direct them to its source. Thus, for maize and rice weevils, the need for the chiral centers to have opposite configurations is nearly as important to receptor binding as the specific orientation at each center.

The major component of the pheromone of both *S. zeamais* and *S. oryzae* has herein been shown to be (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone. While the differential attractiveness of the other stereoisomers may simply reflect imperfect receptor specificity, it is possible that these responses reflect differences in the makeup of the maize and rice weevil pheromones, which could have important implications in reproductive isolation, as well as interspecific competition.

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